Angionesis and the inception of pregnancy

Kapiteijn, C.J.

ANGIOGENESIS AND THE INCEPTION OF PREGNANCY

ISBN-10: 90-6464-133-1
ISBN-13: 978-90-6464-133-6

Cover: Dick Bensdorp
Lay-out: Grafisch bureau Christine van der Ven, Voorschoten
Printed by: Grafische Producties, Universitair Facilitair Bedrijf, Leiden

Financial support for the publication of this thesis was provided by Ferring BV, J.E. Jurriaanse Stichting and Bronovo Research Fonds.

ANGIOGENESIS AND THE INCEPTION OF PREGNANCY

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op maandag 12 juni 2006
te klokke 14.15 uur

door

Catharina Johanna Kapiteijn

geboren te Sassenheim in 1971

PROMOTIECOMMISSIE

Promotores:	Prof. Dr. F.M. Helmerhorst Prof. Dr. V.W.M. van Hinsbergh (Vrije Universiteit Amsterdam)
Copromotor:	Dr. P. Koolwijk
Referent:	Prof. Dr. E.A.P. Steegers (Erasmus Universitair Medisch Centrum Rotterdam)
Overige leden:	Prof. Dr. J.P. Vandenbroucke Prof. Dr. F.J. Walther Prof. Dr. G.G. Kenter

CONTENTS

1

GENERAL INTRODUCTION

1. Background

A pregnancy rate of approximately 15% per cycle renders the process of human reproduction inefficient[1]. From assisted procreation studies we have learned that fertilization is not the major problem as this succeeds in about 70-80%. However, the next phase, the implantation, seems to be the biggest challenge. Embryo selection in these procedures can somewhat increase the chance of implantation.

Controlled ovarian hyperstimulation (COHS), used in assisted procreation, adversely affects perinatal outcome. Singleton pregnancies from assisted reproduction have a significantly worse outcome (birth weight and gestational age) compared with spontaneous singleton pregnancies[2] whereas, birth weights of singletons conceived by implanting a cryopreserved embryo tend to be normal or even above average[3]. The difference in these procedures is that embryo transfer of a cryopreserved embryo occurs predominantly in a natural menstrual cycle, whereas embryo transfer after IVF/ICSI occurs directly in an environment that was exposed to COHS.

Whether the factor subfertility confounds the association between COHS and a worse perinatal outcome is still unclear. However, the fact that neonates born after cryopreserved embryo transfer tend to have normal/higher birth weights, suggests that subfertility does not influence perinatal outcome. The adverse effect of COHS on perinatal outcome may be caused by its negative effect on the endometrium[4] and as such on the implantation process. An adverse effect on implantation may lead to worse perinatal outcome, like low birth weight.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is thought to play an important role in the process of implantation. In rodents, it was shown that stimulation with urinary gonadotrophins (a form of COHS), in contrast to recombinant gonadotrophins, negatively affected parameters important in angiogenesis[5,6].

2. Human implantation

Implantation is a series of events, which is initiated when the blastocyst starts to interact with its implantation site, leading to placentation. Of all mammalian physiological processes, implantation involves very species-specific mechanisms, which make comparisons with other species difficult. Therefore research on human implantation has to, at least in part, be performed on our own species by designing *in vitro* assays simulating parts of the *in vivo* process.

Normally, implantation occurs intra-uterine, in the endometrium. However, advanced ectopic pregnancies have been described, for example on the intestinal mucosa. This

shows that the embryo does not solely relies on the endometrium as an implantation site but is able to create its own implantation site even extra-uterine.

2.1 Pre-implantation

As most pregnancies develop intra-uterine, the implantation process in the endometrium is described here.

The endometrium is the mucosa that forms the inner lining of the uterus (Fig. 1). Its growth, differentiation and breakdown is cyclical regulated by the ovarian steroids during the female's fertile life span. Every month the 2/3 upper part of the endometrium, called the functional endometrium, is shed (menstruation). Subsequently, a new functional layer growths from the basal endometrium under the influence of estradiol during the proliferative phase of the cycle. After ovulation, differentiation of the endometrium takes place under the influence of progesterone produced by the corpus luteum (Fig. 2). Both steroids influence processes directly and indirectly via various factors like growth factors and cytokines.

Uterine blood supply is facilitated by the uterine arteries, which give rise to arcuate arteries. From these arteries arise the radial arteries, which divide at the endo-myometrial junction into straight arterioles supplying the basal layer of the endometrium and spiral end-arterioles supplying the functional layer (Fig. 1). Arterioles in the basal layer

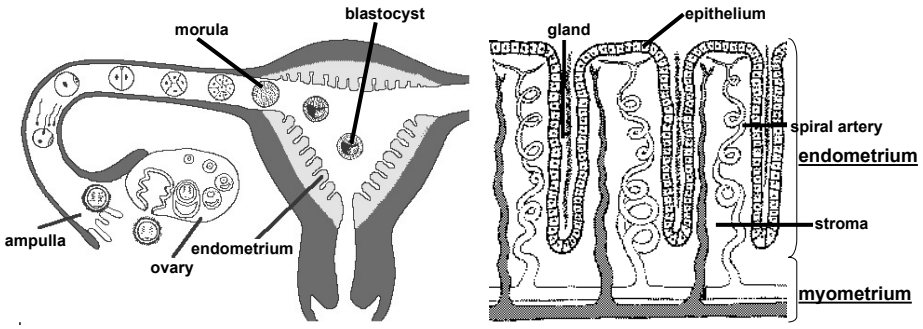


Figure 1. The endometrium.
The inner lining of the uterus, the endometrium, is monthly prepared for the implantation and placentation of a blastocyst. From the basal layer, which is not shed during menstruation, a new functional layer of endometrium develops. Angiogenesis is indispensable for the proliferation and differentiation of the epithelial cells and cells in the stromal compartment. Spiral arteries descend from stumps in the basal layer and from these arteries a new sub-epithelial capillary complex develops.

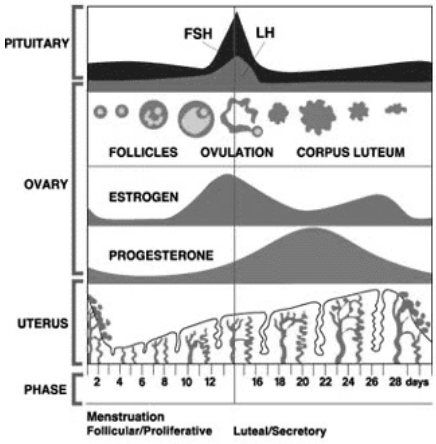


Figure 2. The menstrual cycle.
Hormonal, ovarian, and endometrial changes and relations throughout the normal menstrual cycle.

are surrounded by a vascular smooth muscle coat. Smooth muscle and pericytes are reduced in the superficial layer and the most superficial vessels consist only of endothelial cells[7]. The vessels form a capillary plexus under the epithelium. Endothelial cells in the functional layer show cyclical variation in proliferation, while the endothelial cells in the basal layer do not vary with the menstrual cycle[8,9].

Angiogenesis is required to support the proliferation and differentiation of glandular and surface epithelial cells, and stromal cells, of which the endometrium is composed[10,11]. Together with the changes in vascular permeability throughout the menstrual cycle a transformation of a thin, dense endometrium into a thick, highly edematous secretory endometrium takes place[12].

The morphological changes in the endometrial stroma seen after ovulation can be described as pre-decidualization. Decidualization is a reaction of the endometrium to support and regulate implantation and pregnancy. Further decidualization only occurs in the presence of a pregnancy.

Under normal conditions, when fertilization has occurred, the conceptus travels through the oviduct to the uterus proceeding cellular divisions (Fig. 1). Between its 4-8 cell stage it becomes transcriptionally active and genes of the conceptus itself start to contribute to its development. The metabolic activity and growth of the pre-implanted conceptus is stimulated by a number of growth factors for which it has receptors. On its turn the conceptus is able to synthesize several growth factors. These factors likely act as autocrine and/or paracrine factors, to promote its development and implantation.

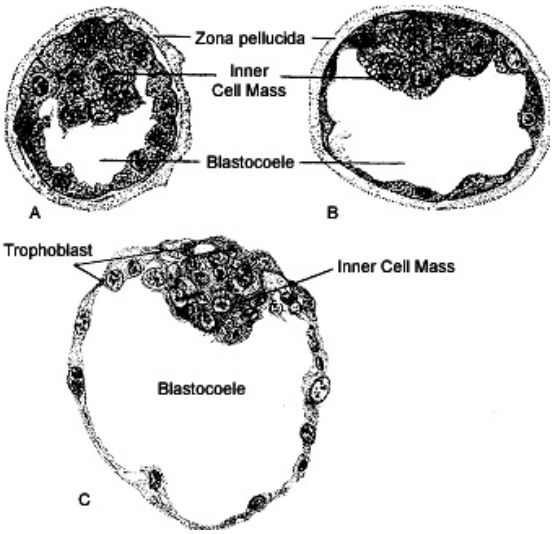


Figure 3. Formation of a blastocyst.
4-5 days after fertilization the embryo has differentiated into two distinct cell types: inner cell mass, which will develop into the fetus, and trophoblasts, which will develop into the placenta. In the blastocyst a cavity has developed called the blastocoele. Around day 6-9 the conceptus loosens its zona pellucida.

3-4 days after fertilization the conceptus enters the uterine cavity and changes from a morula stage (compact 12-16-cell stage) to the blastocyst stage (Fig. 1). The blastocyst contains an outer cell layer called trophoblast which surrounds a cavity called the blastocoele. The extra-embryonic tissue is concerned with the nutrition of the embryo and gives rise to part of the placenta. The group of centrally located cells, know as the inner cell mass, forms the embryo (Fig. 3).

When floating freely in the uterine cavity, the blastocyst derives its nourishment from the secretions of the uterine glands. However, this source becomes inadequate and implantation in highly vascularized endometrium is necessary for its further survival.

Implantation can only take place in a very narrow window of time (48h, 7-10 days after ovulation) during the menstrual cycle, the so-called "implantation window". During this period the endometrial epithelium is receptive to the implanting embryo. Receptive epithelium has specific characteristics that facilitate the conceptus to position and adhere for further implantation (apposition). These characteristics are the expression of small apical protusions called pinopodes and specific cell adhesion molecules called integrins[13-15]. Before and after the receptive period the endometrium resists attachment of the embryo.

For the embryo to survive, its early development and transport must be coordinated

precisely with the changing receptivity of the endometrium. The ovarian steroids play an important role in this coordination.

2.2 Attachment

Implantation involves an initial process of attachment which starts around day 6-9 with the conceptus loosening its zona pellucida (Fig. 3). During attachment close apposition and adherence of the trophoblast cells of the blastocyst to the luminal epithelium of the endometrium occurs.

Evidence derived from *in vitro* experiments and animal studies suggests that successful implantation and placentation depend on the interaction between the conceptus and endometrium[16]. Highly localized signals from the conceptus during apposition, attachment, and later during invasion enhance further decidualization of the endometrium. These processes initiate the development of the maternal part of the placenta. Important features of decidualization are an increase in vascular permeability causing edema, changes in the extracellular matrix (ECM) composition and stromal cell morphology, and angiogenesis. The signaling molecules responsible for decidualization are cytokines, growth factors and hormones[13,17-25]. Some of these signaling molecules and their role(s) are known, others remain unidentified. Studies have shown that the human blastocyst produces activin, colony stimulating factor (CSF)-1, epidermal growth factor (EGF), interferon (IFN) γ, insulin-like growth factor (IGF) I and II, interleukin (IL) 1α and -β, IL-6, IL-10, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) α and β, tumor necrosis factor (TNF) α, vascular endothelial growth factor (VEGF)-A, and hCG[26-33]. The elaborate interaction between the conceptus and the mother has two important distinctive components. First, the conceptus establishes physical and nutritional contact with the maternal endometrium. And second, the conceptus announces its presence to the maternal pituitary-ovarian axis by producing hCG; failure to do so would result in the regression of the corpus luteum, causing progesterone levels to fall, and subsequent loss of the conceptus.

2.3 Invasion

After attachment, controlled invasion takes place. To this end the (syncytio-)trophoblast uses various proteolytic enzymes. By eroding the surface epithelium and larger maternal vessels the trophoblast cells come into contact with maternal blood. This creates a new nutritional source and the basis of placental development (placentation). Decidualization of the endometrium proceeds due to trophoblastic growth factors, cytokines and

steroids. First trimester human trophoblast produces EGF, IGF-II, placental growth factor (PLGF), TGFα, TGFβ, TNFα, hCG, estradiol and progesterone[26-29,34-41 30-33,42].

In these early stages of pregnancy, intact capillaries grow and surround the (syncytio-) trophoblast. These capillaries form a capillary plexus connected to the (syncytio-) trophoblast lacunae and constitute the first very simple vascular system supplying the embryo. A close relationship between embryonic development and the state of vascularization of the chorionic villi has been demonstrated[43]. Nevertheless, the maternal circulation to the human placenta is not well established until the beginning of the second trimester of pregnancy[44]. The main nutritional source remain the uterine glands who deliver secretions into the intervillous space until 10 weeks of gestation.

Implantation is completed two weeks after fertilization. Around this time, the embryo itself synthesizes the hormones required for the continuation of pregnancy and becomes therefore independent of the maternal endocrine condition.

Flaws early in life, during implantation, may result in pregnancy loss or aberrant fetal development, such as intra uterine growth retardation (IUGR), resulting in low birth weight. A low birth weight, on its turn, might have serious consequences later in life as Barker describes in his hypothesis[45,46].

3. Barker hypothesis

In 1989 Barker published the first results from a cohort study of men and women born in Hertfordshire which suggested that cardiovascular disease was inversely related with birth weight[47-49]. Since then, this association has been confirmed by others in different countries[50-54]. Individuals who had low birth weight or were thinner at birth show, besides the increased rate of coronary heart disease, an increased risk for hypertension, (non-insulin-dependent) diabetes, abnormal lipid metabolism, renal disease and coagulation disorders[45,55-59]. Critics doubted the validity of these studies; they were concerned about genetic influences and the influence of socio-economic/environmental confounders on birth weight and cause of death later in life[46,46,60-64]. Several investigators adjusted for socio-economic/environmental factors and they still found, however less strong, an association between birth weight and coronary heart disease. This confirms that socio-economic circumstances at birth and in adult life cannot completely explain the association[51,52,54,65-67].

Out of Barker's observations arose the fetal origin hypothesis or Barker hypothesis, which proposes that several diseases in later life originate in utero from the persistence of physiological, endocrine and metabolic adaptations generated by the fetus (biological programming) when it is undernourished during critical periods of development[45].

These fetal adaptations may be protective in the short term, but may give rise to overt disease later in life. Barker acknowledges that besides the mechanism of programming, genetic and environmental factors play a role in this phenomenon[68]. But, to emphasize the role of biological programming, he believes that what appears to be due to socio-economic or genetic factors may in fact represent a perpetuation of a programming influence through several generations (intergenerational programming)[68].

The underlying biological mechanisms behind the association of low birth weight and adult disease have not been explained yet. Recent studies in animals and man try to elucidate this relationship. Markedly, in most diseases, which have been described to be related to low birth weight, the endothelium is involved. It has been shown that individuals with low birth weight exhibit endothelial dysfunction already at very young ages persisting into childhood and adult life[69-75]. Smith *et al*.[61] found that mothers, who once gave birth to babies with low birth weights, have a higher risk of developing ischemic heart diseases later in life. If this condition of endothelial dysfunction already existed during the time of implantation it might have led to an inadequate placental formation and subsequent IUGR. This suggests that endothelial dysfunction might represent the link between low birth weight and diseases later in life.

4. Angiogenesis

In the process of implantation and placentation, angiogenesis is crucial[1]. It is the result of a delicate balance between stimulators and inhibitors. This balance is influenced by the interaction of endothelial cells with their ECM, by growth factors and cytokines, and by environmental factors such as hypoxia and hormonal status[76-82].

Several steps are involved in angiogenesis. First the endothelial cells need to be activated by angiogenic factors. Secondly, the endothelial cells penetrate their basal membrane and subsequently invade and migrate into the underlying ECM. For this purpose the cells require proteolytic activity, which they obtain by the expression of proteolytic enzymes. Thirdly, the cells proliferate under the influence of angiogenic factors into the underlying interstitial matrix and form new capillary structures. Vessel stabilization is achieved by interaction with pericytes (larger vessels) and reconstitution of the basement membrane (BM)[83,84] (Fig. 4).

4.1 Proteolytic enzymes

Proteolytic enzymes and their inhibitors play an important role in the process of degradation of the BM and ECM and capillary lumen formation[85-88]. They are expressed by the

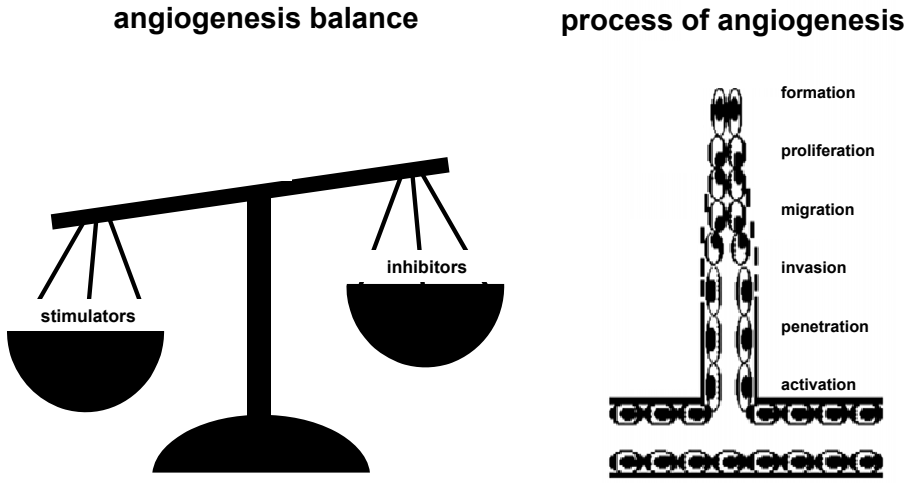


Figure 4. Angiogenesis.
Angiogenesis is a balance between stimulating and inhibiting factors. When resting endothelial cells in an existing capillary get activated by stimulating angiogenic factors they degrade the basement membrane and extracellular matrix (ECM), by expression of proteolytic enzymes. As such, the cells are able to penetrate, invade and migrate into the surrounding interstitium. Subsequently these endothelial cells proliferate, elongate and capillaries are formed.

endothelial cells and act in focal areas at the cell surface, and as such facilitate in a controlled balanced manner cell invasion and migration without loss of the bulk of matrix which is needed as structural support[84]. The proteolytic enzymes can also influence the angiogenic process by generating angiogenesis stimulating or inhibiting ECM fragments and by the activation or release of growth factors.

At least two proteolytic cascades are generally thought to play a major role in cell migration and invasion, namely the urokinase-type plasminogen activator (u-PA)/plasmin cascade and the matrix metalloproteinases (MMPs)[78,79,84,89-92].

4.1-1 The urokinase-type plasminogen activator (u-PA)/plasmin cascade

U-PA converts the inactive plasminogen into the broadly-acting serine protease plasmin. Plasmin is able to cleave fibrin, to degrade several matrix proteins such as thrombospondin and collagens and to activate several MMPs[93-96]. Like plasmin, u-PA is secreted as an inactive single-chain zymogen and can get activated to two-chain u-PA by plasmin or kallikrein to obtain proteolytic activity[97,98]. U-PA is primarily involved in proteolytic processes during cell migration and matrix remodeling. Inhibition of two-chain u-PA occurs by plasminogen activator inhibitors, of which PAI-1 is the predominant physiological inhibitor, secreted, among other cells, by endothelial cells[99,100].

Plasmin(ogen), single-chain u-PA and two-chain u-PA bind with high affinity to their cell surface receptors on endothelial cells. Binding of plasmin(ogen) and two-chain u-PA accelerates the conversion of single-chain u-PA into two-chain u-PA and u-PA-induced plasmin formation[101-108]. The u-PA receptor (u-PAR) acts both as a site for local pericellular proteolysis by u-PA and as a clearance receptor for the u-PA:PAI-1 complex which gets internalized after binding. After internalization the u-PA:PAI-1 complex is degraded and u-PAR is recycled to the cell surface[105,109]. By this process and on a transcriptional level (after stimulation with angiogenic factors) the cell is able to regulate u-PAR density on the cell surface and thus u-PA activity[110,111]. The u-PAR density can also be regulated by the cleavage of u-PAR from the cell membrane and as such generating an soluble form of u-PAR[112-114].

The u-PA expression has been observed to be low in resting endothelial cells[115,116]. The expression is induced in the endothelial cells by e.g. angiogenic factors when migration is induced such is the case during angiogenesis and inflammation[90,117,118].

4.1-2 The MMPs

MMPs are a still expanding, tightly regulated family of zinc-requiring enzymes that play a role in matrix remodeling and many cell-matrix interactions[119]. They have been evidently shown to play a role in angiogenesis both *in vitro* and *in vivo*[84,120-122]. MMPs can also have an inhibitory effect on angiogenesis by cleaving the u-PA, this way disabling its binding to the receptor[123]. Furthermore, MMPs can inactivate plasminogen or cleave plasminogen resulting in the product angiostatin, an angiogenesis inhibitor[124-126]. In the endometrium MMPs are known to play a role in tissue degradation and menstrual bleeding. MMPs have a high affinity for fibronectin, laminins and collagens, which are major ECM components of the endometrium (BM and interstitium). Some MMPs (e.g. MT1-MMP) can, independent of the plasminogen activator pathway, act as a fibrinolysin[127].

MMPs are either secreted from the cell as latent pro-enzymes or they are membrane bound enzymes. Six membrane-type MMPs (MT-MMPs) have been described, 4 transmembrane proteins and 2 GPI-anchored ones. The membrane-associated localization of MT-MMPs makes them particularly suited to function in pericellular proteolysis[128].

Growth factors, cytokines, plasmin but also activated MMPs or MT-MMPS can modulate the expression and activation of MMPs[85,129]. Specific inhibitors are the tissue inhibitors of MMPs (TIMPs) and α-macroglobulins. The TIMP family consists of 4 members, which differ in expression patterns, regulation and ability to interact specifically with latent MMPs[130]. TIMPs are secreted as soluble proteins (e.g. TIMP-1 and -2) or as proteins associated with the matrix components (e.g. TIMP-3)[131].

The relations between the u-PA/plasmin system, MMPs, and their inhibitors is schematically shown in Figure 5.

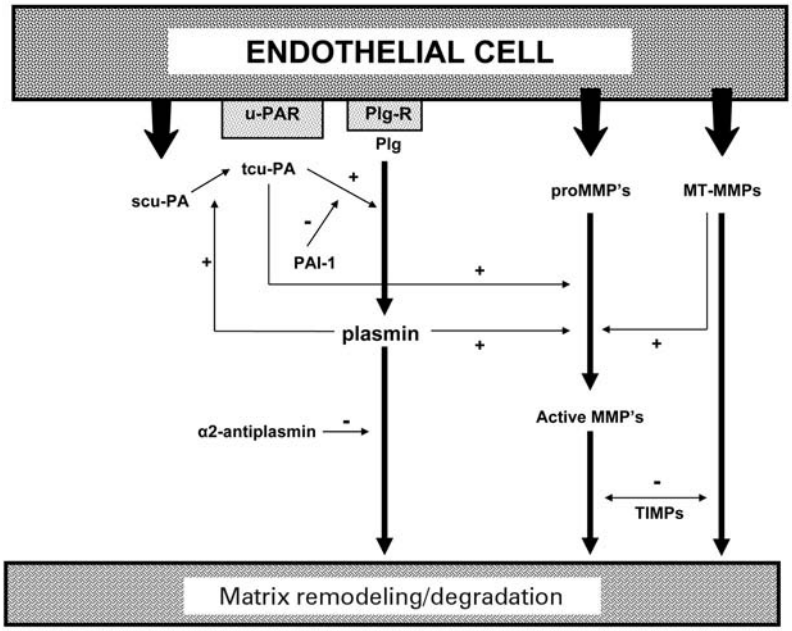


Figure 5. Schematic representation of the relations between the u-PA/plasmin system, MMPs and their inhibitors.

Abbreviations: u-PA: urokinase-type plasminogen activator, sc-u-PA: single-chain u-PA, tc-u-PA: two-chain u-PA, u-PAR: u-PA receptor, Plg: plasminogen, Plg-R: Plg receptor, PAI: PA inhibitor, MT-MMP: membrane-type MMP, TIMP: tissue inhibitor of MMP.

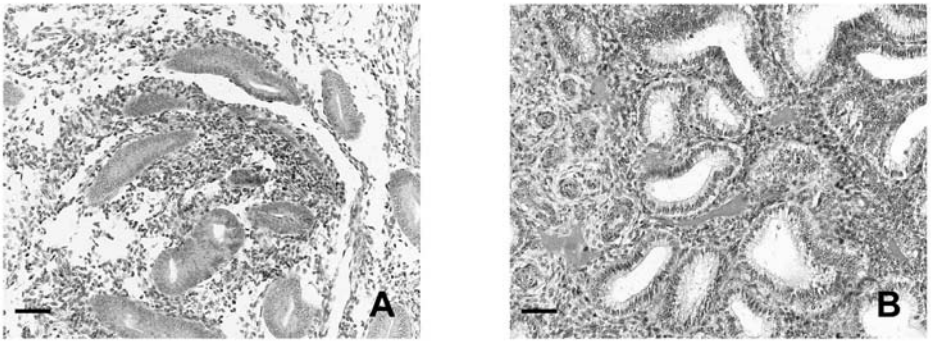


Figure 6. Fibrin staining in secretory endometrium.

In vivo the ECM of the endometrium consists of a number of proteins, such as laminin, fibrin, collagen type I, II, IV and VI, fibronectin and heparan sulphate proteogycan. The composition of the ECM varies during the cycle, as is shown here on paraffin sections of late-proliferative (A) and secretory (B) endometrium of two patients with a Martius Scarlet Blue staining. This staining stains fibrin red, collagen blue and erythrocytes yellow. During the secretory phase an increase of fibrin deposition is seen in the ECM of the endometrium. Bar = 100 μM. *[See appendix: color figures]*

4.2 Extra cellular matrix

In relation to the proteolytic enzymes, the matrix composition also plays an important regulatory role in the process of angiogenesis[132-134]. The composition of the endometrial ECM is subject to cyclic changes. Collagen and fibrin, which have been shown to be a stimulatory factor for endothelial cells and angiogenesis, are components of the endometrial ECM (Fig. 6)[47,135-138,138-140]. Fibrinogen deposition in the endometrium likely results from increased vascular permeability (probably due to VEGF, see below) which is observed during the secretory phase of the cycle and during implantation[138,141].

4.3 Angiogenic growth factors

The growth factor, which is generally assumed to play an important role in both physiological and pathological angiogenesis, is VEGF-A. In addition to inducing endothelial proliferation, VEGF-A modulates the expression of many genes including proteolytic enzymes, it affects endothelial permeability, and it is involved in the maintenance of immature blood vessels[142-145]. It is a homodimeric protein with great homology with placental derived growth factor and the other members of the VEGF family, VEGF-B, C and D[144]. Four forms arise from alternative splicing of the mRNA from a single gene, coding for the proteins of 121, 165, 189 and 206 amino acids (VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}). The two larger forms, and VEGF-A_{165} to some extent, apparently stay cell bound via proteoglycans. Less frequent splice variants have also been reported, including VEGF-A_{145}, VEGF-A_{183}, VEGF-A_{162} and VEGF-A_{165b}[146].

In the human endometrium the epithelial and stromal cells produce VEGF-A, with a higher expression in the epithelial cells than in the stroma. The predominant isoforms in the human endometrium are VEGF-A_{121} and VEGF-A_{165}, whereas VEGF-A_{189} and VEGF-A_{145} are only weakly detectable[12,147-149]. Endometrial macrophages and leukocytes also produce VEGF-A[148-155]. By diffusion into the endometrial interstitium, VEGF-A binds to the endometrial endothelial cells. Whether epithelial derived VEGF-A becomes available for the endothelial cells is doubtful, as a mainly apical secretion by epithelial cells has been described[156]. A positive correlation between stromal VEGF immunostaining and endothelial cell density has been found[152].

Several studies have reported a cyclic or a steroid-dependent variation in the expression of VEGF and VEGF receptors in the endometrium[12,151,157-164]. Furthermore, it has been shown that hypoxia, a major driving force for angiogenesis, can regulate the expression of VEGF[80,161,165].

Three VEGF-specific tyrosine kinase receptors are known: VEGFR-1 (flt-1), VEGFR-2 (KDR) and VEGFR-3 (flt-4). Activation of the VEGFR-2 by VEGF results in a mitogenic response as well as migration[166,167]; whereas the VEGFR-1 has been shown to be important for cell migration but not mitogenesis[167,168]. The high affinity receptors VEGFR-1

and VEGFR-2 were mainly found on endothelial cells in the endometrium[141]. Endothelial strands, which have not yet formed a lumen, strongly stained for both receptors. Inhibition of VEGF activity using soluble-VEGFR-1 prevents endometrial maturation[169].

Whereas VEGF-A has been associated with capillary permeability[170] it is suggested to be responsible for the increased endometrial microvascular permeability. This idea is further supported by a high expression of VEGFR-1 and -2 on capillaries during the mid-secretory period. During this period subepithelial microvascular complexes and spiral arteries are formed and hence the VEGF receptors might be expressed for regulation of the microvascular permeability.

VEGFR-3 is thought to be involved in lymphangiogenesis and acts in concert with VEGFR-2. VEGFR-3 binds VEGF-C and VEGF-D, two gene products of the VEGF family.

5. Ovarian steroids

Markee[171] and Abel[172] were the first to shown that the ovarian steroids, 17β-estradiol and progesterone, are the overall regulators of endometrial angiogenesis. In the menstrual cycle, angiogenesis is seen during the early proliferative phase as a process of post-menstrual repair; during the mid-proliferative phase under the influence of estradiol; and during the estradiol and progesterone mediated secretory phase, when the coiled arteries grow and an extensive subepithelial capillary network is formed[173].

17β-estradiol and progesterone can pass through the cell membrane and bind to their specific (nuclear) receptors. These receptors can control the activity of target genes through direct association with specific DNA sequences known as hormone response elements (HREs)[174,175]. Two estrogen receptors (ERs) are known, ERα and ERβ. Both are different in that the receptors are derived from different genes and they have their own tissue distribution and specific functions. They show similarities in the fact that they share a high level of homology in the DNA-binding and ligand-binding domains and that both receptors bind estradiol with high affinity[176-178]. Estrogens may also act via receptors on the cell surface to achieve rapid, non-genomic effects[179,180].

ERα is suggested to be mainly responsible for the uterotrophic response upon estrogen exposure[181]. The precise physiological function and importance of ERβ in the endometrium is still unclear[182]. ERα knockout mice have a uterus that shows a lack of cell proliferation[181], and ERβ knockout mice demonstrate diminished reproductive capacity (small litter size, multiple resorbed fetuses)[182]. It has been suggested that a role of ERβ may be antagonizing and/or modulating ERα mediated actions.

Progesterone receptor (PR) knockout mice develop an inflammatory response to estradiol in the uterus, with no decidual response[181]. Estrogen induces ER and PR during

the proliferative phase; progesterone has therefore mainly an effect on an estrogen-primed endometrium[181]. In addition, progesterone by itself and steroid withdrawal down regulate the PR and ER expression[183,184].

PR reaches highest concentrations around mid-cycle, and ER mid-proliferative, correlating with the plasma peak of estradiol and the maximum mitotic rate of the endometrial cells[184,185]. The receptors decrease during the secretory phase[186].

6. Outline of this thesis

Defects during the process of implantation may lead to pregnancy loss, or aberrant fetal development which may give rise to diseases in later life. To increase the "take-home-baby-rate" in assisted procreation and to be able to prevent possible consequences of defective implantation (due to for example COHS?), it is important to understand more about the physiological process of implantation. As angiogenesis plays a key role in the process of implantation and placentation, and as endothelial (dys-) function might represent a link in fetal programming (Barker hypothesis), we wanted to elucidate more of the processes involved in angiogenesis. Our main focus was the maternal vessels at the endometrial implantation site, as these likely form the basis for successful implantation and subsequent formation of a healthy environment for the developing fetus.

Barker described the relation of low birth weight with diseases in later life in many cohort studies done in different countries. We wanted to know whether his hypothesis also applied to a case control study among Dutch women. In Chapter 2 we investigated the association between low birth weight and myocardial infarction.

COHS is widely used in assisted procreation in subfertile couples. It is of interest to know whether or not COHS might adversely effect the intra-uterine environment leading to a higher risk of low birth weight and/or preterm birth in these patients. We investigated the effect of subfertility and COHS on perinatal outcome. The results are described in Chapter 3.

Endothelial cells in different organs are heterogeneous. Physiologic processes involving the endothelium could therefore be best addressed by studies of endothelial cells derived from the organ of interest. To learn more about the maternal vasculature at the site of implantation, which is most often the endometrium, human endometrial endothelial cells were isolated and examined in an *in vitro* angiogenesis model consisting of a three-dimensional fibrin and/or collagen type I matrix. These studies are described in Chapter 4.

Several MMPs and MT-MMPs are present in endometrial tissue. However, little information is available on the expression and role of specific MMPs and MT-MMPs in

endometrial endothelial cells and their role in endometrial angiogenesis. The role of proteases was studied to obtain more insight in the factors that might act as key regulators in the process of endometrial angiogenesis. The results of these studies are given in Chapter 5.

Chapter 6 describes a literature search on the influence of steroids on factors important in the process of angiogenesis.

It is unknown how ovarian steroids exactly regulate the process of endometrial angiogenesis. They might exert a direct influence on the endometrial endothelial cells or act indirectly via for example the stromal or epithelial cells which are known to express the angiogenic factor VEGF. Crucial in this respect is the expression of steroid receptors by endometrial cells. As overall regulators of endometrial angiogenesis their influence on endometrial endothelial and stromal cells was examined in Chapter 7.

The early embryo (blastocyst, trophoblast) expresses several cytokines, growth factors and hormones by which it can optimize its own implantation site. These factors might induce, directly or indirectly, local angiogenesis at the place of implantation. Chapter 8 describes studies on the influence of the human embryo on human endometrial endothelial cells.

In Chapter 9, the results of the studies are summarized and discussed in a broader perspective.

References

1. Hoozemans DA, Schats R, Lambalk CB, Homburg R, Hompes PG. Human embryo implantation: current knowledge and clinical implications in assisted reproductive technology. Reprod Biomed Online. 2004;9:692-715.
2. Helmerhorst FM, Perquin DA, Donker D, Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. BMJ. 2004;328:261.
3. Heijnsbroek I, Helmerhorst FM, van den Berg-Helder AF, van der Zwan KJ, Naaktgeboren N, Keirse MJ. Follow-up of 30 pregnancies after embryo cryopreservation. Eur J Obstet Gynecol Reprod Biol. 1995;59:201-204.
4. Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. Hum Reprod Update. 2003;9:515-522.
5. Sibug RM, Helmerhorst FM, Tijssen AM, de Kloet ER, de Koning J. Gonadotrophin stimulation reduces VEGF(120) expression in the mouse uterus during the peri-implantation period. Hum Reprod. 2002;17:1643-1648.
6. Sibug RM, de Koning J, Tijssen AM, de Ruiter MC, de Kloet ER, Helmerhorst FM. Urinary gonadotrophins but not recombinant gonadotrophins reduce expression of VEGF120 and its receptors flt-1 and flk-1 in the mouse uterus during the peri-implantation period. Hum Reprod. 2005;20:649-656.
7. Kohnen G, Campbell S, Jeffers MD, Cameron IT. Spatially regulated differentiation of endometrial vascular smooth muscle cells. Hum Reprod. 2000;15:284-292.
8. Gargett CE, Rogers PA. Human endometrial angiogenesis. Reproduction 2001;121:181-186.
9. Weston G, Rogers PA. Endometrial angiogenesis. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14:919-936.
10. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992;267:10931-10934.
11. Nikitenko LL, Mackenzie IZ, Rees MC, Bicknell R. Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. Mol Hum Reprod. 2000;6:811-819.
12. Bausero P, Cavaille F, Meduri G, Freitas S, Perrot-Applanat M. Paracrine action of vascular endothelial growth factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis.1998;2:167-182.
13. Tabibzadeh S, Babaknia A. The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. Hum Reprod. 1995;10:1579-1602.
14. Nardo LG, Nikas G, Makrigiannakis A, Sinatra F, Nardo F. Synchronous expression of pinopodes and alpha v beta 3 and alpha 4 beta 1 integrins in the endometrial surface epithelium of normally menstruating women during the implantation window. J Reprod Med. 2003;48:355-361.
15. Lessey BA. The role of the endometrium during embryo implantation. Hum Reprod. 2000;15 Suppl:39-50.
16. Licht P, Russu V, Lehmeyer S, Wildt L. Molecular aspects of direct LH/hCG effects on human endometrium--lessons from intrauterine microdialysis in the human female in vivo. Reprod Biol. 2001;1:10-19.
17. Fazleabas AT, Kim JJ, Strakova Z. Implantation: embryonic signals and the modulation of the uterine environment--a review. Placenta. 2004;25 Suppl A:S26-S31.
18. Herrler A, von Rango U, Beier HM. Embryo-maternal signalling: how the embryo starts talking to its mother to accomplish implantation. Reprod Biomed Online. 2003;6:244-256.
19. van der Weiden RM, Wisse LJ, Helmerhorst FM, Keirse MJ, Poelmann RE. Immunohistochemical and ultrastructural localization of prostaglandin H synthase in the preimplantation mouse embryo. J Reprod Fertil. 1996;107:161-166.
20. Armant DR, Wang J, Liu Z. Intracellular signaling in the developing blastocyst as a consequence of the maternal-embryonic dialogue. Semin Reprod Med. 2000;18:273 -87.
21. Salamonsen LA, Dimitriadis E, Robb L. Cytokines in implantation. Semin Reprod Med. 2000;18:299 -310.
22. Kimber SJ. Molecular interactions at the maternal-embryonic interface during the early phase of implantation. Semin Reprod Med. 2000;18:237-53.
23. Krussel JS, Huang HY, Hirchenhain J, Bielfeld P, Cupisti S, Jeremias L, Polan ML. Is there a place for biochemical embryonic preimplantational screening? J Reprod Fertil Suppl. 2000;55:147-159.
24. Vailhe B, Kapp M, Dietl J, Arck P. Human first-trimester decidua vascular density: an immunohistochemical study using VE-cadherin and endoglin as endothelial cell markers. Am J Reprod Immunol. 2000;44:9-15.
25. van der Weiden RM, Helmerhorst FM, Keirse MJ. Influence of prostaglandins and platelet activating factor on implantation. Hum Reprod. 1991;6:436-442.
26. Krussel JS, Simon C, Rubio MC, Pape AR, Wen Y, Huang HY, Bielfeld P, Polan ML. Expression of interleukin-1 system mRNA in single blastomeres from human preimplantation embryos. Hum Reprod. 1998;13:2206-2211.

27. Austgulen R, Arntzen KJ, Vatten LJ, Kahn J, Sunde A. Detection of cytokines (interleukin-1, interleukin-6, transforming growth factor-beta) and soluble tumour necrosis factor receptors in embryo culture fluids during in-vitro fertilization. Hum Reprod. 1995;10:171-176.
28. Zolti M, Ben-Rafael Z, Meirom R, Shemesh M, Bider D, Mashiach S, Apte RN. Cytokine involvement in oocytes and early embryos. Fertil Steril. 1991;56:265-272.
29. Chia CM, Winston RM, Handyside AH. EGF, TGF-alpha and EGFR expression in human preimplantation embryos. Development. 1995;121:299-307.
30. Krussel J, Behr B, Hirchenhain J et al. Expression of vascular endothelial growth factor mRNA in human preimplantation embryos derived from tripronuclear zygotes. Fertil Steril. 2000;74:1220-1226.
31. Krussel JS, Behr B, Milki AA et al. Vascular endothelial growth factor (VEGF) mRNA splice variants are differentially expressed in human blastocysts. Mol Hum Reprod. 2001;7:57-63.
32. Ozornek MH, Bielfeld P, Krussel JS et al. Interferon gamma and interleukin 10 levels in preimplantation embryo culture media. J Assist Reprod Genet. 1995;12:590-593.
33. Svalander PC, Holmes PV, Olovsson M et al. Platelet-derived growth factor is detected in human blastocyst culture medium but not in human follicular fluid--a preliminary report. Fertil Steril. 1991;56:367-369.
34. Hofmann GE, Horowitz GM, Scott RTJ, Navot D. Transforming growth factor-alpha in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. J Clin Endocrinol Metab. 1993;76:781-785.
35. King A, Jokhi PP, Smith SK, Sharkey AM, Loke YW. Screening for cytokine mRNA in human villous and extravillous trophoblasts using the reverse-transcriptase polymerase chain reaction (RT-PCR). Cytokine. 1995;7:364-371.
36. Hofmann GE, Drews MR, Scott RTJ et al. Epidermal growth factor and its receptor in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. J Clin Endocrinol Metab. 1992;74:981-988.
37. Hofmann GE, Scott RTJ, Bergh PA, Deligdisch L. Immunohistochemical localization of epidermal growth factor in human endometrium, decidua, and placenta. J Clin Endocrinol Metab. 1991;73:882-887.
38. Katsuragawa H, Kanzaki H, Inoue T et al. Endometrial stromal cell decidualization inhibits human chorionic gonadotrophin and human placental lactogen secretion by co-cultured trophoblasts. Hum Reprod. 1995;10:3028-3034.
39. Sunder S, Lenton EA. Endocrinology of the peri-implantation period. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14:789-800.
40. Sheth KV, Roca GL, al-Sedairy ST et al. Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertil Steril. 1991;55:952-957.
41. Polan ML, Simon C, Frances A, Lee BY, Prichard LE. Role of embryonic factors in human implantation. Hum Reprod. 1995;10 Suppl 2:22-29.
42. Sharkey AM, Dellow K, Blayney M et al. Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. Biol Reprod. 1995;53:974-981.
43. te Velde EA, Exalto N, Hesseling P, van der Linden HC. First trimester development of human chorionic villous vascularization studied with CD34 immunohistochemistry. Hum Reprod. 1997;12:1577-1581.
44. Jauniaux E, Watson AL, Hempstock J et al. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol. 2000;157:2111-2122.
45. Barker DJ. Fetal origins of coronary heart disease. BMJ. 1995;311:171-174.
46. Paneth N, Susser M. Early origin of coronary heart disease (the "Barker hypothesis"). BMJ. 1995;310:411-412.
47. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;2:577-580.
48. Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. BMJ. 1993;307:1519-1524.
49. Barker DJ, Osmond C, Simmonds SJ, Wield GA. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. BMJ. 1993;306:422-426.
50. Stein CE, Fall CH, Kumaran K et al. Fetal growth and coronary heart disease in south India. Lancet. 1996;348:1269-1273.
51. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD. Birthweight, body-mass index in middle age, and incident coronary heart disease. Lancet. 1996;348:1478-1480.
52. Rich-Edwards JW, Stampfer MJ, Manson JE et al. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. BMJ. 1997;315:396-400.
53. Forsen T, Eriksson JG, Tuomilehto J et al. Mother's weight in pregnancy and coronary heart disease in a cohort of Finnish men: follow up study. BMJ. 1997;315:837-840.
54. Leon DA, Lithell HO, Vagero D et al. Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. BMJ. 1998;317:241-245.

55. Hales CN, Barker DJ, Clark PM et al. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ. 1991;303:1019-1022.
56. Law CM, Shiell AW. Is blood pressure inversely related to birth weight? The strength of evidence from a systematic review of the literature. J Hypertens. 1996;14:935-941.
57. Lithell HO, McKeigue PM, Berglund L et al. Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. BMJ. 1996;312:406-410.
58. Phillips DI, Hirst S, Clark PM, Hales CN, Osmond C. Fetal growth and insulin secretion in adult life. Diabetologia. 1994;37:592-596.
59. Hoy WE, Rees M, Kile E, Mathews JD, Wang Z. A new dimension to the Barker hypothesis: low birthweight and susceptibility to renal disease. Kidney Int. 1999;56:1072-1077.
60. Ben Shlomo Y, Smith GD. Deprivation in infancy or in adult life: which is more important for mortality risk? Lancet. 1991;337:530-534.
61. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. Lancet. 2001;357:2002-2006.
62. Morris JA. Fetal origin of maturity-onset diabetes mellitus: genetic or environmental cause? Med Hypotheses. 1998;51:285-288.
63. Hubinette A, Cnattingius S, Ekbom A et al. Birthweight, early environment, and genetics: a study of twins discordant for acute myocardial infarction. Lancet. 2001;357:1997-2001.
64. Susser M, Levin B. Ordeals for the fetal programming hypothesis. The hypothesis largely survives one ordeal but not another. BMJ. 1999;318:885-886.
65. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD. Birthweight, adult risk factors and incident coronary heart disease: the Caerphilly Study. Public Health. 1996;110:139-143.
66. Barker DJ, Gluckman PD, Godfrey KM et al. Fetal nutrition and cardiovascular disease in adult life [see comments]. Lancet. 1993;341:938-941.
67. Poulter NR. Birthweights, maternal cardiovascular events, and Barker hypothesis. Lancet. 2001;357:1990-1991.
68. Drake AJ, Walker BR. The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk. J Endocrinol. 2004;180:1-16.
69. Martin H, Gazelius B, Norman M. Impaired acetylcholine-induced vascular relaxation in low birth weight infants: implications for adult hypertension? Pediatr Res. 2000;47:457-462.
70. Goh KL, Shore AC, Quinn M, Tooke JE. Impaired microvascular vasodilatory function in 3-month-old infants of low birth weight. Diabetes Care. 2001;24:1102-1107.
71. Leeson CP, Whincup PH, Cook DG et al. Flow-mediated dilation in 9- to 11-year-old children: the influence of intrauterine and childhood factors. Circulation. 1997;96:2233-2238.
72. Martin H, Hu J, Gennser G, Norman M. Impaired endothelial function and increased carotid stiffness in 9-year-old children with low birthweight. Circulation. 2000;102:2739-2744.
73. Leeson CP, Kattenhorn M, Morley R, Lucas A, Deanfield JE. Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life. Circulation. 2001;103:1264-1268.
74. Goodfellow J, Bellamy MF, Gorman ST et al. Endothelial function is impaired in fit young adults of low birth weight. Cardiovasc Res. 1998;40:600-606.
75. McAllister AS, Atkinson AB, Johnston GD, McCance DR. Relationship of endothelial function to birth weight in humans. Diabetes Care. 1999;22:2061-2066.
76. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. Annu Rev Physiol. 1991;53:217-239.
77. Iruela-Arispe ML, Dvorak HF. Angiogenesis: a dynamic balance of stimulators and inhibitors. Thromb Haemost. 1997;78:672-677.
78. Koolwijk P, van Erck MG, de Vree WJ et al. Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol. 1996;132:1177-1188.
79. Kroon ME, Koolwijk P, van Goor H et al. Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. Am J Pathol. 1999;154:1731-1742.
80. Sharkey AM, Day K, McPherson A et al. Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. J Clin Endocrinol Metab. 2000;85:402-409.
81. Lansink M, Koolwijk P, van H, V, Kooistra T. Effect of steroid hormones and retinoids on the formation of capillary- like tubular structures of human microvascular endothelial cells in fibrin matrices is related to urokinase expression. Blood. 1998;92:927-938.
82. Folkman J, Ingber DE. Angiostatic steroids. Method of discovery and mechanism of action. Ann Surg. 1987;206:374-383.
83. Risau W. Mechanisms of angiogenesis. Nature. 1997;386:671-674.
84. Pepper MS. Manipulating angiogenesis. From basic science to the bedside. Arterioscler Thromb Vasc Biol. 1997;17:605-619.

85. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein. 1996;49:117-137.
86. Pintucci G, Bikfalvi A, Klein S, Rifkin DB. Angiogenesis and the fibrinolytic system. Semin Thromb Hemost. 1996;22:517-524.
87. Rabbani SA. Metalloproteases and urokinase in angiogenesis and tumor progression. In Vivo. 1998;12:135-142.
88. Iivanainen E, Kahari VM, Heino J, Elenius K. Endothelial cell-matrix interactions. Microsc Res Tech. 2003;60:13-22.
89. Pepper MS, Belin D, Montesano R, Orci L, Vassalli JD. Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells *in vitro*. J Cell Biol. 1990;111:743-755.
90. Bacharach E, Itin A, Keshet E. In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc Natl Acad Sci U S A. 1992;89:10686-10690.
91. Van Hinsbergh VWM. Impact of endothelial activation on fibrinolysis and local proteolysis in tissue repair. Ann N Y Acad Sci. 1992;667:151-162.
92. Vassalli JD, Pepper MS. Tumour biology. Membrane proteases in focus. Nature. 1994;370:14-15.
93. Okumura Y, Sato H, Seiki M, Kido H. Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin. A possible cell surface activator. FEBS Lett. 1997;402:181-184.
94. Santibanez JF, Martinez J. Membrane-associated procollagenase of leukemic cells is activated by urokinase-type plasminogen activator. Leuk Res. 1993;17:1057-1062.
95. Murphy G, Stanton H, Cowell S et al. Mechanisms for pro matrix metalloproteinase activation. APMIS. 1999;107:38-44.
96. Liotta LA, Goldfarb RH, Brundage R et al. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. Cancer Res. 1981;41:4629-4636.
97. List K, Jensen ON, Bugge TH et al. Plasminogen-independent initiation of the pro-urokinase activation cascade in vivo. Activation of pro-urokinase by glandular kallikrein (mGK-6) in plasminogen-deficient mice. Biochemistry. 2000.39:508-515.
98. Ichinose A, Fujikawa K, Suyama T. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. J Biol Chem. 1986;261:3486-3489.
99. Erickson LA, Hekman CM, Loskutoff DJ. The primary plasminogen-activator inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related. Proc Natl Acad Sci U S A. 1985;82:8710-8714.
100. van Mourik JA, Lawrence DA, Loskutoff DJ. Purification of an inhibitor of plasminogen activator (antiactivator.synthesized by endothelial cells. J Biol Chem. 1984;259:14914-14921.
101. Hajjar KA, Hamel NM. Identification and characterization of human endothelial cell membrane binding sites for tissue plasminogen activator and urokinase. J Biol Chem. 1990;265:2908-2916.
102. Miles LA, Levin EG, Plescia J, Collen D, Plow EF. Plasminogen receptors, urokinase receptors, and their modulation on human endothelial cells. Blood. 1988;72:628-635.
103. Hall SW, Humphries JE, Gonias SL. Inhibition of cell surface receptor-bound plasmin by alpha 2-antiplasmin and alpha 2-macroglobulin. J Biol Chem. 1991;266:12329-12336.
104. Vassalli JD, Baccino D, Belin D. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J Cell Biol. 1985;100:86-92.
105. Cubellis MV, Nolli ML, Cassani G, Blasi F. Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. J Biol Chem. 1986;261:15819-15822.
106. Ellis V, Behrendt N, Dano K. Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. J Biol Chem. 1991;266:12752-12758.
107. Quax PH, Pedersen N, Masucci MT et al. Complementation between urokinase-producing and receptor-producing cells in extracellular matrix degradation. Cell Regul. 1991;2:793-803.
108. Duval-Jobe C, Parmely MJ. Regulation of plasminogen activation by human U937 promonocytic cells. J Biol Chem. 1994;269:21353-21357.
109. Nykjaer A, Conese M, Christensen EI et al. Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. EMBO J. 1997;16:2610-2620.
110. Mandriota SJ, Seghezzi G, Vassalli JD et al. Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. J Biol Chem. 1995;270:9709-9716.
111. Mignatti P, Mazzieri R, Rifkin DB. Expression of the urokinase receptor in vascular endothelial cells is stimulated by basic fibroblast growth factor. J Cell Biol. 1991;113:1193-1201.
112. Koolwijk P, Sidenius N, Peters E et al. Proteolysis of the urokinase-type plasminogen activator receptor by metalloproteinase-12: implication for angiogenesis in fibrin matrices. Blood. 2001;97:3123-3131.
113. Solberg H, Romer J, Brunner N et al. A cleaved form of the receptor for urokinase-type plasminogen activator in invasive transplanted human and murine tumors. Int J Cancer. 1994;58:877-881.
114. Andolfo A, English WR, Resnati M et al. Metalloproteases cleave the urokinase-type plasminogen activator

receptor in the D1-D2 linker region and expose epitopes not present in the intact soluble receptor. Thromb Haemost. 2002;88:298-306.
115. Kristensen P, Larsson LI, Nielsen LS et al. Human endothelial cells contain one type of plasminogen activator. FEBS Lett. 1984;168:33-37.
116. Larsson LI, Skriver L, Nielsen LS et al. Distribution of urokinase-type plasminogen activator immunoreactivity in the mouse. J Cell Biol. 1984;98:894-903.
117. Grondahl-Hansen J, Kirkeby LT, Ralfkiaer E et al. Urokinase-type plasminogen activator in endothelial cells during acute inflammation of the appendix. Am J Pathol. 1989;135:631-636.
118. Pepper MS, Sappino AP, Stocklin R et al. Upregulation of urokinase receptor expression on migrating endothelial cells. J Cell Biol. 1993;122:673-684.
119. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003;92:827-839.
120. Montesano R. 1992 Mack Forster Award Lecture. Review. Regulation of angiogenesis *in vitro*. Eur J Clin Invest. 1992;22:504-515.
121. Anand-Apte B, Pepper MS, Voest E et al. Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. Invest Ophthalmol Vis Sci. 1997;38:817-823.
122. Schnaper HW, Grant DS, Stetler-Stevenson WG et al. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis *in vitro*. J Cell Physiol. 1993;156:235-246.
123. Ugwu F, Van Hoef B, Bini A, Collen D, Lijnen HR. Proteolytic cleavage of urokinase-type plasminogen activator by stromelysin-1 (MMP-3). Biochemistry. 1998;37:7231-7236.
124. Ugwu F, Lemmens G, Collen D, Lijnen HR. Modulation of cell-associated plasminogen activation by stromelysin-1 (MMP-3). Thromb Haemost. 1999;82:1127-1131.
125. Cornelius LA, Nehring LC, Harding E et al. Matrix metalloproteinases generate angiostatin: effects on neovascularization. J Immunol. 1998;161:6845-6852.
126. Lijnen HR, Ugwu F, Bini A, Collen D. Generation of an angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). Biochemistry. 1998;37:4699-4702.
127. Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell. 1998;95:365-377.
128. Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. J Cell Biol. 2000;149:1309-1323.
129. Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem. 1997;378:151-160.
130. Woessner JFJ. That impish TIMP: the tissue inhibitor of metalloproteinases-3. J Clin Invest. 2001;108:799-800.
131. Li H, Lindenmeyer F, Grenet C et al. AdTIMP-2 inhibits tumor growth, angiogenesis, and metastasis, and prolongs survival in mice. Hum Gene Ther. 2001;12:515-526.
132. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol. 1988;107:1589-1598.
133. Montesano R, Orci L, Vassalli P. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J Cell Biol. 1983;97:1648-1652.
134. Nicosia RF, Bonanno E, Smith M. Fibronectin promotes the elongation of microvessels during angiogenesis *in vitro*. J Cell Physiol. 1993;154:654-661.
135. Dvorak HF, Harvey VS, Estrella P et al. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab Invest. 1987;57:673-686.
136. Olander JV, Bremer ME, Marasa JC, Feder J. Fibrin-enhanced endothelial cell organization. J Cell Physiol. 1985;125:1-9.
137. Madri JA, Williams SK. Capillary endothelial cell cultures: phenotypic modulation by matrix components. J Cell Biol. 1983;97:153-165.
138. Okada Y, Asahina T, Kobayashi T, Goto J, Terao T. Studies on the mechanism of edematous changes at the endometrial stroma for implantation. Semin Thromb Hemost. 2001;27:67-77.
139. Iwahashi M, Muragaki Y, Ooshima A, Yamoto M, Nakano R. Alterations in distribution and composition of the extracellular matrix during decidualization of the human endometrium. J Reprod Fertil. 1996;108:147-155.
140. Aplin JD, Charlton AK, Ayad S. An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. Cell Tissue Res. 1988;253:231-240.
141. Meduri G, Bausero P, Perrot-Applanat M. Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. Biol Reprod. 2000;62:439-447.
142. Smith SK. Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update. 1998;4:509-519.
143. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in estab-

lished human tumors follows vascular endothelial growth factor withdrawal [see comments]. J Clin Invest. 1999;103:159-165.
144. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol. 2001;280:C1358-C1366.
145. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol. 1995;146:1029-1039.
146. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev. 2004;25:581-611.
147. Huang JC, Liu DY, Dawood MY. The expression of vascular endothelial growth factor isoforms in cultured human endometrial stromal cells and its regulation by 17beta- oestradiol. Mol Hum Reprod. 1998;4:603-607.
148. Torry DS, Holt VJ, Keenan JA et al. Vascular endothelial growth factor expression in cycling human endometrium. Fertil Steril. 1996;66:72-80.
149. Charnock-Jones DS, Sharkey AM, Rajput-Williams J et al. Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biol Reprod. 1993;48:1120-1128.
150. Gargett CE, Lederman FL, Lau TM, Taylor NH, Rogers PA. Lack of correlation between vascular endothelial growth factor production and endothelial cell proliferation in the human endometrium [In Process Citation]. Hum Reprod. 1999;14:2080-2088.
151. Rogers PA, Gargett CE. Endometrial angiogenesis. Angiogenesis. 1998;2:287-294.
152. Charnock-Jones DS, Macpherson AM, Archer DF et al. The effect of progestins on vascular endothelial growth factor, oestrogen receptor and progesterone receptor immunoreactivity and endothelial cell density in human endometrium. Hum Reprod. 2000;15 Suppl 3:85-95.
153. Mueller MD, Lebovic DI, Garrett E, Taylor RN. Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. Fertil Steril. 2000;74:107-112.
154. Taylor RN, Lebovic DI, Hornung D, Mueller MD. Endocrine and paracrine regulation of endometrial angiogenesis. Ann N Y Acad Sci. 2001;943:109-121.
155. McLaren J, Prentice A, Charnock-Jones DS et al. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest. 1996;98:482-489.
156. Hornung D, Lebovic DI, Shifren JL, Vigne JL, Taylor RN. Vectorial secretion of vascular endothelial growth factor by polarized human endometrial epithelial cells. Fertil Steril. 1998;69:909-915.
157. Wheeler T, Evans PW, Anthony FW et al. Relationship between maternal serum vascular endothelial growth factor concentration in early pregnancy and fetal and placental growth. Hum Reprod. 1999;14:1619-1623.
158. Zhang L, Rees MC, Bicknell R. The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges. J Cell Sci. 1995;108:323-331.
159. Shifren JL, Tseng JF, Zaloudek CJ et al. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab. 1996;81:3112-3118.
160. Rogers PA, Lederman F, Taylor N. Endometrial microvascular growth in normal and dysfunctional states. Hum Reprod Update. 1998;4:503-508.
161. Popovici RM, Irwin JC, Giaccia AJ, Giudice LC. Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration. J Clin Endocrinol Metab. 1999;84:2245-2248.
162. Cullinan-Bove K, Koos RD. Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. Endocrinology. 1993;133:829-837.
163. Li XF, Gregory J, Ahmed A. Immunolocalisation of vascular endothelial growth factor in human endometrium. Growth Factors. 1994;11:277-282.
164. Perrot-Applanat M, Ancelin M, Buteau-Lozano H, Meduri G, Bausero P. Ovarian steroids in endometrial angiogenesis. Steroids. 2000;65:599-603.
165. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med. 2003;54:17-28.
166. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994;269:26988-26995.
167. Kanno S, Oda N, Abe M et al. Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. Oncogene. 2000;19:2138-2146.
168. Barleon B, Sozzani S, Zhou D et al. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood. 1996;87:3336-3343.
169. Ferrara N, Chen H, Davis-Smyth T et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med. 1998;4:336-340.

170. Ancelin M, Buteau-Lozano H, Meduri G et al. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proc Natl Acad Sci U S A. 2002;99:6023-6028.
171. Markee JE. Menstruation in intraocular endometrial transplants in the rhesus monkey. Contrib.Embryol. 1940;177:221-308.
172. Abel MH. Prostanoids and menstruation. Mechanisms of menstrual bleeding. 1985;25:139-156.
173. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol. 1975;122:262-263.
174. Berg JM. DNA binding specificity of steroid receptors. Cell. 1989;57:1065-1068.
175. Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Update. 2000;6:225-236.
176. Nilsson S, Kuiper GGJM, Gustafsson J-A. ERb: a novel estrogen receptor offers the potential for new drug development. Trends in Endocrinological Metabolism. 1998;9:387-395.
177. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 1996;392:49-53.
178. Kuiper GG, Carlsson B, Grandien K et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997;138:863-870.
179. Revelli A, Massobrio M, Tesarik J. Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev. 1998;19:3-17.
180. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci U S A. 2000;97:5930-5935.
181. Curtis SH, Korach KS. Steroid receptor knockout models: phenotypes and responses illustrate interactions between receptor signaling pathways in vivo. Adv Pharmacol. 2000;47:357-380.
182. Weihua Z, Saji S, Makinen S et al. Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A. 2000;97:5936-5941.
183. Classen-Linke I, Kusche M, Knauthe R, Beier HM. Establishment of a human endometrial cell culture system and characterization of its polarized hormone responsive epithelial cells. Cell Tissue Res. 1997;287:171-185.
184. Classen-Linke I, Alfer J, Hey S et al. Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. Hum Reprod Update. 1998;4:539-549.
185. Ferenczy A, Bertrand G, Gelfand MM. Proliferation kinetics of human endometrium during the normal menstrual cycle. Am J Obstet Gynecol. 1979;133:859-867.
186. Nisolle M, Casanas-Roux F, Wyns C et al. Immunohistochemical analysis of estrogen and progesterone receptors in endometrium and peritoneal endometriosis: a new quantitative method. Fertil Steril. 1994;62:751-759.

2

DUTCH WOMEN WITH A LOW BIRTH WEIGHT HAVE AN INCREASED RISK OF MYOCARDIAL INFARCTION LATER IN LIFE: A CASE CONTROL STUDY

B.C. Tanis
K. Kapiteijn
R.M. Hage
F.R. Rosendaal
F.M. Helmerhorst

Reproductive Health 2005,2:1

Background

Intrauterine malnutrition, as reflected by birth weight and abnormal thinness at birth, has been associated with an increased incidence of risk factors for arterial disease, i.e. hypertension, impaired glucose tolerance, diabetes and to a lesser extent hyperlipidemia and body fat distribution in adulthood[1-10]. This observation has become known as the 'fetal origins of adult disease' or 'Barker hypothesis', which suggests that several of the major diseases of later life, including coronary heart disease, stroke and cardiovascular death, originate in impaired intrauterine growth and development[11,12]. In cohort studies, Barker[13-15] in England and Finland, Rich-Edwards *et al*.[16] as part of the Nurses' Health Study in the USA and Leon *et al.*[17] from Uppsala in Sweden showed an inverse relationship between birth weight and the clinical endpoint ischemic heart disease. Leon *et al.*[17] found a significant relationship only among male singletons and adjusted their results for gestational age and socioeconomic confounding. The association was not found in a cohort study from Gothenburg[18].

Our aim was to investigate the association in a case control study among Dutch women.

Methods

The RATIO (Risk of Arterial Thrombosis In relation to Oral contraceptives) study is a population-based case-control study on myocardial infarction in relation to oral contraceptive use among women aged 18 to 49 years in the Netherlands[19]. An additional standardized questionnaire was sent to all 218 patients and 769 controls from whom also blood samples had been taken for determination of metabolic risk factors (diabetes and hypercholesterolemia). Questions elicited information on birth weight, waist and hip circumference and data on the menstrual cycle. For 13 women no current address could be found (12 patients, 1 control). Four women had died since the index date (2 patients, 2 controls), which was the date of the first myocardial infarction for the patients and the midyear for the controls. One hundred and fifty two patients (71%) and 568 controls (75%) responded to the questionnaire and women were asked to measure their waist and hip circumference. Body mass index (BMI) was calculated as body weight (kg) divided by height squared (m^2). Waist-hip-ratio was calculated as waist circumference divided by hip circumference.

Multiple linear and unconditional logistic regression were used to analyze the data. Odds ratios for the relationship between birth weight and myocardial infarction were calculated and 95% confidence intervals (95%CI) were derived from the models. Birth weights were categorized according to quintiles in control women in order to investigate

an association between birth weight and the risk of myocardial infarction later in life. These were <3000 g, 3000 to 3199 g, 3200 to 3499 g, 3500 to 3883 g, and >3884 g, respectively. To determine whether women with a lower birth weight had a higher risk for a myocardial infarction, patients were divided in a group with a birth weight equally or higher than 2000 g and a group with a birth weight lower 2000 g[20]. Odds ratios were adjusted for age, education level, body mass index, waist-hip ratio, hypertension, diabetes, hypercholesterolemia, smoking, and family history of cardiovascular disease, when appropriate. Interaction between low birth weight and low education level was investigated by computing a dummy variable.

Results

The characteristics of 152 women with myocardial infarction and 568 control women at the index date are shown in Table 1. At the moment of completing the questionnaire, patients were aged 32-59 years (mean 50), and control women 25-60 years (mean 47). The mean body mass index was 25.1 kg/m^2 for the patients and 23.4 kg/m^2 for control women, mean difference 1.76 kg/m^2 (95%CI 1.05-2.47), $p<0.001$. Ninety-seven patients (64%) and 415 (73%) controls could give their birth weight. Compared with control women, patients had a significantly lower mean birth weight (3214 vs. 3370 g, mean difference -156.3 g (95%CI -9.5 to -303.1). The odds ratio for myocardial infarction for children with a low birth weight (< 2000 g) compared to a birth weight $\geq$ 2000 g was 2.4 (95%CI 1.0 to 5.8). After adjustment for putative confounders (age, education level, body mass index, waist-hip ratio, hypertension, diabetes, hypercholesterolemia, smoking, and family history of cardiovascular disease) the odds ratio did not change. Odds ratios for myocardial infarction in different categories of birth weight as compared to the reference category (birth weight higher than 3884 g) were 1.3 (95%CI 0.5-3.3) for a birth weight 3500 to 3883 g, 1.4 (95%CI 0.6-3.4) for a birth weight 3200 to 3499 g, 1.7 (95%CI 0.6-5.1) for a birth weight 3000 to 3199 g, and 2.3 (95%CI 1.0-5.4) for a birth weight lower than 3000 g (Table 2). The risk of myocardial infarction was 6.2 fold increased (95%CI 2.7-13.9) among women with low birth weight and a low educational level compared to women with a high birth weight and a high educational level (reference category).

Discussion

In this case control study we have found that women with a low birth weight had a higher risk of myocardial infarction than women with a higher birth weight. The data

Table 1. Characteristics of patients with a first myocardial infarction and control women.

Characteristic	Patients (N=152)	Control women (N=568)
Age – yr (SD)	42.1 (0.5)	38.6 (0.3)
Caucasian ethnicity (%)	142 (93)	538 (95)
Educational level		
- Primary school or less (%)	83 (55)	160 (28)
- Secondary school (%)	52 (34)	257 (45)
- Higher education or university (%)	17 (11)	149 (26)
Current smokers (%)	128 (84)	218 (39)
History of hypertension (%)	35 (23)	35 (6)
History of hypercholesterolemia (%)	16 (11)	14 (3)
History of diabetes (%)	8 (5)	7 (1)
Family history of cardiovascular disease (%)	98 (66)	194 (36)
Birth weight -gram		
- Mean (SD)	3214 (676)	3370 (659)
- Median (range)	3150 (1500-5010)	3500 (1500-5800)
Body Mass Index – kg/m^2 - Mean (SD)	25.1 (0.4)	23.4 (0.2)
Waist circumference – cm (SD)	89.5 (13.1)	83.0 (10.2)
Waist/hip ratio (SD)	0.85 (0.006)	0.81 (0.008)
Premenopausal (%)	132 (87)	480 (85)

Table 2. Odds ratios (95% CI) for myocardial infarction in quintiles of birth weight as compared to the reference category.

Birth weight (g)	Odds Ratio (95% CI)
> 3884	1*
3500-3883	1.3 (0.5-3.3)
3200-3499	1.4 (0.6-3.4)
3000-3199	1.7 (0.6-3.4)
< 3000	2.3 (1.0-5.4)

* Reference category

confirm the association found in cohort studies[13-17] and as such support the 'fetal origins of adult disease' or 'Barker hypothesis'.

A limitation of the study is that we have no information on gestational age. However, recently it has been demonstrated that both children who had been born prematurely and children who are small for gestational age had a reduction in insulin resistance[21,22]. The self-report of birth weights as well as the rather high percentage of missing values for birth weight may also limit the study, but the random events among cases and controls cannot explain our results. Socio-economic factors associated with low birth weight are also associated to risk factors for arterial disease later in life. As pointed out by several others, it will be nearly impossible to disentangle these effects[16,17].

In the present study we confirmed an interactive effect between low birth weight and a low educational level on the risk of myocardial infarction in women. However, when we adjusted for age, education level, body mass index, waist-hip ratio, hypertension, diabetes, hypercholesterolemia, smoking, and family history of cardiovascular disease, factors of which some may partly been seen as (the result of) socio-economic/environmental and genetic factors, we still observed an association between low birth weight and a higher risk of myocardial infarction. This is in agreement with others who also found that genetic and socio-economic circumstances at birth and in adult life can not completely explain the association between low birth weight and disease late in life[10,16,17,23-25].

Among the proposed underlying biological mechanisms to explain the association is impaired endothelial development. Already at very young age, individuals with low birth weight exhibit endothelial dysfunction that persists into childhood and adult life, suggesting that endothelial dysfunction precedes the development of vascular related diseases later in life and represents the link between low birth weight and these diseases[26-32]. Furthermore, Smith *et al*.[33] found that mothers, who once gave birth to thin babies, have a higher risk of developing ischemic heart disease later in life. Therefore, these mothers seem to have, just as their children, an impaired endothelial function. If the impaired endothelial function is already manifest during the process of implantation, it might lead to inadequate development of the vasculature in the maternal part of the placenta, which enfeebles the function of the placenta resulting in low birth weight.

Conclusions

In conclusion, our study shows that a low birth weight (<2000 g) is associated with a 2.4 fold higher risk of myocardial infarction before the age of 50 as compared with a birth weight $\geq$ 2000 g. Because the risk of cardiovascular disease is known to increase

with an increasing number of risk factors, women with a low birth weight should try to avoid acquired risk factors, like smoking and obesity. In addition, extra attention should be given in detecting diabetes or hypertension at a later stage in life and in detecting exaggerated growth during childhood, as these individuals seem most prone to develop disease later on in life[34-36].

Acknowledgements

This work was supported by the Netherlands Heart Foundation (97-063).

References

1. Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME: Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989,298:564-567.
2. Barker DJ, Bull AR, Osmond C, Simmonds SJ: Fetal and placental size and risk of hypertension in adult life. *BMJ* 1990,301:259-262.
3. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD: Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* 1991,303:1019-1022.
4. Barker DJ, Meade TW, Fall CH, Lee A, Osmond C, Phipps K, Stirling Y: Relation of fetal and infant growth to plasma fibrinogen and factor VII concentrations in adult life. *BMJ* 1992,304:148-152.
5. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM: Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 1993,36:62-67.
6. Valdez R, Athens MA, Thompson GH, Bradshaw BS, Stern MP: Birthweight and adult health outcomes in a biethnic population in the USA. *Diabetologia* 1994,37:624-631.
7. McCance DR, Pettitt DJ, Hanson RL, Jacobsson LT, Knowler WC, Bennett PH: Birth weight and non-insulin dependent diabetes: thrifty genotype, thrifty phenotype, or surviving small baby genotype? *BMJ* 1994,308:942-945.
8. Whincup P, Cook D, Papacosta O, Walker M: Birth weight and blood pressure: cross sectional and longitudinal relations in childhood. *BMJ* 1995,311:773-776.
9. Stein CE, Fall CH, Kumaran K, Osmond C, Cox V, Barker DJ: Fetal growth and coronary heart disease in south India. *Lancet* 1996,348:1269-1273.
10. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD: Birthweight, body-mass index in middle age, and incident coronary heart disease. *Lancet* 1996,348:1478-1480.
11. Barker DJ: Fetal origins of coronary heart disease. *BMJ* 1995,311:171-174.
12. Paneth N, Susser M: Early origin of coronary heart disease (the "Barker hypothesis"). *BMJ* 1995,310:411-412.
13. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ: Weight in infancy and death from ischaemic heart disease. *Lancet* 1989,2:577-580.
14. Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ: Early growth and death from cardiovascular disease in women. *BMJ* 1993,307:1519-1524.
15. Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJ: Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *BMJ* 1999,318:427-431.
16. Rich-Edwards JW, Stampfer MJ, Manson JE, Rosner B, Hankinson SE, Colditz GA, Willett WC, Hennekens CN: Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ* 1997,315:396-400.
17. Leon DA, Lithell HO, Vagero D, Koupilova I, Mohsen R, Berglund L, Lithell UB, McKeigue PM: Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. *BMJ* 1998,317:241-245.
18. Eriksson M, Tibblin G, Cnattingius S: Low birthweight and ischaemic heart disease. *Lancet* 1994,343:731.
19. Tanis BC, Van Den Bosch MA, Kemmeren JM, Cats VM, Helmerhorst FM, Algra A, Van Der Graaf Y, Rosendaal FR: Oral contraceptives and the risk of myocardial infarction. *N Engl J Med* 2001,345:1787-1793.
20. World Health Organization Low birth weight. A tabulation of available information. *WHO* Geneva, 1992 (WHO/MCH/92.2).
21. Bazaes RA, Alegria A, Pittaluga E, Avila A, Iniguez G, Mericq V: Determinants of insulin sensitivity and secretion in very-low-birth-weight children. *J Clin Endocrinol Metab*. 2004,89:267-272.
22. Hofman PL, Regan F, Jackson WE, Jefferies C, Knight DB, Robinson EM, Cutfield WS: Premature birth and later insulin resistance. *N Engl J Med*. 2004,351:2179-2186.
23. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD: Birthweight, adult risk factors and incident coronary heart disease: the Caerphilly Study. *Public Health* 1996,110:139-143.
24. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS: Fetal nutrition and cardiovascular disease in adult life. *Lancet* 1993,341:938-941.
25. Poulter NR: Birthweights, maternal cardiovascular events, and Barker hypothesis. *Lancet* 2001,357:1990-1991.
26. Martin H, Gazelius B, Norman M: Impaired acetylcholine-induced vascular relaxation in low birth weight infants: implications for adult hypertension? *Pediatr Res* 2000,47:457-462.
27. Goh KL, Shore AC, Quinn M, Tooke JE: Impaired microvascular vasodilatory function in 3-month-old infants of low birth weight. *Diabetes Care* 2001,24:1102-1107.

28. Leeson CP, Whincup PH, Cook DG, Donald AE, Papacosta O, Lucas A, Deanfield JE: Flow-mediated dilation in 9- to 11-year-old children: the influence of intrauterine and childhood factors. *Circulation* 1997,96:2233-2238.
29. Martin H, Hu J, Gennser G, Norman M: Impaired endothelial function and increased carotid stiffness in 9-year-old children with low birthweight. *Circulation* 2000,102:2739-2744.
30. Leeson CP, Kattenhorn M, Morley R, Lucas A, Deanfield JE: Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life. *Circulation* 2001,103:1264-1268.
31. Goodfellow J, Bellamy MF, Gorman ST, Brownlee M, Ramsey MW, Lewis MJ, Davies DP, Henderson AH: Endothelial function is impaired in fit young adults of low birth weight. *Cardiovasc Res* 1998,40:600-606.
32. McAllister AS, Atkinson AB, Johnston GD, McCance DR: Relationship of endothelial function to birth weight in humans. *Diabetes Care* 1999,22:2061-2066.
33. Smith GC, Pell JP, Walsh D: Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. *Lancet* 2001,357:2002-2006.
34. Oren A, Vos LE, Uiterwaal CS, Gorissen WH, Grobbee DE, Bots ML: Birth weight and carotid intima-media thickness: new perspectives from the atherosclerosis risk in young adults (ARYA) study. *Ann Epidemiol* 2004,14:8-16.
35. Forsen T, Eriksson J, Tuomilehto J, Reunanen A, Osmond C, Barker DJ: The fetal and childhood growth of persons who develop type 2 diabetes. *Ann Intern Med* 2000,133:176-182.
36. Yajnik C: Interactions of perturbations in intrauterine growth and growth during childhood on the risk of adult-onset disease. *Proc Nutr Soc* 2000,59:257-265.

3

DOES SUBFERTILITY EXPLAIN THE RISK OF POOR PERINATAL OUTCOME AFTER IVF AND OVARIAN HYPERSTIMULATION?

K. Kapiteijn
C.S. de Bruijn
E. de Boer
A.J.M. de Craen
C.W. Burger
F.E. van Leeuwen
F.M. Helmerhorst

Submitted

Introduction

Singleton pregnancies from assisted conception have a significantly higher risk of (very) low birth weight and (very) preterm birth as compared to naturally conceived controls, although the prevalence of very low birth weight and very preterm birth is low. Confounding factors such as maternal age and parity did not change this outcome[1]. Factors as social economic status, sex of the fetus, delivery date and site can probably not explain the difference[1,2]. However, history of subfertility, irrespective of infertility treatment, has also been found to be associated with perinatal death in a case-control study[3]. Jackson *et al.*[2] summarized the different trials and advised for future research in which treatment biases can be addressed.

Poor perinatal outcome was also observed in 263 singletons born of subfertile patients conceived after only controlled ovarian hyperstimulation (COHS) relative to 5,096 spontaneously conceived controls delivered in the same hospital and period; no difference was seen when the comparison was made between COHS and 162 IVF singletons[4]. After stratification for the number of years of involuntary childlessness, Källén *et al.*[5] still found a significant increased risk of preterm birth (<37 wks) and of low birth weight (<2500 g) in singletons conceived after just COHS as compared to naturally conceived singletons. Gaudoin *et al.*,[6] concluded that "infertility" should be added to the list of recognized factors associated with low birth weight by comparing 97 singletons whose subfertile mothers were treated with COHS & IUI with 35 singletons whose mothers were treated with COHS (although with normal reproductive health) as well as artificial insemination with donor sperm.

In the present study we investigate whether subfertility explains the poor perinatal outcome after assisted conception. We used data from a nation-wide historical cohort of 26,428 women treated for subfertility in the Netherlands between 1980 and 1995 (Klip *et al.*)[7]. Furthermore we tried to answer the question whether COHS with or without IVF adversely affects perinatal outcome.

The observation that birth weight of singletons conceived by implanting a cryopreserved embryo is significantly higher than birth weight after a fresh embryo transfer (ET)[8,9], suggests that the cryopreservation and thawing procedure might essentially differ from the IVF and ICSI procedure, in which the embryo(s) are directly and freshly transferred to the uterus. Since ET of cryopreserved embryos occurs predominantly in a natural cycle, one may hypothesize that COHS it self might influence uterine receptivity. Using the same database as mentioned above, we compared the birth weight and preterm birth of singletons conceived after transfer of thawed embryos with and without COHS.

Materials and methods

Study population

Data were obtained from an historical nation-wide cohort study (OMEGA study) of 26,428 women diagnosed with subfertility in all 12 Dutch IVF clinics between 1st of January 1980 and 1st of January 1995. Approval of the ethics committees of all institutions was obtained. Inclusion criteria were at least one year of subfertility[10] and an age older than 18 years at the time of admission to one of the clinics. Women were included in the IVF group when they had completed at least one treatment cycle with COHS and IVF before 1st of January 1995 (n=19,840). A group of 6,588 women unexposed to COHS, whose subfertility was diagnosed after 1980, was recruited from existing computerized databases of 4 out of the 12 clinics. All clinics provided a minimal data set with names, birth dates and addresses of all eligible women. After tracing of the current addresses of all women, they were contacted at their home address and were asked to fill in and return a questionnaire, as well as a written informed consent, asking each participant for permission for data abstraction from their medical records. From the initial 26,428 women, 1,105 women (4.2%) were not approachable for several reasons (for more details see Klip *et al.*[7]. Of the remaining 25,323 women nearly 67% agreed to participate in the study[11].

Some women delivered more than once during the study period. Initially, 29,148 pregnancies were reported in the returned questionnaires. As described in detail by Klip *et al.*[7], 12,148 pregnancies were directly excluded (intrauterine mortality n=10,815; pregnant at the time of returning questionnaire n=404; missing data n=929). This resulted in a total of 17,000 deliveries with a minimal gestational age of 24 weeks. This group consisted of 9,479 pregnancies following assisted reproductive techniques (IVF, ICSI, inseminations and fertility drug use not for IVF/inseminations), 5,862 pregnancies achieved with only IVF; 2,239 of them were IVF singletons. Eighty four singletons were recruited from the group of mothers who were treated with COHS (COHS-only). From the 7,521 subfertile controls, 6,343 were singletons with a full data set.

For the first comparison in this study between the IVF group, the subfertile group and the group with only ovarian hyperstimulation as treatment, pregnancies which resulted from transfer of frozen embryos were excluded. The remaining "ART deliveries" were divided into two groups. The first group consisted of singleton pregnancies which developed after ovarian hyperstimulation with IVF (IVF+COHS; n= 2,239). The second group was the control group and consisted of singleton pregnancies among women with a history of subfertility who conceived spontaneously (n=6,343). The

third group consisted of singleton pregnancies which developed after ovarian hyperstimulation without IVF (COHS-only, n=84).

From the OMEGA data base, 139 pregnancies were the result of cryo-preserved ET. In 66 cases the ET took place after COHS and/or ovulation induction with hCG (Stim+Cryo group). In 56 cases, only human choriongonadotrophin (hCG) was administered and 8 received human menopausal gonadotrophin (hMG) or clomiphene citrate alone or in combination with hCG. In 2 cases the specific type of COHS and/or ovulation induction was not known. In 73 cases (Stim–Cryo group), the ET was performed in an apparently ovulatory cycle or before progesterone administration.

In each participating clinic, research assistants specifically trained for data collection for the OMEGA study abstracted detailed information from the medical records. For each reported child, the questionnaire, completed by the study participants, provided detailed information on the maternal characteristics, method of conception, the duration of gestation in weeks, data of birth, gender and birth weight.

Definitions

Although the National Institute for Clinical Excellence[34] defines subfertility as failure to conceive after regular unprotected sexual intercourse for 2 years in the absence of known reproductive pathology, the definition currently used in the Netherlands during the period of the cohort followed the one given in the textbook Clinical gynaecologic endocrinology and infertility[10]: 'one year unprotected coitus without conception'.

Gestational age (duration of pregnancy) at birth in case of IVF pregnancies was determined by adding 14 days to the interval between LH administration and delivery. For the control pregnancies, it was calculated as the interval between the first day of the last menstrual period and delivery. International definitions were followed for preterm (<37 weeks), very preterm (< 32 weeks), low birth weight (<2500 g) and very low birth weight (<1500 g).

Statistics

Differences between groups were assessed by *t*-tests for continuous variables and by χ-square tests for ordinal variables. Multivariate logistic regression analysis was used to determine the odds ratios of (very) low birth weight and (very) preterm birth between the groups. Odds ratios were first adjusted for the confounders, maternal age and primiparity, and thereafter for each of the following potential confounders: BMI, race, education level, smoking, diabetes mellitus and sex of infant. Significance level was set at 5% two-tailed. Analyses were performed with SPSS 12.0.

Results

Complete perinatal data were obtained from 2,239 singleton IVF+COHS pregnancies, from 6,343 pregnancies in subfertile controls and from 84 COHS-only pregnancies.

Maternal characteristics (Table I)

In the IVF+COHS group the mean maternal age was significantly higher as compared to COHS-only and subfertile controls. The proportion of women with pre-existing diabetes mellitus (for which women used medication during pregnancy) was found significantly higher in the IVF+COHS group as compared to the subfertile control group (Table Ia). The mean BMI was significantly lower and the education level was significantly higher in the IVF+COHS group as compared to the control group. In the COHS-only group, primiparity was significantly more prevalent as compared to the IVF+COHS group and the subfertile controls, whereas there were significantly less women who smoked as compared to the IVF+COHS and subfertile control group. To rule out the possibility that the women with multiple births influenced our results, we also made a comparison (Table 1b) in which we only looked at the first pregnancies of women. No significant changes in the maternal characteristics were seen.

Table Ia. Maternal characteristics at the onset of and during pregnancy in IVF+COHS, subfertile control and COHS-only pregnancies. Values are means (±SD) or percentages.

	IVF+COHS (n=2,239)	Subfertile controls (n=6,343)	COHS-only (n=84)
Age (years)	34.2 (3.7)*)	30.7 (6.0)	31.9 (4.3)
Height (cm)	168.8 (6.5)*)	168.4 (6.5)	168.5 (5.4)
Weight (kg)	67.6 (11.2)*)	68.3 (12.0)	68.4 (12.7)
BMI[1]	23.7 (3.7)*)	24.1 (4.1)	24.1 (4.2)
Caucasian (%)	97.9	97.8	98.8
Low education level[2] (%)	46.6*)	50.2	43.4
Primiparous (%)	55.1*)	44.1	66.7
Smoking[3] (%)	64.1	65.7	51.8
Pre-existent DM[4](%)	1.1*)	0.5	1.2

[1] body mass index (weight (kg) divided by height (m) squared)
[2] only primary school
[3] during pregnancy
[4] diabetes mellitus for which medication was needed also during pregnancy
*) $p<0.05$, IVF+COHS group versus control group
COHS: controlled ovarian hyperstimulation

Table Ib. Maternal characteristics at the onset of and during first pregnancy of subfertile controls and of women receiving IVF+COHS or COHS-only. Values are means (±SD) or percentages.

	IVF+COHS (n=1,576)	Subfertile controls (n=3,754)	COHS-only (n=68)
Age (years)	33.9 (3.7)	29.6 (5.9)	31.2 (4.1)
Height (cm)	169 (7)	169 (7)	168 (6)
Weight (kg)	67.8 (11.2)	68.4 (12.1)	68.6 (13.7)
BMI[1]	23.8 (3.8)	24.0 (4.1)	24.2 (4.5)
Caucasian (%)	97.3	97.7	98.5
Low education level[2] (%)	46.8	50.6	44.2
Primiparous (%)	70.4	68.5	77.9
Smoking[3] (%)	68.3	57.1	80.9
Pre-existent DM[4](%)	1.0	0.5	1.5

[1] body mass index (weight (kg) divided by height (m) squared)
[2] only primary school
[3] during pregnancy
[4] diabetes mellitus for which medication was needed also during pregnancy
COHS: controlled ovarian hyperstimulation

Table II. Birth weight and gestational age of singletons conceived after IVF compared with naturally conceived singletons of subfertile women

		IVF+COHS (n=2,239)	Subfertile controls (n=6,343)		
				OR (95%CI)	OR_{adj} (95% CI)[a]
Birth weight (g)					
mean	g (SD)	3,199 (664)*	3,351 (600)		
> 2500	n (%)	1,955 (87.3)	5,848 (92.2)	1	1
1500 – 2500	n (%)	223 (10.0)	429 (6.8)	1.6 (1.3 – 1.8)	1.7 (1.4 – 2.0)
< 1500	n (%)	61 (2.7)	66 (1.0)	2.8 (1.9 – 3.9)	2.7 (1.8 – 4.0)
Gestational age (wks)					
mean	wks (SD)	38.9 (2.5)*	39.4 (2.2)		
≥ 37	n (%)	1,972 (88.0)	5,842 (92.1)	1	1
32 – 37	n (%)	218 (9,8)	428 (6,7)	1.5 (1.3 – 1.8)	1.6 (1.3 – 1.9)
< 32	n (%)	49 (2.2)	73 (1.2)	2.0 (1.4 – 2.9)	2.2 (1.5 – 3.3)

* $p<0.05$
[a] Adjusted for maternal age and primiparity
COHS: controlled ovarian hyperstimulation

Perinatal outcome

The mean birth weight and the mean gestational age of the singletons born to the IVF+COHS group were significantly lower and shorter, respectively, as compared to children born to the subfertile control group (Table II). The ORs for of very preterm birth and very low birth weight in the IVF+COHS group in comparison with the subfertile controls were 2.0 and 2.8 respectively, while the ORs of the preterm birth and low birth weight groups were increased to a lesser extent: 1.5 and 1.6 respectively. Only minor changes in the aforementioned ORs were seen after adjustment for potential confounders (maternal age and primiparity and also BMI, race, education, smoking, diabetes mellitus and sex of infant) that may influence birth weight and/or gestational age. When we excluded the multiple births of women and only looked at their first pregnancies, no material changes in the ORs were seen (data not shown). The OR for very low birth weight in the COHS-only group in comparison with the subfertile controls was 3.5 (95%CI 1.1-11.4); however after adjustment for maternal age and primiparity, the association became slightly weaker (Table IIIa).

ORs for preterm birth and low birth weight in the IVF+COHS group compared to the COHS-only group were not significantly different (Table IIIb).

Cryopreservation

Complete perinatal data were obtained from 66 singleton pregnancies derived from cryopreserved ET after COHS (Stim+Cryo group) and from 73 singleton pregnancies in which the ET was performed in a natural cycle (Stim-Cryo group). When we compared maternal characteristics of the Cryo+ and Cryo- group, no significant differences were found: mean age 34.1 yr vs 33.8 yr, mean height 168.3 cm vs 167.5 cm, mean weight 67.7 kg vs 65.2 kg, mean BMI 24.0 vs 23.3, mean % Caucasian 97.0 vs 98.6, mean % women who enjoyed only primary school 43.9 vs 47.9, mean % primiparous women 36.4 vs 41.1, mean % of women who smoked during pregnancy 37.9 vs 30.1 and in both groups nobody indicated to suffer from pre-existent diabetes mellitus.

The group Stim+Cryo treated women did not have a significantly higher risk of singleton birth with a low birth weight and/or of preterm delivery as compared to when ET had taken place in a natural or progesterone treated cycle (Table IIIc). Correction for the already mentioned confounders did not materially change the results. In the Stim+Cryo group significantly less boys were born (48.5% versus 65.8%, $p=0.03$).

Table IIIa. Birth weight and gestational age of singletons conceived after controlled ovarian hyperstimulation only compared with naturally conceived singletons of subfertile women

		COHS-only (n=84)	Subfertile controls (n=6,343)		
				OR (95%CI)	OR_{adj} (95% CI)[a]
Birth weight (g)					
mean	g (SD)	3,226 (597)	3,351 (600)		
> 2500	n (%)	76 (90.5)	5,848 (92.2)	1	1
1500 – 2500	n (%)	5 (6.0)	429 (6.8)	0.9 (0.4 – 2.2)	0.9 (0.4 – 2.2)
< 1500	n (%)	3 (3.5)	66 (1.0)	3.5 (1.1 – 11.4)	3.1 (0.9 – 10.2)
Gestational age (wks)					
mean	wks (SD)	39.6 (2.3)	39.4 (2.2)		
≥ 37	n (%)	79 (94.0)	5,842 (92.1)	1	1
32 – 37	n (%)	3 (3.6)	428 (6.7)	0.5 (0.2 – 1.7)	0.5 (0.2 – 1.7)
< 32	n (%)	2 (2.4)	73 (1.2)	2.0 (0.5 – 8.4)	1.9 (0.5 – 8.0)

[a] Adjusted for maternal age and primiparity
COHS: controlled ovarian hyperstimulation

Table IIIb. Birth weight and gestational age of singletons conceived after controlled ovarian hyperstimulation and IVF compared with singletons conceived after controlled ovarian hyperstimulation alone

		IVF+COHS (n=2,239)	COHS-only (n=84)		
				OR (95%CI)	OR_{adj} (95% CI)[a]
Birth weight (g)					
mean	g (SD)	3,199 (664)	3,226 (597)		
> 2500	n (%)	1,955 (87.3)	76 (90.5)	1	1
1500 – 2500	n (%)	223 (10.0)	5 (6.0)	1.7 (0.7 – 4.3)	1.7 (0.7 – 4.4)
< 1500	n (%)	61 (2.7)	3 (3.5)	0.8 (0.2 – 2.6)	0.8 (0.3 – 2.7)
Gestational age (wks)					
mean	wks (SD)	38.9 (2.5)	39.6 (2.3)		
≥ 37	n (%)	1,972 (88.0)	79 (94.0)	1	1
32 – 37	n (%)	218 (9.8)	3 (3.6)	2.9 (0.9 – 9.3)	2.7 (0.9 – 8.7)
< 32	n (%)	49 (2.2)	2 (2.4)	1.0 (0.2 – 4.1)	0.9 (0.2 – 3.8)

[a] Adjusted for maternal age and primiparity
COHS: controlled ovarian hyperstimulation

Table IIIc. Birth weight and gestational age of singleton pregnancies after ET of thawed embryo's in a treated cycle (Stim+Cryo) versus an untreated cycle (Stim-Cryo)

		Stim+Cryo (n=66)	Stim-Cryo (n=73)		
				OR (95%CI)	OR_{adj} (95% CI)[a]
Birth weight (g)					
mean	g (SD)	3,396 (621)	3,319 (641)		
> 2500	n (%)	62 (93.9)	67 (91.8)	1	1
1500 – 2500	n (%)	3 (4.6)	5 (6.8)	0.7 (0.2 – 2.9)	0.7 (0.2 –2.9)
< 1500	n (%)	1 (1.5)	1 (1.4)	1.1 (0.1 – 16.7)	1.0 (0.1 – 16.7)
Gestational age (wks)					
mean	wks (SD)	39.2 (2.3)	39.2 (2.1)		
≥ 37	n (%)	59 (89.4)	68 (93.1)	1	1
32 – 37	n (%)	6 (9.1)	4 (5.5)	1.7 (0.5 – 6.3)	1.7 (0.5 – 6.3)
< 32	n (%)	1 (1.5)	1 (1.4)	1.2 (0.1 – 20.0)	1.1 (0.1 – 16.7)
Sex of infant					
male	(%)	48.5*)	65.8		

* $p<0.05$
[a] Adjusted for maternal age and primiparity
COHS: controlled ovarian hyperstimulation

Discussion

In this large database of Dutch IVF clinics, singleton IVF pregnancies have significantly worse perinatal outcomes than spontaneously conceived pregnancies in subfertile women. The risk is more pronounced for very preterm and very low birth weight than for preterm and low birth weight. The estimates did not materially change after adjustment for maternal age, primiparity, or other potential confounders.

Randomization is the proper way to evaluate the effect of treatment for subfertility on the perinatal outcome[12]; however, it is difficult and unethical[2] to conduct. Alternative methodological approaches have been followed with different outcomes. In a population-based case-control study, Draper *et al.*[3] showed that history of subfertility, irrespective of treatment, increased the risk of perinatal death, while Basso and Olsen[13] found that the odds of neonatal (and not intra-uterine) death among firstborn singletons was significantly increased in the group of non-treated mothers with >12 months of subfecundity, relative to mothers who became pregnant within 3 months. McElrath *et al.*[14], using logistic regression models, found the risk of very low birth weight among

subfertile, non-treated women to be 1.4 (95% CI 1.1-1.9) and among subfertile, treated women 2.6 (95% CI 2.1-3.2) compared to a national US control group gathered in 1988. Both estimates were slightly lower, but still significantly increased when they were adjusted for effects of multiple gestation, maternal age and a history of miscarriage.

Another approach is to compare different treatments among subfertile couples, with the difficulty of the difference in treatment. Olivennes *et al.*[4] found no difference in the prevalence of (very) preterm and (very) low birth weight among 162 IVF and 263 COHS singletons, Bonduelle *et al.*[15] showed the same results among 1499 ICSI and 1556 IVF singletons. However, the analysis of Ombelet *et al*.[16] showed only a significantly higher risk of preterm birth among 3974 IVF singletons relative to 1655 ICSI singletons. The authors hypothesize that the indication for ICSI is predominantly a male factor. Remarkable is that the two latter studies have been conducted in the relatively circumscriptive, Dutch speaking part of Belgium. The Ombelet study[16] gathered all deliveries in Flanders in the period 1997-2003, whereas the Bonduelle study[15] collected the data of one reproductive centre in the period 1991-1999 for ICSI and 1983-1999 for IVF. Part of these data has been included in the Ombelet study[16]. An explanation for the difference has not been offered by Ombelet *et al.*[16] In the Wang study[17] preterm birth was significantly more often observed in the high technology group (IVF, ICSI, GIFT) than in the low technology group (IUI, donor insemination), with ORs of 2.39 and 1.50, respectively, compared to naturally conceived controls. Two similar studies comparing IVF with IUI singletons[18,19] found no differences in the prevalence of preterm birth and low birth weight.

Our study might suffer from some information bias through the use of a mailed questionnaire to collect perinatal outcome and might be limited by not taking into account pregnancy complications, fetal malformations and a history of previous pregnancy loss, factors that may be associated with adverse pregnancy outcome. However it is unlikely that the IVF+COHS group would report systematically different from the subfertile controls. Therefore we conclude, also based on the data from the above mentioned literature that subfertility might explain part of the association between assisted conception and poor perinatal outcome of singletons, but that still there remains an important effect of assisted reproduction it self.

Is the controlled ovarian hyperstimulation, as part of the assisted technology methods, the culprit or the technique itself as suggested by Olivennes *et al.*[4]? In our study the risk estimates comparing singletons conceived after COHS & IVF versus singletons conceived after COHS only did not differ significantly. Preterm birth and low weight birth were more likely to occur among singletons conceived by transfer of fresh embryos, relative to those conceived by with transfer of frozen embryos, as reported by Wang *et al.*[9] in a retrospective cohort study of Australian data of infants conceived through assisted reproduction. We are not informed in their study whether the transfer of thawed

embryo(s) was performed in a natural or stimulated cycle. Unfortunately, in our study we were not able to test the hypothesis that COHS prior to embryo transfer affects uterine receptivity due to the small number of patients in this database. As female-factor subfertility increased the likelihood of preterm birth and low birth weight significantly more than male-factor subfertility, Wang *et al*.[9] suggested that uterine receptivity might offer us an biological plausibility for the phenomenon, however no difference in prevalence of preterm birth and low birth weight among singletons were seen born after ICSI, representing the male subfertility factor[15,16].

If ovarian stimulation by itself has a negative effect on the pregnancy outcome it may influence oocyte/embryo quality, resulting in impaired implantation and embryonic/fetal development[20]. Other studies suggest that ovarian stimulation is rather associated with an unbalanced endometrium and/or oviductal environment[21,22,23,24]. Supra-physiologic concentrations of estradiol and progesterone during ovarian stimulation may modulate growth factors, cell adhesion molecule profiles, steroid receptors and expression of pinopodes in the endometrium[25,26,28], influencing endometrial receptivity[25,27,28].

Sibug *et al*.[29,30] suggested that the effect of ovarian stimulation on pregnancy outcome might be explained by the modulation of vascular endothelial growth factor (VEGF) affecting angiogenesis during implantation and placentation[31,32,33].

In conclusion, our study shows that the association between assisted conception and poor perinatal outcome can not be explained by subfertility.

Acknowledgements

The OMEGA-project group included the following persons: M.Kortman and E.R.te Velde (University Medical Center Utrecht), N.Macklon (Academic Hospital Dijkzigt, Rotterdam), C.A.M.Jansen (Diaconessenhuis, Voorburg), R.A.Leerentveld (Isala clinics, Zwolle), W.N.P.Willemsen (Academic Hospital Nijmegen, St. Radboud), R.Schats (Academic Hospital Free University, Amsterdam), N.Naaktgeboren and F.M.Helmerhorst (Leiden University Medical Center), R.S.G.M.Bots (St. Elisabeth Hospital, Tilburg), A.H.M. Simons (Academic Hospital Groningen), H.V.Hogerzeil (Academic Medical Center, Amsterdam), J.L.H.Evers (Academic Hospital Maastricht), P.A.van Dop (Catharina Hospital, Eindhoven).

References

1 Helmerhorst FM, Perquin DA, Donker D and Keirse MJ (2004) Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. BMJ 328,261-265.
2 Jackson RA, Gibson KA, Wu YW and Croughan MS (2004) Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. Obstet Gynecol 103,551-563.
3 Draper ES, Kurinczuk JJ, Abrams KR and Clarke M (1999) Assessment of separate contributions to perinatal mortality of infertility history and treatment: a case-control analysis. Lancet 353,1746-1749.
4 Olivennes F, Rufat P, Andre B, Pourade A, Quiros MC and Frydman R (1993) The increased risk of complication observed in singleton pregnancies resulting from in-vitro fertilization (IVF) does not seem to be related to the IVF method itself. Hum Reprod 8,1297-1300.
5 Källén B, Olausson PO and Nygren KG (2002) Neonatal outcome in pregnancies from ovarian stimulation. Obstet Gynecol 100,414-419.
6 Gaudoin M, Dobbie R, Finlayson A, Chalmers J, Cameron IT and Fleming R (2003) Ovulation induction/intra-uterine insemination in infertile couples is associated with low-birth-weight infants. Am J Obstet Gynecol 188,611-616.
7 Klip H, Burger CW, de Kraker J and van Leeuwen FE (2001) Risk of cancer in the offspring of women who underwent ovarian stimulation for IVF. Hum Reprod 16,2451-2458.
8 Wennerholm UB, Bergh C, Hamberger L, Westlander G, Wikland M and Wood M (2000) Obstetric outcome of pregnancies following ICSI, classified according to sperm origin and quality. Hum Reprod 15,1189-1194.
9 Wang YP, Sullivan EA, Black D, Eng JDM, Bryant J and Chapman M (2005) Preterm birth and low birth weight after assisted reproductive technology-related pregnancy in Australia between 1996 and 2000. Fertil Steril 83,1650-1658.
10 Speroff L, Glass RH, Kase NG. 6 ed.1999 Lippincott Williams & Wilkins, Baltimore, USA, page 1013.
11 De Boer EJ, den Tonkelaar I, Burger CW and van Leeuwen FE; OMEGA Project Group (2005) Validity of self-reported causes of subfertility. Am J Epidemiol 15,978-986.
12 Buck Louis GM, Schisterman EF, Dukic VM and Schieve LA (2005) Research hurdles complicating the analysis of infertility treatment and child health. Hum Reprod 20,12-18.
13 Basso O and Olsen J (2005) Subfecundity and neonatal mortality: longitudinal study within the Danish national birth cohort. BMJ 330,393-394.
14 McElrath T and Wise PH (1997) Fertility therapy and the risk of very low birth weight. Am J Obstet Gynecol 90,600-605.
15 Bonduelle M, Liebaers I, Deketelare V, Derde MP, camus M, Devroey P, van Steirteghem (2002) Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999). Hum Reprod 17,671-694.
16 Ombelet W, Cadron I, Gerris J, de Sutter P, Bosmans E, Martens G, Ruyssinck G, Defoort P, Molenberghs G and Gyselaers W. (2005) obstetric and perinatal outcome of 1655 ICSI and 3974 INF singleton and 1102 ICSI and 2901 IVF twin births: a comparative analysis. Reprod Biomed Online 11,76-85.
17 Wang JX, Norman RJ and Kristiansson P (2002) The effect of various infertility treatments on the risk of preterm birth. Hum Reprod 17,945-949.
18 De Sutter P, Veldeman L, Kok P, Szymczak N, van der Elst J, Dhont M. (2005) Comparison of outcome of pregnancy after intra-uterine insemination (IUI) and IVF. Hum Reprod 20,1642-1646.
19 Nuojua-Huttunen S, Gissler M, Martikainen H and Tuomivaara L (1999) obstetric and perinatal outcome of pregnancies after intrauterine insemination. Hum Reprod 14,2110-2115.
20 Rao CV (2001) Tropic effects of LH and hCG on early pregnancy events in women's reproductive tract. Early Pregnancy 5,18-19.
21 Ertzeid G, Storeng R and Lyberg T (1993) Treatment with gonadotropins impaired implantation and fetal development in mice. J Assist Reprod Genet 10,286-291.
22 Fossum GT, Davidson A and Paulson RJ (1989) Ovarian hyperstimulation inhibits embryo implantation in the mouse. J In Vitro Fert Embryo Transf 6,7-10.
23 Walton EA, Huntley S, Kennedy TG and Armstrong DT (1982) Possible causes of implantation failure in superovulated immature rats. Biol Reprod 27,847-852.
24 Walton EA and Armstrong DT (1983) Oocyte normality after superovulation in immature rats. J Reprod Fertil 67,309-314.
25 Kolb BA, Najmabadi S and Paulson RJ (1997) Ultrastructural characteristics of the luteal phase endometrium in patients undergoing controlled ovarian hyperstimulation. Fertil Steril 67,625-630.
26 Macklon NS and Fauser BC (2000) Impact of ovarian hyperstimulation on the luteal phase. J Reprod Fertil Suppl 55,101-108.

27 Paulson RJ, Sauer MV and Lobo RA (1990) Embryo implantation after human in vitro fertilization: importance of endometrial receptivity. Fertil Steril 53,870-874.
28 Paulson RJ, Sauer MV and Lobo RA (1997) Potential enhancement of endometrial receptivity in cycles using controlled ovarian hyperstimulation with antiprogestins: a hypothesis. Fertil Steril 67,321-325.
29 Sibug RM, Helmerhorst FM, Tijssen AM, de Kloet ER and de Koning J (2002) Gonadotrophin stimulation reduces VEGF(120) expression in the mouse uterus during the peri-implantation period. Hum Reprod 17,1643-1648.
30 Sibug RM, de Koning J, Tijssen AM, de Ruiter MC, de Kloet ER and Helmerhorst FM (2005) Urinary gonadotrophins but not recombinant gonadotrophins reduce expression of VEGF120 and its receptors flt-1 and flk-1 in the mouse uterus during the peri-implantation period. Hum Reprod 20,649-656.
31 Kapiteijn K, Koolwijk P, Van Der Weiden RM, Helmerhorst FM, Kooistra T and van Hinsbergh VW (2001) Steroids and cytokines in endometrial angiogenesis. Anticancer Res 21,4231-4242.
32 Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van der Vecht B, Helmerhorst FM and van Hinsbergh VW (2001) Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. J Clin Endocrinol Metab 86,3359-3367.
33 Smith SK (1998) Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 4,509-519.
34 National Institute for Clinical Excellence, NHS (2004) Fertility: assessment and treatment for people with fertility problems. London, RCOG Press. http://www.rcog.org.uk/index.asp?PageID=696

4

ENHANCED ANGIOGENIC CAPACITY AND UROKINASE-TYPE PLASMINOGEN ACTIVATOR EXPRESSION BY ENDOTHELIAL CELLS ISOLATED FROM HUMAN ENDOMETRIUM

P. Koolwijk
K. Kapiteijn
B. Molenaar
E. van Spronsen
B. van der Vecht
F.M. Helmerhorst
V.W.M. van Hinsbergh

Journal of Clinical Endocrinology & Metabolism 2001;86,3359-3367

Introduction

Human reproduction depends on the rapid cyclical development of a receptive maternal environment necessary for implantation and placentation. Indispensable for this physiological process is angiogenesis, the forming of new blood vessels. It is required for supporting the proliferation and differentiation of glandular and surface epithelial cells and stromal cells, of which the endometrium is mainly composed[1].

Studies have indicated that there are three successive episodes of physiological angiogenesis in the endometrium during the menstrual cycle[2]. The first episode can be seen as post-menstrual repair and occurs during the early proliferative phase, the second episode takes place during the mid-proliferative phase under the influence of estradiol, and the third occurs during the estradiol- and progesterone-mediated secretory phase, when the coiled arteries grow. Together with the changes in vascular permeability throughout the menstrual cycle a transformation of a thin, dense endometrium into a thick, highly edematous secretory endometrium takes place[3].

Regulation of the outgrowth of new vessels is the result of a delicate balance between stimulators and inhibitors and involves several steps. After stimulation of the endothelial cells by angiogenic factors, the basement membrane is degraded by proteolytic enzymes, in particular matrix-degrading metalloproteinases (MMPs) and enzymes of the plasminogen activator system[4]. The cells will then invade, migrate and proliferate under the influence of angiogenic factors into the underlying interstitial matrix and will form new capillary structures[5,6]. It has been suggested that angiogenesis in the endometrium may occur by a process of elongation and expansion of pre-existing vessels[7], a process that differs from the traditional concept of angiogenesis[5,6].

It is generally assumed that urokinase-type plasminogen activator (u-PA) and its inhibitor, the plasminogen activator inhibitor I (PAI-1), are involved in regulation of the first steps of angiogenesis, *i.e.* local proteolytic remodeling of matrix proteins and migration of endothelial cells[6,8,9]. U-PA converts plasminogen into the broadly acting serine protease plasmin, which, in turn, is able to both degrade matrix proteins and activate several MMPs[10-12].

Studies have shown organ-specific characteristics among microvascular endothelial cells[13]. Physiology and pathology involving the endothelium could therefore be best addressed by studies of endothelial cells of the affected organ. As the endometrium is a tissue unique for its cyclic destruction and rapid regeneration of blood vessels, the angiogenic behavior of its endothelial cells is expected to differ from that of endothelial cells in other tissues (e.g. human microvascular foreskin endothelial cells (hFMVEC)). To date, only a few reports describe the isolation and characterization of endothelial cells of the endometrium[14-16]. These isolated human endometrial microvascular endothelial cells

(hEMVEC) express estradiol and progesterone receptors and display an enhanced expression of the VEGF receptor type 2 (VEGFR-2)[14]. Furthermore, the expression of extracellular matrix proteins elastin, collagens and fibronectin by hEMVEC was not detectable, whereas endothelial cells from the human umbilical vein (HUVEC) do express these proteins[15].

The aim of this study was to examine the growth characteristics of hEMVEC and to study the fibrinolytic capacity of these cells and their ability to form capillary-like tubular structures in a three-dimensional (3-D) fibrin matrix. A comparison was made with hFMVEC and HUVEC.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and medium 199 (M199) with or without phenol red, and supplemented with 20 mM HEPES was obtained from BioWhittaker (Ver viers, Belgium); new born calf serum (NBCS) and collagenase type II was obtained from Life Technologies (Grand Island, NY). Tissue culture plastics came from Costar/Corning (Cambridge, MA) and Falcon (Becton Dickinson, Bedford, MA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al*.[17] Human serum was obtained from a local blood bank and was prepared from fresh blood from 10-20 healthy donors, pooled and stored at 4°C. It was heat-inactivated before use. Heparin and thrombin was obtained from Leo Pharmaceutics Products (Weesp, the Netherlands). Human fibrinogen came from Chromogenics AB (Mölndal, Sweden). Dr. H. Metzner and Dr. G. Seemann (Aventis Behring, Marburg, Germany) generously provided factor XIII (Fibrogammin-P). Fibronectin was a gift from Dr. J. van Mourik (Sanquin, Amsterdam, The Netherlands). In addition, the mouse antihuman CD31 antibodies (clone CLB-HEC/75) and human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Recombinant human vascular endothelial growth factor (VEGF-A) and human placenta growth factor-2 (PlGF-2) were commercially obtained from RELIATech (Braunschweig, Germany) and tumor necrosis factor-α (TNFα), containing 2.45 x 10^7 U/mg protein and less than 40 ng lipopolysacharide/mg protein, a gift from Dr. J. Travernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ). Tosyl-activated Dynabeads M450 and goat antimouse IgG coated Dynabeads were from Dynal AS (Oslo, Norway). Ulex europaeus agglutinin-1 (UEA-1) was from Sigma Chemical Co. (St. Louis, USA), and UEA-1 conjugated with fluorescein isothiocyanate (UEA-1-FITC) was from Vector Laboratories (Burlingame, CA). Monoclonal antibodies (mAbs) against cytokeratin 8 (mAb M20) and cytokeratin 18 (mAb M9) were gifts from Dr. G. van Muijen (Dept. Pa-

thology, Academic Hospital Nijmegen, The Netherlands), and the u-PA receptor-blocking mAb H-2 from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany)[18]. Rabbit polyclonal anti-u-PA antibodies were prepared in our laboratory. Mouse mAb against smooth muscle cell actin was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). Rabbit antihuman Von Willebrand Factor (vWF) antibodies, FITC-conjugated swine anti-rabbit Ig, FITC-conjugated rabbit antimouse Ig and horseradish peroxidase (HRP)-conjugated goat antirabbit Ig came from Dako Immunoglobulins (Glostrup, Denmark). The rabbit polyclonal antibodies specific for u-PA were prepared in our laboratory[19].

Complementary DNA (cDNA) probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.02 kb fragment of the human u-PA cDNA[20], a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (provided by Dr. R Offringa, Leiden University, Leiden, The Netherlands), a 1.05 kb fragment of the human VEGFR-1 cDNA[21], and a 1.4 kb fragment of the human VEGFR-2 cDNA[22].

Cell culture

HUVEC and hFMVEC were isolated and characterized as previously described[23,24]. The HUVEC and hFMVEC were cultured on fibronectin- or gelatin-coated dishes in M199 supplemented with 20 mM HEPES (pH 7.3), 10% human serum, 10% heat-inactivated NBCS, 150 mg/mL ECGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin (*i.e.* culture medium). HEMVEC were isolated from endometrial tissue as described below and maintained in the above-described culture medium supplemented with 10% human serum and 5 ng/mL VEGF-A (*i.e.* hEMVEC culture medium). Cells were cultured on fibronectin-coated wells at 5% CO_2/95% air until confluence was reached and were subsequently detached with 0.05% trypsin/0.025% ethylenediamine tetraacetate (EDTA) and transferred into fibronectin-coated or gelatin-coated dishes at a split ratio of 1:3. Fresh medium was given three times a week, twice at 2-day intervals and once after a weekend interval. All of the experiments described below were performed in M199 and 20% human serum with HUVEC between passage 1-3, hFMVEC between passage 9-11, and hEMVEC between passage 3-7, respectively.

Isolation and purification of hEMVEC

Endometrial tissue was obtained from pre-menopausal women who had had their uteri removed for benign pathology. All of the patients gave their informed consent according

to the guidelines of the Medical Ethical Review Boards of the Leiden University Medical Center (Leiden, The Netherlands), Bronovo Hospital (The Hague, The Netherlands), and St. Franciscus Gasthuis (Rotterdam, The Netherlands). After removal of the uterus, the endometrial tissue was scraped off and stored into ice-cold storage buffer (140 mM NaCl, 4 mM KCl, 11 mM D-glucose, 10 mM HEPES and 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.3) at 4°C overnight. The endometrium was minced and incubated in M199/penicillin/streptomycin containing 0.2% collagenase type II at 37°C for two h. Adding the same amount of culture medium stopped the reaction, and all remaining tissue was dissolved by powerful resuspension, resulting in a homogenous solution. After centrifugation (1200 rpm for 5 min at room temperature) the pellet obtained was resuspended in culture medium, and transferred into a fibronectin-coated culture dish. Two to four h later, the nonadhered cells were removed, and the adherent cells were cultured in hEMVEC culture medium.

The primary heterogeneous cell population was grown until near confluence before selection of the endothelial cells using UEA-1-coated Dynabeads. After detachment using trypsin and centrifugation, the cells were resuspended in M199 containing 0.1% HSA with the UEA-1-coated beads (20 beads / target cell). A 15- to 30-min end-over-end rotation was performed at 4°C before the cells that bound to the beads were selected by the use of a magnet (Dynal). The positively selected cell population was cultured in hEMVEC culture medium until confluence and then further isolated using mouse antihuman CD31 antibodies and goat antimouse IgG-coated Dynabeads. Trypsinized cells were incubated with antihuman CD31 antibodies (2 μg/mL in M199/0.1% HSA) for 30 min and kept on ice while being stirred occasionally. The nonbound antibodies were washed away with M199/0.1% HSA before the addition of the goat antimouse IgG-coated Dynabeads. After 15-30 min of incubation with the beads at 4°C, the cells were separated by the use of a magnet. After this selection the CD31-positive cells were cultured in hEMVEC culture medium in fibronectin-coated culture dishes till confluence.

The isolation procedure with anti-CD31 antibodies and antimouse Dynabeads was repeated until a homogeneous culture of endometrial endothelial cells was obtained (as determined after immunofluorescent characterization; see below).

Characterization of the isolated hEMVEC

Immunofluorescent characterization of the hEMVEC was performed on cell monolayers that had been cultured on special optics 96-wells black plates with an ultra-thin clear bottom (Costar/Corning). After washing with M199 containing penicillin/streptomycin, the cells were fixated by the addition of 80% (vol/vol) acetone or 4% formaldehyde for

10 min. The cells were washed with PBS before they were incubated for 30 min with various primary monoclonal or polyclonal antibodies diluted in PBS and 0.3% HSA (PBS/HSA). The control wells were incubated with PBS/HSA only. After washing with PBS/HSA, the cells were incubated with the appropriate second antibody, either FITC-labeled rabbit antimouse (50 μg/mL in PBS/HSA) or FITC-labeled swine antirabbit IgG (20 μg/mL in PBS/HSA).

Incorporation of [^{3}H]thymidine

Incorporation of [^{3}H]thymidine in DNA was determined as the measurement of endothelial cell proliferation. Confluent cultures of endothelial cells were detached by trypsin/EDTA solution and allowed to adhere and spread at a density of 10^4 cells/cm^2 on gelatin-coated dishes in M199-HEPES medium supplemented with 10% heat-inactivated NBCS and penicillin/streptomycin for 18 h. Then the cells were stimulated with bFGF, VEGF-A, or PlGF-2. After an incubation period of 42 h, a tracer amount of [^{3}H]thymidine (0.5 μCi/2 cm^2 well, added in a 10 μL volume) was added to the wells and the cells were incubated for another 6-h period. Subsequently, the cells were washed with PBS and fixated with 100% methanol, ^{3}H-labeled DNA was precipitated in 5% trichloroacetic-acid, and the cells were dissolved in 0.5 mL (0.3 mol/L) NaOH and counted in a liquid scintillation counter. The stimulation index was calculated as follows:

$$\text{Stimulation index} = \frac{(\text{dpm}_{\text{stimulated condition}}) - (\text{dpm}_{\text{background}})}{(\text{dpm}_{\text{control condition}}) - (\text{dpm}_{\text{background}})}$$

Determination of specific u-PA binding

Determination of specific u-PA binding was determined as previously described by Kroon *et al*.[25] In short, cells were incubated for 10 min on ice with 50 mmol/L glycine/HCl buffer (pH 3.0) to remove receptor-bound endogenous u-PA. Subsequently, the cells were incubated on ice with 8 nmol/L ^{125}I-labeled diisopropylfluorophosphate-treated (DIP)-u-PA in endothelial-cell conditioned medium (M199 supplemented with 1% human serum albumin, conditioned for 24 h) for 3 h. After the incubation period, unbound ligand was removed by extensive washing with ice-cold M199. Cell-bound ligand was solubilized with 0.3 mol/L NaOH, and the radioactivity was determined in a γ-counter (Cobra Auto γ, Packard, Meriden, CT). Specific binding was calculated by the subtraction of nonspecific binding from the total binding.

Northern blotting

Total ribonucleic acid (RNA) from hFMVEC and hEMVEC (30 cm^2/condition) was isolated 8 and 24 h after stimulation using the isothiocyanate/phenol acid extraction method described by Chomczynski *et al*.[26] The RNA was dissolved in formamide, and the concentration was determined spectrophotometrically. Equal amounts (7.5 μg) of RNA were separated on a formaldehyde/agarose gel. Subsequently, the separated RNA was transferred to a Hybond-N membrane through capillary force according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Arlington Heights, IL). Hybridization was performed in 7% (wt/vol) SDS, 1 mmol/L EDTA, and 0.5 mol/L NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2) overnight at 63°C with 25 ng of probe labeled with a random primer (Megaprime kit, Amersham Pharmacia Biotech). Thereafter the Hybond membrane was washed twice for 20 min each time with 2x SSC/1% SDS (wt/vol) and three times with 1x SSC/1% SDS (SSC contains 0.15 mol/L NaCl, 0.015 mol/L sodium citrate). Finally, the filters were exposed to a phosphoimager screen and analyzed using a computer.

Enzyme-linked immunosorbent assays

U-PA, tissue-type PA (t-PA), and PAI-1 antigen determinations were performed using commercially available immunoassay kits: u-PA EIA HS Taurus (Leiden, The Netherlands); Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium), and IMULYSE PAI-1 (Biopool, Umea, Sweden).

Immunohistochemistry

Human endometrial tissues were embedded in paraffin and cut in sections of 4 μm. The cross-sections were prewashed in PBS containing 1% NaN_3 and 0.3% H_2O_2 solution. Subsequently, they were incubated for 15 min in a block buffer (5% BSA in PBS) to reduce background staining. After three wash steps in PBS, the polyclonal anti-u-PA antibody was added (0.01 μg/mL in PBS supplemented with 0.05 % Tween-20 and 0.1% BSA), followed by an overnight incubation at 4°C. The next day, the sections were washed in PBS, horseradish peroxidase-conjugated goat antirabbit Ig (1:1,000 in PBS supplemented with 1% BSA) was added, and the sections were incubated for 1 h at 37°C. Thereafter the sections were washed, and the peroxidase activity was developed with diaminobenzidine. The sections were counterstained with Mayer's haematoxylin and mounted in malinol.

In vitro angiogenesis model

Human fibrin matrices were prepared by the addition of 0.1 U/mL thrombin to a mixture of 2.5 U/mL factor XIII (final concentrations), 2 mg fibrinogen, 2 mg sodium citrate, 0.8 mg NaCl, and 3 μg plasminogen/mL M199 medium. Three hundred microliters of this mixture were added to the wells of 48-wells (1 cm^2) plates. After clotting at 37°C, the fibrin matrices were soaked with M199 supplemented with 10% human serum and 10% NBCS for 2 h at 37°C to inactivate the thrombin.

Type I collagen was solubilized by stirring adult rat tail tendons for 48 h at 4°C in a sterile 1:1,000 (vol/vol) acetic solution (300 mL for 1 g collagen). The resulting solution was extensively dialyzed against 1:10,000 (vol/vol) acetic acid and stored at 4°C[27]. For the collagen gels, 8 volumes of rat tail collagen type I were mixed with 1 volume of 10x M199 and 1 volume of 2% (wt/vol) Na_2CO_3 (mixture pH 7.4). Three hundred-microliter aliquots were added to each well and allowed to gel at 37°C in the absence of CO_2.

Highly confluent hFMVEC and hEMVEC were detached, seeded in a split ratio of 1.25:1 and 2.5:1, respectively, on the surface of the fibrin or type I collagen matrices, and cultured for 24 h in M199 medium without indicator supplemented with 20% human serum, 10% NBCS, and penicillin/streptomycin. Then the endothelial cells were cultured with the mediators indicated for 3-7 days. The culture medium was collected and replaced every 2 or 3 days. Invading cells and the formation of tubular structures of endothelial cells in the 3-D fibrin or collagen matrix were analyzed by phase contrast microscopy.

Statistics

Data for three experiments per well are expressed as the mean ± SEM, and data for duplicate experiments per well are expressed as the mean, with the range between the error bars. Statistical analyses of the data (paired-samples t tests) were calculated using the statistic program SPSS (version 10.0, SPSS, Inc., Chicago, IL).

Results

Isolation and characterization of human endothelial cells from endometrium tissue

HEMVEC were isolated by repeated selection of UEA-1 and CD31-positive cells by means of lectin- or antibody-coated magnetic beads. After two to four rounds of selection, all growing cells were characterized as endothelial cells on the basis of the expression of the classical endothelial markers CD31 and vWF and the capacity to bind UEA-1 (Fig. 1). In

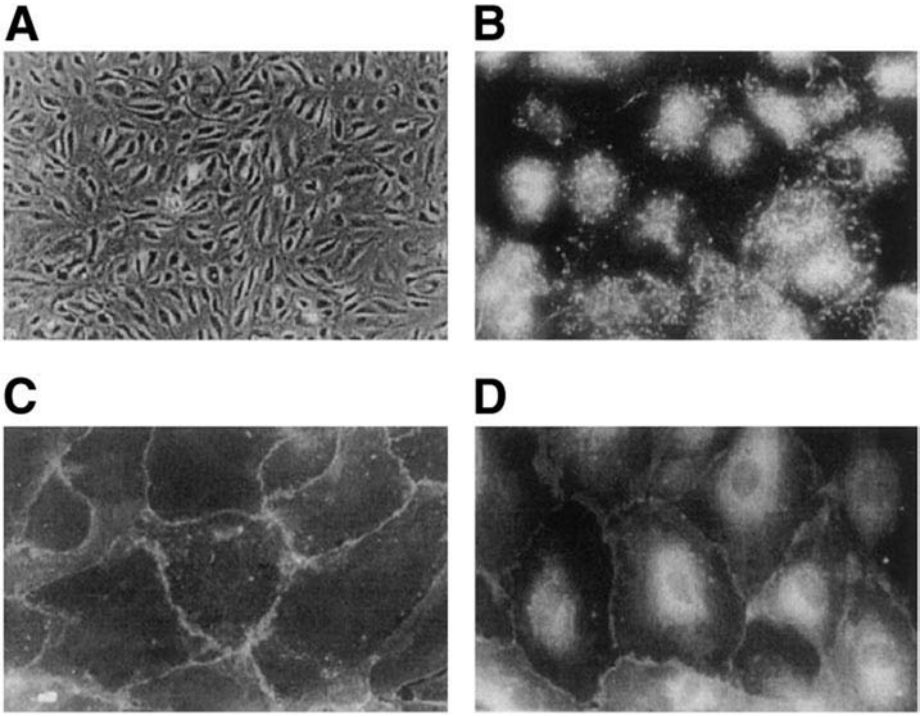


Figure 1. Characterization of hEMVEC.
HEMVEC (passage 5) were cultured to confluence and immunostained as described in *Materials and Methods*. A, Phase-contrast photomicrograph; B-D, indirect immunofluorescent staining of vWF antigen (B) and CD31 antigen (C) or UEA-1-FITC (D) binding.

addition, the cells were negative when stained with antibodies recognizing the epithelial cell markers cytokeratin 8 and 18 and smooth muscle cell actin (data not shown).
In the first instance the isolated hEMVEC were cultured in the presence of high level of serum (20% inactivated human serum and 10% NBCS) and the addition of a crude ECGF preparation on gelatin- or fibronectin-coated culture dishes. This high level of human serum was essential for the maintenance of hEMVEC in culture. Lower amounts of human serum (<20%) resulted in the death of the hEMVEC. Later, after the evaluation of the growth characteristics (see below), the hEMVEC were grown in M199 supplemented with the indicated amount of serum and the combination of 150 μg/mL ECGF and 5 ng/mL VEGF-A. The hEMVEC could be maintained until passage 6-15, varying between the different isolations.

Using the method described, we succeeded in isolating 13 different hEMVEC isolations in 33 attempts. The phase of the menstrual cycle of the women who underwent hysterectomy did not influence the success rate. We succeeded in isolating hEMVEC from proliferative phase tissue as well as from secretory tissue. We

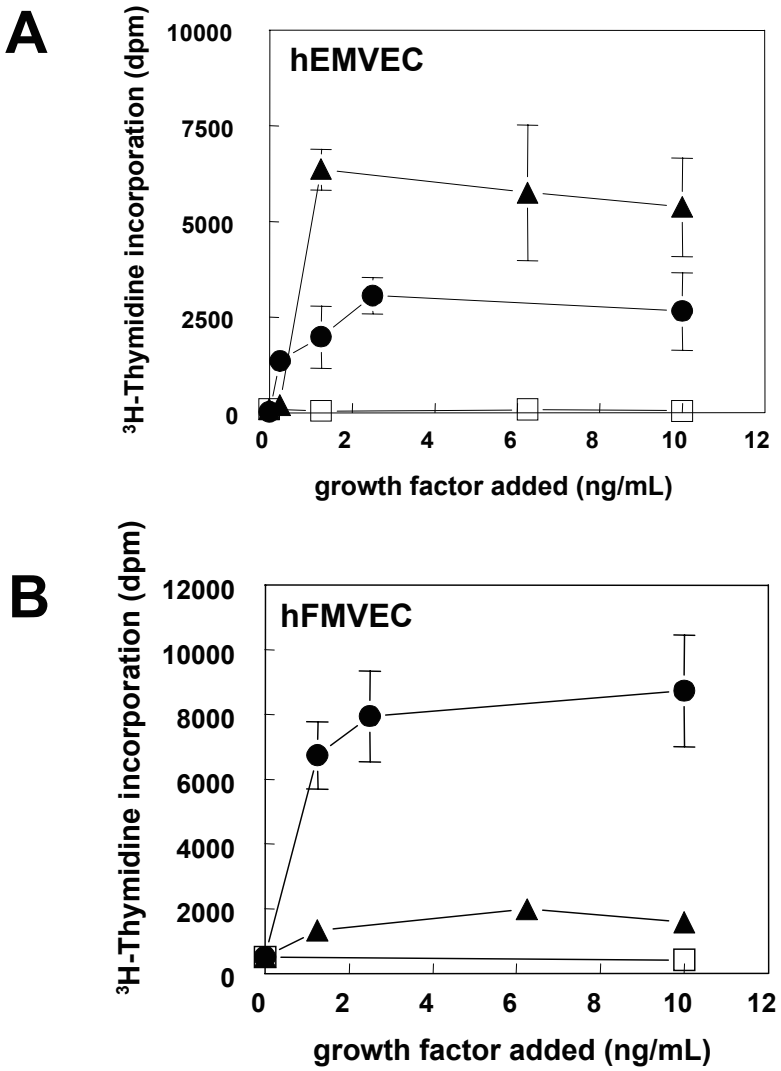


Figure 2. VEGF-A-induced hEMVEC proliferation.
Nonconfluent hEMVEC (A) and hFMVEC (B) were cultured for 48 h in the absence or presence of increasing amount of VEGF-A (▲), bFGF (●), or PlGF-2 (□) in M199 supplemented with 10% NBCS. After 48 h, a tracer amount of [^{3}H]thymidine was added to the medium, the incubation was continued in the same medium for another 6 h, and [^{3}H]thymidine incorporation was determined as described in *Materials and Methods.* The data are expressed as mean ± SEM of triplicate wells and are representative of four experiments performed with different hEMVEC isolations.

never succeeded in isolating hEMVEC from menstruation phase endometrium. Probably the amount of tissue, especially that obtained from the thin (basal) endometrial layer toward the end of the menstruation, was too little to isolate hEMVEC.

Growth characteristics of hEMVEC

The growth characteristics of hEMVEC were compared with those of hFMVEC. HFMVEC (as well as HUVEC, data not shown) were stimulated to proliferate by the addition of bFGF and VEGF-A. These types of human EC react better to bFGF compared with VEGF-A, as determined by the incorporation of 3[H]-thymidine (Fig. 2B). The overall stimulation indexes for HUVEC were 10.9 ± 1.2 and 5.3 ± 0.8 induced by 2.5 ng/mL bFGF and 6.25 ng/mL VEGF-A, respectively ($p=0.00004$; $n=16$), and those for hFMVEC were 12.1 ± 2.0 and. 3.9 ± 0.5 ($p=0.006$; $n=11$). In contrast, VEGF-A was more potent in stimulating hEMVEC to proliferate compared with bFGF (Fig. 2A). The mean stimulation index of hEMVEC was 16.8 ± 4.8 using 2.5 ng/mL bFGF and 30.0 ± 8.3 using 6.25 ng/mL VEGF-A ($p=017$; $n=7$, performed with hEMVEC from four different donors). The stimulation index for hEMVEC was higher than those of HUVEC and hFMVEC due to the lower proliferative capacity of the hEMVEC under control condition (only in the presence of 10% NBCS). Neither hEMVEC nor hFMVEC responded to PlGF-2 (Fig. 2).

The enhanced responsiveness of hEMVEC to VEGF-A was probably due to an enhanced basal expression of the messenger RNA (mRNA) of VEGFR-2 (Fig. 3). Densitometric analysis of the blots revealed a 2.4 fold increase in VEGFR-2 mRNA in hEMVEC compared with hFMVEC. The expression of VEGFR-1 mRNA was very low and comparable in the different endothelial cell types (data not shown).

Production of plasminogen activators and expression of u-PAR by hEMVEC

Compared with other types of humane endothelial cells, unstimulated hEMVEC produced considerably more u-PA. In 24 h, they accumulated 33.8 ± 6.9 ng/10^5 cells ($n=5$) compared with 0.1 ± 0.1 in HUVEC ($n=4$, data not shown). This was not due to a difference in reuptake of u-PA:PAI-1 complexes by the cellular u-PA receptor, because after blockade of the u-PA/u-PAR interaction by monoclonal antibody H-2, the levels of u-PA antigen were 43.2 ± 7.0 (Table 1) and 6.2 ± 2.3 ng/10^5 cells for hEMVEC and HFMVEC (data not shown), respectively. The overall production of u-PA by hEMVEC was enhanced by 1.3-, 1.5- and 1.8-fold after a 24-h incubation with bFGF, VEGF-A or their combination (Table 1). TNFα further enhanced the production of u-PA antigen by hEMVEC. The relatively high u-PA production by hEMVEC compared with hFMVEC was also found at the mRNA level (Fig. 3).

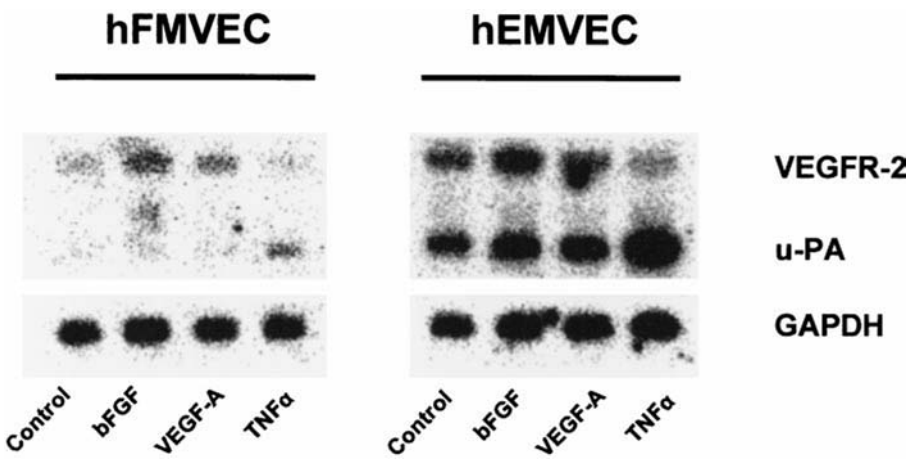


Figure 3. Expression of u-PA, and VEGFR-2 mRNA in hEMVEC.
Confluent (passage 6) hEMVEC and (passage 11) hFMVEC, cultured on fibronectin-coated wells, were stimulated with or without bFGF (10 ng/mL), VEGF-A (100 ng/mL), or TNFα (10 ng/mL). Total RNA was isolated at 24 h and analyzed by Northern blotting for u-PA and VEGFR-2 mRNA. Equal loading was checked by hybridization with a glyceraldehydes-3-phosphate dehydrogenase mRNA. This experiment was performed with two different hEMVEC isolations with similar results.

Table 1. Production of u-PA, t-PA, and PAI-1 by hEMVEC

Addition	u-PA (ng/10^5 cells)		t-PA (ng/10^5 cells)	PAI-1 (ng/10^5 cells)
	- anti-u-PAR mAb	+ anti-u-PAR mAb	- anti-u-PAR mAb	- anti-u-PAR mAb
None	33.8 ± 6.9	43.2 ± 7.0	1.3 ± 0.2	186 ± 42
bFGF	39.1 ± 7.5	54.9 ± 7.7*	1.5 ± 0.3	209 ± 43
VEGF-A	38.8 ± 7.9	64.2 ± 10.1*	2.3 ± 0.4*	218 ± 50
bFGF/VEGF-A	45.8 ± 12.3	78.5 ± 11.2*	2.2 ± 0.3*	196 ± 56
TNFα	91.6 ± 9.8*	nd	1.5 ± 0.3	249 ± 40*
bFGF/TNFα	77.4 ± 8.1*	nd	1.4 ± 0.5	282 ± 58*

HEMVEC were cultured on gelatin-coated wells till confluence in hEMVEC culture medium and preincubated with M199 supplemented with 20% human serum for 24 h. Then the hEMVEC were stimulated with 10 ng/mL bFGF, 100 ng/mL VEGF-A, 10 ng/mL TNFα, or the combination of these mediators in M199 plus 20% human serum. After 24 h, supernatants were collected, and the cells were counted. The u-PA, t-PA, and PAI-1 amounts were determined by ELISA as described in *Materials and Methods*. The data are expressed as the mean ± SEM of 5 experiments/isolations in nanograms per 24 h/10^5 cells. ND, not done.
* $p < 0.05$ compared to control (no addition).

The overall production of t-PA and PAI-1 antigens was 1.3 ± 0.2 and 186 ± 42 ng/10^5 cells, respectively. The production of t-PA antigen was increased by VEGF-A, whereas PAI-1 antigen was enhanced by TNFα (Table 1).

Binding of [^{125}I]DIP-u-PA to cellular u-PA receptor on hEMVEC and hFMVEC revealed comparable binding of u-PA to its receptor and a similar increase induced by VEGF-A, bFGF and simultaneous addition of bFGF/VEGF-A, bFGF/TNFα or VEGF/TNFα (Fig. 4). The average basal u-PA binding to hEMVEC was 4.2 ± 1.4 (n=3) fmol/10^5 cells, whereas that to hFMVEC was 3.3 ± 0.8 fmol/10^5 cells (triplicate wells, n=1, Fig. 4B).

These data indicate that hEMVEC produce very high amounts of u-PA compared with other types of human endothelial cells, whereas their expression and regulation of other fibrinolytic regulators, *i.e.* u-PAR, t-PA and PAI-1, are similar to those of other human endothelial cell types.

Immunolocalization of u-PA in human endometrium tissue samples

To compare the high amount of u-PA accumulation *in vitro* with the *in vivo* situation, sections of endometrium and myometrium were studied. Immunostaining for u-PA was found on the vessels of the endometrium and myometrium (Fig. 5) and in the stroma of the endometrium, whereas surface and glandular epithelial cells were negative for the u-PA antigen (Fig. 5). A negative staining procedure (the same staining procedure but without the primary antibody) showed no staining of the vessels and stroma (data not shown).

In vitro capillary-like tube formation by hEMVEC in 3-D fibrin matrices

Subsequently, we evaluated the ability of hEMVEC to form capillary-like tubular structures in an *in vitro* angiogenesis assay, consisting of human endothelial cells cultured on 3-D fibrin matrices[28]. As we had previously found, hFMVEC form capillary-like tubular structures when stimulated with the combination of VEGF-A (or bFGF) and TNFα after a culture period of 7 days (Fig. 6E)[28,29]. The addition of growth factor alone did not result in the formation of tube-like structures, but induced holes in the monolayer of endothelial cells due to uncontrolled lysis of the fibrin matrix (Fig. 6D)[29]. When hEMVEC were cultured on top of the fibrin matrix in the presence of 20% human serum, they began forming capillary-like tubes within 3 days (Fig. 6A). This spontaneous tube formation was enhanced by the addition of VEGF-A (Fig. 6B). Further enhancement and stabilization of the tube-like structures were seen when the growth factors were added in combination with TNFα (data not shown). Maximal induction of the formation of tube-like structures by hEMVEC was seen on days 2-3, whereas those formed by hFMVEC were maximal on days 7-10. The extent of tube formation was markedly inhibited by polyclo-

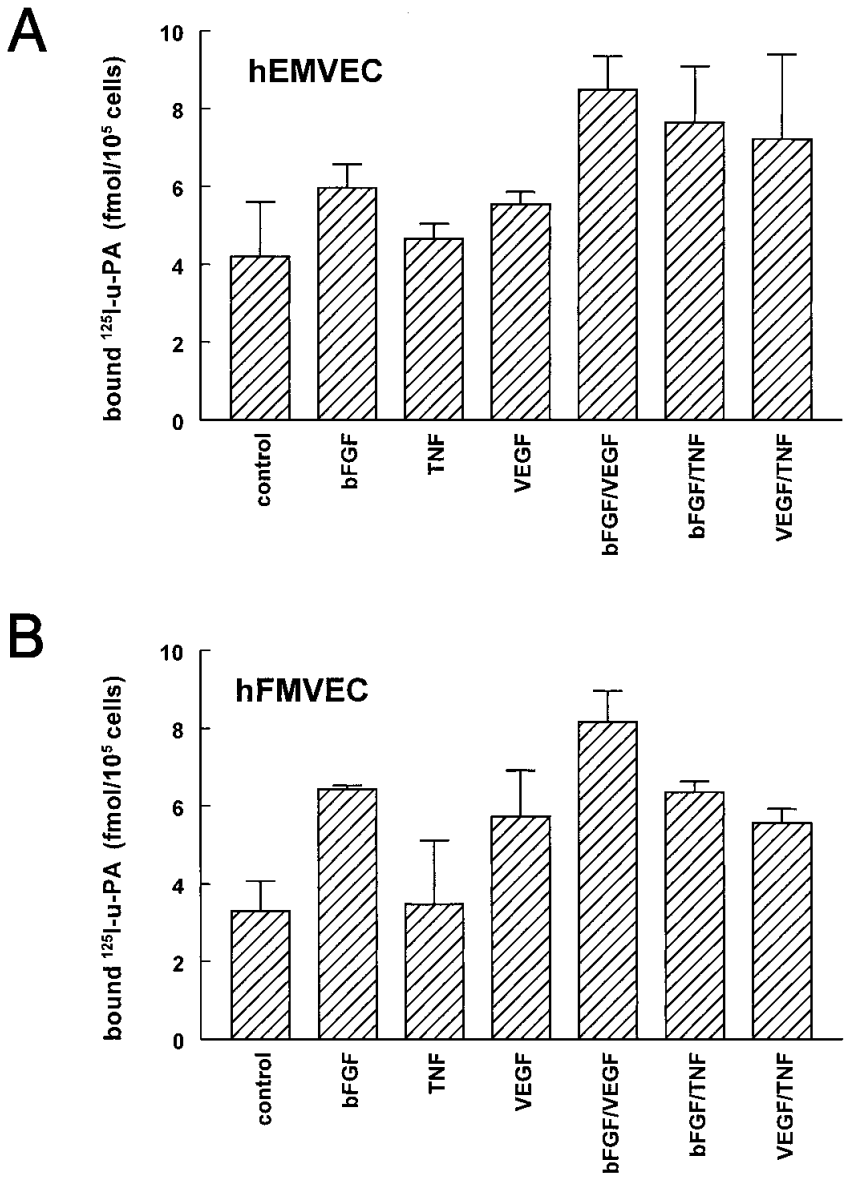


Figure 4. u-PA binding to hEMVEC and hFMVEC.
HEMVEC and hFMVEC were preincubated for 24 h in M199 supplemented with 20% human serum with or without bFGF (10 ng/mL), VEGF-A (100 ng/mL), TNFα (10 ng/mL), or the combination of these mediators. Subsequently, the cells were cooled on ice, and the specific binding of [^{125}I]DIP-u-PA was determined in triplicate wells as described in *Materials and Methods*. A, Data for u-PA binding to hEMVEC expressed as mean ± SEM in femtomoles per 10^5 cells of three independent experiments performed in triplicate wells. B, A representative experiment showing u-PA binding to hFMVEC. The binding is expressed as mean ± SEM in femtomoles per 10^5 cells of triplicate wells.

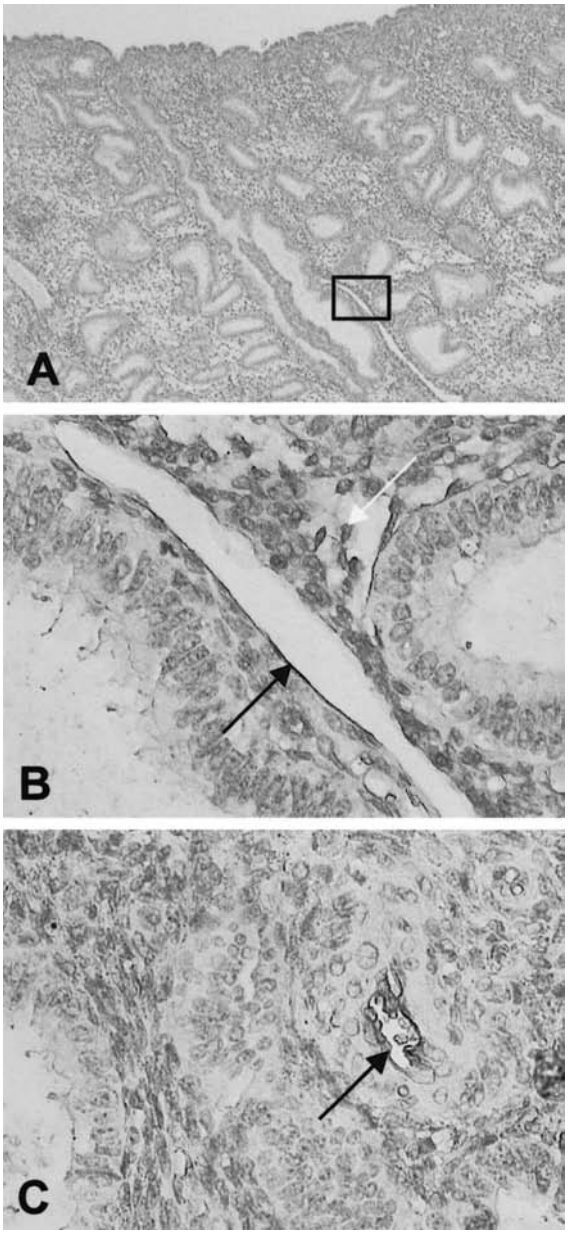


Figure 5. Expression of u-PA in blood vessels in human secretory phase endometrial tissue. Immunohistochemistry was performed with labeled antibody to u-PA on paraffin sections of human endometrium, as described in *Materials and Methods*. A, Brown staining shows accumulation of u-PA in the stromal endometrium; the surface and glandular epithelium are negative for the u-PA antigen (20x magnification). The *rectangle area* is enlarged in B. B and C, *Black arrows* indicate examples of positive endothelial cells, and a *white arrowhead* indicate positive stromal cells (200x magnification). The same results were obtained using proliferative phase endometrium. *[See appendix: color figures]*

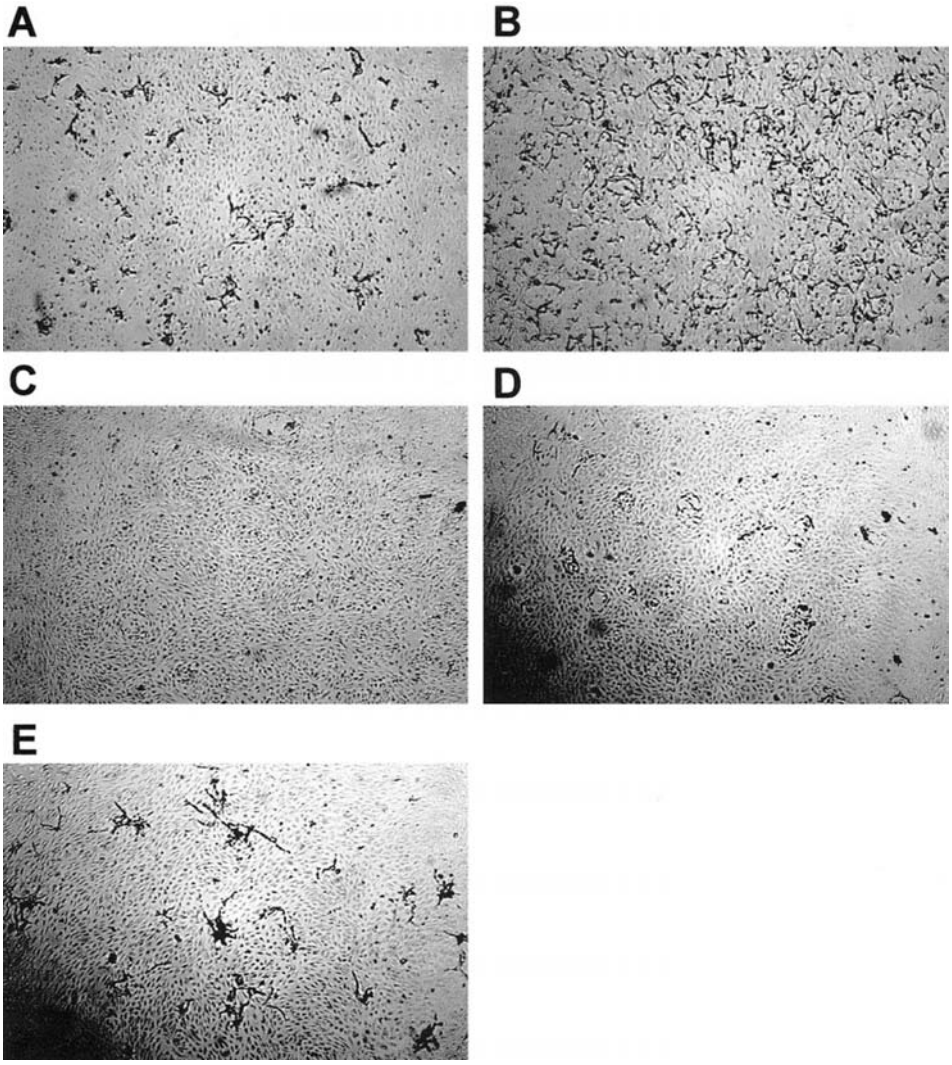


Figure 6. Enhanced capillary-like tube formation by hEMVEC.
HEMVEC (passage 7) were cultured on top of a 3-D fibrin matrix in M199 supplemented with 20% human serum and 10% NBCS and stimulated without (A) or with 10 ng/mL VEGF-A (B). After 2 days of culture, nonphase contrast views were taken, which show in both panels growth of capillary-like tube structures. In contrast, hFMVEC (passage 11) stimulated without (C) or with 25 ng/mL VEGF-A (D) cultured for 6 days show no capillary-like tube structures. E, hFMVEC cultured on fibrin for 6 days in the presence of the combination of 25 ng/mL VEGF-A and 1 ng/mL TNFα. The data are representative of four different hEMVEC isolations.

nal anti-u-PA antibodies under both unstimulated as well as VEGF-A-stimulated (71.2 ± 5.3%; n=4) conditions.

A similar increased angiogenic capacity of the hEMVEC was observed when hEMVEC were cultured on top of rat tail collagen type I matrices. Under our culture conditions, unstimulated hFMVEC did not or hardly invaded the collagen matrix, which was slightly enhanced by addition of the combination of bFGF or VEGF-A and TNFα (data not shown). However, those few hFMVEC that were able to invade the matrix did not form tube-like structures with lumens surrounded by endothelial cells, but formed sprouts at or just beneath the collagen surface[45]. HEMVEC displayed an increased invasion and sprout formation in the collagen matrix that was increased by the addition of VEGF-A alone. However, sprouts of this type of human endothelial cells did not contain clear lumen-like structures (data not shown).

Discussion

Here we describe the isolation and characterization of hEMVEC from various donors. These endothelial cells displayed an enhanced responsiveness to VEGF-A compared with hFMVEC due to an enhanced expression of VEGFR-2. In addition, hEMVEC are more angiogenic when cultured in the presence of 20% human serum on top of a 3-D fibrin matrix or 3-D collagen matrix compared with hFMVEC. The hEMVEC formed tube-like structures within 2-4 days that were enhanced by the addition of VEGF-A alone. This in contrast to hFMVEC, which had to be stimulated with the combination of VEGF-A and TNFα for a period of 7 days to form tubes. The enhanced angiogenic behavior of the hEMVEC was probably due to an increase in expression of u-PA, facilitating an enhanced proteolytic capacity to the hEMVEC.

Overt angiogenesis takes place in the endometrium throughout the reproductive life of the female during each menstrual cycle. Therefore, the endothelial cells of the endometrium must be able to respond quickly to changes in steroids and environmental conditions and to angiogenic mediators to form a new vascular bed to allow proper implantation and placentation. To study the process of angiogenesis in the endometrium we isolated hEMVEC using a standard isolation procedure of endothelial cells consisting of a digestion of the endometrial tissue and selection of endothelial cells using UEA-1 or anti-CD31-coated magnetic beads. HEMVEC were difficult to maintain in culture. Only in the presence of 20% human serum and VEGF-A (and/or ECGF) were the cells were able to proliferate and to be passed up to passages 6-15. The sensitivity of the cells to the culture condition may be a reflection of the *in vivo* situation, where rapid apoptosis of the cells in the endometrium starts in the secretory phase and peaks in the menstrual phase[30].

The family of the VEGF growth factors is thought to play an important role in the process of angiogenesis[31,32]. Both the expression of VEGF[3,33,34] and the specific VEGF receptors VEGFR-1 and VEGFR-2 are found in the endometrium during the three stages of the menstrual cycle[35,36]. In particular, the expression of VEGFR-2 on the vessels was increased during the proliferative phase[35]. Cultured hEMVEC also display enhanced expression of VEGFR-2 compared with hFMVEC or HUVEC, whereas the expression of VEGFR-1 is comparable among these EC types. As it is generally accepted that VEGF-A-induced endothelial proliferation is mediated *via* VEGFR-2[37,38], the enhanced expression of this VEGFR may be an explanation for why hEMVEC are more reactive toward VEGF-A than to bFGF compared with hFMVEC. These data are in accordance with recently published data by Iruela-Arispe *et al.*[14], who also showed that hEMVEC expressed increased levels of VEGFR-2 and an increased proliferation in response to VEGF-A.

Most striking was the finding of the relative high u-PA expression by hEMVEC, whereas the levels of the other compounds of the plasminogen system, t-PA and PAI-1, were not significantly enhanced compared with those in hFMVEC or HUVEC[28,39]. The average u-PA binding to the u-PAR was also similar (4.2 ± 1.4 (n=3) fmol/10^5 cells vs. 6.4 ± 3.2 (n=17)[28], and 2.6 ± 1.8 (n=8)[28] fmol/10^5 cells for hEMVEC, HUVEC, and hFMVEC, respectively). Under basal conditions, hFMVEC do not express such high levels of u-PA (0.2 ± 0.1 ng/10^5 cells; n=9)[28,40]. Only when stimulated with the inflammatory mediator TNFα, but not with VEGF-A or bFGF, do both hFMVEC and HUVEC start to secrete considerable levels of u-PA (up to 1 ng/10^5 cells)[28,39,41]. However, production of u-PA by activated hFMVEC and HUVEC is still a magnitude lower than basal or TNFα-stimulated u-PA production by hEMVEC. In contrast to that by hFMVEC[28], u-PA production by hEMVEC increased after addition of the angiogenic growth factors bFGF, VEGF-A, or the combination of these mediators with TNFα. The high expression of u-PA by the hEMVEC *in vitro* was confirmed by immunohistochemical staining of endometrial tissue obtained from healthy premenopausal women. The endothelial cells of the vessels in the myometrium as well as the vessels of the endometrium *in vivo* showed expression of the u-PA antigen. In normal human tissue the expression of endothelium-associated u-PA is hardly detectable, but there is an increase in endothelial cell expression of u-PA detectable in inflamed tissues, such as during appendicitis[42], and tumor angiogenesis[43], in atherosclerotic vessels[44], and in vessels in atherosclerotic plaques[25].

Simultaneously with the enhanced u-PA expression, hEMVEC displayed an enhanced capacity to form tube-like structures in 3-D fibrin matrices and sprout formation in collagen matrices compared to hFMVEC. The formation of tube-like structures of hFMVEC in fibrin matrices depends on u-PAR-localized u-PA activity[25] and occurs only after stimulation of the cells with the combination of a growth factor (either bFGF or VEGF-A) and the inflammatory mediator TNFα[28], which is a potent stimulator of the production of u-PA

by human EC. It is possible that the spontaneous tube formation by the hEMVEC may be due to a response of the hEMVEC (mediated *via*, for instance, the increased VEGFR-2 expression) to growth factors in human serum in combination with the enhanced basal u-PA expression, as shown by the inhibition of tube formation after the addition of neutralizing anti-u-PA antibodies. The enhanced u-PA/plasmin activity, which is also able to activate several MMPs, such as MMP-1, MMP-3, and MMP-9 *in vitro*[10-12], may provide endothelial cells in the endometrium with enhanced angiogenic capacity, as shown *in vivo*.

In conclusion, we show that human endometrium-derived endothelial cells display an enhanced proteolytic capacity and an enhanced angiogenic capacity. These data provide us with a better understanding of the regulation, production, and physiological responses of the vasculature in the endometrium and may lead to new insight into pathology during pregnancy, which may be related to diseases later in life and therapeutic strategies in the future.

Acknowledgements

We thank Dr. R.M.F. van der Weiden (St. Franciscus Gasthuis, Rotterdam, The Netherlands) for valuable discussions and the endometrial tissues, and Dr. R.A. Verwey and colleagues of the Bronovo Hospital (The Hague, The Netherlands), who provided us with endometrial tissues.

References

1. Rees MCP, Bicknell R. 1998 Angiogenesis in the endometrium. Angiogenesis. 2:29-35.
2. Rogers PAW, Abberton KM, Susil B. 1992 Endothelial cell migratory signal produced by human endometrium during the menstrual cycle. Hum Reprod. 7:1061-1066.
3. Bausero P, Cavaillé F, Méduri G, Freitas S, Perrot-Applanat M. 1998 Paracrine action of vascular endothelial growth factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis. 2:167-182.
4. Van Hinsbergh VWM, Koolwijk P, Hanemaaijer R. 1997 Role of fibrin and plasminogen activators in repair-associated angiogenesis: *in vitro* studies with human endothelial cells. Regulation of Angiogenesis, EXS. 79:391-411.
5. Folkman J, Shing Y. 1992 Angiogenesis. J Biol Chem. 267:10931-10934.
6. Pepper MS. 1997 Manipulating angiogenesis - From basic science to the bedside. Arterioscler Thromb Vasc Biol. 17:605-619.
7. Rogers PAW, Gargett CE. 1999 Endometrial angiogenesis. Angiogenesis. 2:287-294.
8. Van Hinsbergh VWM. 1992 Impact of endothelial activation on fibrinolysis and local proteolysis in tissue repair. Ann N Y Acad Sci. 667:151-162.
9. Vassalli J-D, Pepper MS. 1994 Membrane proteases in focus. Nature. 370:14-15.
10. Okumura Y, Sato H, Seiki M, Kido H. 1997 Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin - A possible cell surface activator. FEBS Lett. 402:181-184.
11. Santibanez JF, Martinez J. 1993 Membrane-associated procollagenase of leukemic cells Is activated by urokinase-type plasminogen activator. Leukemia Res. 17:1057-1062.
12. Murphy G, Stanton H, Cowell S *et al*. 1999 Mechanisms for pro matrix metalloproteinase activation. Apmis. 107:38-44.
13. Gerritsen ME. 1987 Functional heterogeneity of vascular endothelial cells. Biochem Pharmacol. 36:2701-2711.
14. Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abujawdeh G. 1999 Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue response to angiogenic growth factors. Microcirculation. 6:127-140.
15. Schatz F, Soderland C, Hendricks-Muñoz KD, Gerrets RP, Lockwood CJ. 2000 Human endometrial endothelial cells: Isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. Biol Reprod. 62:691-697.
16. Nikitenko LL, MacKenzie IZ, Rees MCP, Bicknell R. 2000 Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. Mol Hum Reprod. 6:811-819.
17. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R. 1979 An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci U S A. 76:5674-5678.
18. Bohuslav J, Horejsi V, Hansmann C *et al*. 1995 Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med. 181:1381- 1390.
19. Wijngaards G, Kluft C, Groeneveld E. 1982 Demonstration of urokinase-related fibrinolytic activity in human plasma. Br J Haematol. 51:165-169.
20. Solberg H, Romer J, Brünner N *et al*. 1994 A cleaved form of the receptor for urokinase-type plasminogen activator in invasive transplanted human and murine tumors. Int J Cancer. 58:877-881.
21. Barleon B, Hauser S, Schollmann C *et al*. 1994 Differential expression of the two VEGF receptors Flt and Kdr in placenta and vascular endothelial cells. J Cell Biochem. 54:56-66.
22. Terman BI, Dougher-Vermazen M, Carrion ME *et al*. 1992 Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun. 187:1579- 1586.
23. Van Hinsbergh VWM, Sprengers ED, Kooistra T. 1987 Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. Thromb Haemost. 57:148-153.
24. Defilippi P, Van Hinsbergh VWM, Bertolotto A, Rossino P, Silengo L, Tarone G. 1991 Differential distribution and modulation of expression of alpha 1/beta 1 integrin on human endothelial cells. J Cell Biol. 114:855-863.
25. Kroon ME, Koolwijk P, Van Goor H *et al*. 1999 Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. Am J Pathol. 154:1731-1742.
26. Chomczynski P, Sacchi N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem. 162:156-159.
27. Strom SC, Michalopoulos G. 1982 Collagen as a substrate for cell growth and differentiation. Methods Enzymol. 82 Pt A:544-555.

28. Koolwijk P, Van Erck MGM, De Vree WJA *et al*. 1996 Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol. 132:1177-1188.
29. Collen A, Koolwijk P, Kroon ME, Van Hinsbergh VWM. 1998 Influence of fibrin structure on the formation and maintenance of capillary-like tubules by human microvascular endothelial cells. Angiogenesis. 2:153-165.
30. Shikone T, Kokawa K, Yamoto M, Nakano R. 1997 Apoptosis of human ovary and uterine endometrium during the menstrual cycle. Horm Res. 48 Suppl 3:27-34.
31. Folkman J . 1995 Tumor angiogenesis. In: J Mendelsohn, PM Howley, MA Isreal, LA Liotta, eds. The molecular basis of cancer. Philadelphia: W.B. Saunders Comp.. 206-232.
32. Ferrara N. 1999 Molecular and biological properties of vascular endothelial growth factor. J Mol Med. 77:527-543.
33. Li XF, Gregory J, Ahmed A. 1994 Immunolocalisation of vascular endothelial growth factor in human endometrium. Growth Factors. 11:277-282.
34. Gargett CE, Lederman FL, Lau TM, Taylor NH, Rogers PAW. 1999 Lack of correlation between vascular endothelial growth factor production and endothelial cell proliferation in the human endometrium. Hum Reprod. 14:2080-2088.
35. Meduri G, Bausero P, Perrot-Applanat M. 2000 Expression of vascular endothelial growth factor receptors in the human endometrium: Modulation during the menstrual cycle. Biol Reprod. 62:439-447.
36. Krüssel JS, Casan EM, Raga F *et al*. 1999 Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sFlt in cycling human endometrium. Mol Hum Reprod. 5:452-458.
37. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. 1994 Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 269:26988-26995 .
38. Ortéga N, Jonca F, Vincent S, Favard C, Ruchoux MM, Plouët J. 1997 Systemic activation of the vascular endothelial growth factor receptor KDR/flk-1 selectively triggers endothelial cells with an angiogenic phenotype. Am J Pathol. 151:1215-1224.
39. Van Hinsbergh VWM, Van den Berg EA, Fiers W, Dooijewaard G. 1990 Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells . Blood. 75:1991-1998.
40. Kroon ME, Koolwijk P, Van der Vecht B, Van Hinsbergh VWM. 2000 Urokinase receptor expression on human microvascular endothelial cells is increased by hypoxia: implications for capillary-like tube formation in a fibrin matrix. Blood. 96:2775-2783.
41. Niedbala MJ, Stein-Picarella MS. 1992 Tumor necrosis factor induction of endothelial cell urokinase-type plasminogen activator mediated proteolysis of extracellular matrix and its antagonism by gamma-Interferon. Blood. 79:678-687.
42. Grøndahl-Hansen J, Kirkeby LT, Ralfkiaer E, Kristensen P, Lund LR, Danø K. 1989 Urokinase-type plasminogen activator in endothelial cells during acute inflammation of the appendix. Am J Pathol. 135:631-636.
43. Xu YL, Hagege J, Doublet J-M *et al*. 1997 Endothelial and macrophage upregulation of urokinase receptor expression in human renal cell carcinoma. Hum Pathol. 28:206-213.
44. Falkenberg M, Tjarnstrom J, Ortenwall P, Olausson M, Risberg B. 1996 Localization of fibrinolytic activators and inhibitors in normal and atherosclerotic vessels. Thromb Haemost. 75:933-938.
45. Kroon ME, van Schie ML, van der Vecht B, van Hinsbergh VWM, Koolwijk P. 2002 Collagen type 1 retards tube formation by human microvascular endothelial cells in a fibrin matrix. Angiogenesis. 5,257-265.

5

INVOLVEMENT OF MEMBRANE-TYPE MATRIX METALLOPROTEINASES (MT-MMPS) IN CAPILLARY TUBE FORMATION BY HUMAN ENDOMETRIAL MICROVASCULAR ENDOTHELIAL CELLS: ROLE OF MT3-MMP

K. Kapiteijn
M. Plaisier
P. Koolwijk
C. Fijten
R. Hanemaaijer
J.M. Grimbergen
A. Mulder-Stapel
P.H.A. Quax
F.M. Helmerhorst
V.W.M. van Hinsbergh

Journal of Clinical Endocrinology & Metabolism 2004;89,5828-5836

Introduction

In the adult, angiogenesis plays a role in many pathological conditions, such as the growth of solid tumors, diabetic retinopathy, rheumatoid arthritis, and wound healing[1,2]. Physiological angiogenesis during adulthood is limited to the female reproductive tissue, namely in the ovary and endometrium. Endometrial angiogenesis plays a role in endometrial remodeling during the menstrual cycle and after conception during the implantation of the embryo[3,4,5]. Angiogenesis is initiated by a shift in the balance between pro-angiogenic and anti-angiogenic factors[6,7]. It involves the sprouting of new capillary-like structures from existing vasculature and may involve blood-born cells that intussuscepts in and around the new vascular structures[2]. These newly formed tubes are subsequently stabilized, often by interaction with pericytes. While the general mechanisms of angiogenesis are probably rather similar in various tissues, the individual players, such as growth factors, integrins and proteases, may vary in different tissues. Endothelial cells from different tissues and vessel types have specific properties[8], many of which are conserved *in vitro*[8,9,10]. We previously observed that different types of human microvascular endothelial cells (hMVEC) have different requirements for proliferation and capillary tube formation *in vitro*. While endometrial MVEC (hEMVEC) are highly sensitive to VEGF-A and form capillary tubules after exposure to VEGF-A[9], foreskin MVEC (hFMVEC) are more sensitive to bFGF and only form capillary tubes in a fibrin matrix after simultaneous exposure to bFGF or VEGF-A and the inflammatory cytokine TNFα[11,12].

Among the various processes that regulate angiogenesis, the generation of proteolytic activity is thought to be pivotal in the regulation of cell migration and capillary tube formation[13]. Key regulators of pericellular proteolysis and capillary-like tubule formation by endothelial cells are cell-bound urokinase-type plasminogen activator (u-PA) and plasmin as well as matrix metalloproteinases (MMPs)[5,11-20]. Initial data on the formation of tubular structures by hEMVEC indicated that cell-bound u-PA and plasmin contribute to this process[9]. In addition to the u-PA/plasmin cascade, the rapidly expanding family of MMPs[21] plays an important role in cell migration and invasion, and in angiogenesis *in vivo*[19,22,23]. MMPs are widely expressed in the endometrium and play a role in tissue degradation and menstrual bleeding[24]. Furthermore, a number of them are also detected during the proliferative and early secretory phase[25], which suggests a role in endometrial remodeling and angiogenesis[26,27]. However, the exact role of MMPs in endometrial angiogenesis *in vivo* and tube formation by hEMVEC *in vitro* is unknown.

Membrane-type MMPs (MT-MMPs) have been suggested to play a key role in angiogenesis, in addition to the gelatinases MMP-2 and -9.[17,28,29] The membrane-associated localization of membrane-type MMPs makes this group of MMPs particularly suited to

function in pericellular proteolysis[17]. Six MT-MMPs have been described: four transmembrane proteins and two GPI-anchored ones. Recently, MT1-MMP (MMP-14) received considerable attention as being involved in endothelial cell migration and invasion[14-16]. MT1-MMP contributes to angiogenesis by its capacity to degrade ECM components, thereby promoting cell migration, invasion and possibly the bioavailability of growth factors. Furthermore, it activates pro-MMP-2 (*via* the TIMP-2-MT1-MMP complex), pro-MMP-13, and $\alpha v\beta 3$-integrin, an important integrin in angiogenesis[14,29-31]. MT1-MMP as well as MMP-2 are able to stimulate angiogenesis[32,33]. In hFMVEC, MT1-MMP becomes a key factor in capillary tube formation when collagen is present in the fibrinous matrix[16,17]. MT2-MMP (MMP-15) and MT3-MMP (MMP-16) are also involved in cell migration and invasion, depending on the cell type[17,34]. Their overexpression in endothelial cells can induce capillary-tube formation, similar to MT1-MMP[28]. MT1-MMP and MT2-MMP are present in endometrial tissue during various stages of the menstrual cycle; MT3-MMP mRNA is increased during the proliferative phase of the endometrium[35-38]. It is generally believed that these MMPs also play a role in endometrial angiogenesis[39], but except for the expression and immunolocalization of specific MMPs in endometrial tissue little information is available.

The activity of MMPs and MT-MMPs is regulated by activation of the pro-enzymes and by specific inhibitors, the tissue inhibitors of MMPs (TIMPs) and α-macroglobulins. The TIMP family consists of 4 members, which differ in expression patterns, regulation and ability to interact specifically with latent MMPs and members of the related metalloproteinases of the ADAMs and TACE group[40]. TIMP-1 is secreted as a soluble protein and has a general inhibiting activity on many MMPs, but does not inhibit MT1-MMP. TIMP-3 is associated with the matrix components and has a similar inhibitory spectrum, but also inhibits MT1-MMP[41]. Furthermore, TIMP-3 can induce apoptosis in various cell types[40].

In this study we report on the expression of MMPs and MT-MMPs by hEMVEC and the requirement of these proteases for capillary-like tube formation by these cells. By overexpressing TIMP-1 and TIMP-3 we could demonstrate that different MMPs act as key regulators for tube formation by hEMVEC and hFMVEC.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and tissue culture medium 199 (M199) with 20 mM HEPES with or without phenol red were obtained from BioWhittaker (Verviers, Belgium). Newborn calf serum (NBCS) was obtained from Life Technologies (Grand Is-

land, NY, USA). Human serum (HS), prepared from fresh blood from 10-20 healthy donors, was obtained from a local blood bank and was pooled and stored at 4°C. NBCS and HS were heat-inactivated before use. Pyrogen-free human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Tissue culture plastics and microtiter plates were obtained from Costar/Corning (Cambridge, MA, USA) and Falcon® (Becton Dickinson (BD) Biosciences), Bedford, MA, USA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain as described by Maciag *et al*[42]. Heparin and thrombin were obtained from Leo Pharmaceutics Products (Weesp, the Netherlands). Human fibrinogen was obtained from Chromogenics AB (Mölndal, Sweden). Dr. H. Metzner and Dr. G. Seeman (Aventis Behring GmbH, Marburg, Germany) generously provided factor XIII. Fibronectin was a gift from Dr. J. van Mourik (Sanquin, Amsterdam, The Netherlands). Rat tail collagen type-I was obtained from BD Biosciences. Human recombinant vascular endothelial growth factor-A (VEGF-A) was obtained from RELIATech (Braunschweig, Germany) and tumor necrosis factor alpha (TNFα) was a gift from Dr. J. Travernier (Biogent, Gent, Belgium). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Adenoviral vectors containing LacZ, TIMP-1 and TIMP-3 were previously described[43-45]. Aprotinin was purchased from Pentapharm Ltd (Basel, Switzerland). BB94 (Batimastat) was a kind gift from Dr. E.A. Bone (British Biotech, Oxford, UK). Rabbit-anti-human polyclonal antibodies against u-PA, MMP-9 and MT1-MMP were produced and characterized in our laboratory[11, 16, 46, 47]. Mouse-anti-human monoclonal antibody against MT3-MMP was obtained from Oncogene Research Products (IM50L, Boston, USA), biotinylated horse anti mouse antibody from Vector (BA-2000, Burlingame, UK), avidin-biotin complex from DakoCytomation (Glostrup, Denmark) and NovaRED from Vector (Burlingame, UK). Human recombinant MT1-MMP (pro-domain-catalytic domain-hemopexin domain) was purchased from Chemicon (Temecula, CA, USA) and recombinant pro-MMP-9 from Invitek (Berlin, Germany). PBS/T concentrate was obtained from Organon Teknika (Boxtel, Holland). GAPDH control reagents (VIC-labeled) were purchased from Applied Biosystems (Nieuwerkerk aan de IJssel, The Netherlands). For western blotting, protease inhibitors from Roche Diagnostics (Almere, The Netherlands), Immobilon-P polyvinylidene fluoride transfer membranes from Milipore (Bedford, USA), skim milk powder from Merck (Amsterdam, The Netherlands), goat anti-β-actin antibody (sc-1615) and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz (Heerhugowaard, The Netherlands) were used. The Super Signal West Dura Extended Duration Substrate purchased from Pierce (St. Augustin, Germany) and the luminescent image workstation from Roche Diagnostics (Almere, The Netherlands) were used for visualization.

Cells

Human endometrial microvascular endothelial cells (hEMVEC) were isolated, cultured and characterized as previously described in detail[9]. In short, endometrial tissue was obtained from pre-menopausal women who underwent uterus extirpation for benign pathology. The tissue was collected according to the guidelines of the Institutional Review Board and informed consent was obtained from each patient. Endometrial tissue was scraped from the uterus and stored overnight at 4°C. The following day, tissue was minced and cells extracted using 0.2% collagenase. The primary heterogeneous culture was purified by repeated selections using anti-CD31 and anti-IgG-coated Dynabeads. After purification of the culture, the endothelial cells were characterized as being positive for CD31 and von Willebrand factor and negative for cytokeratin-18 and α-smooth muscle actin. HEMVEC were maintained in hEMVEC culture medium: M199 without phenol-red supplemented with 20 mM HEPES (pH 7.3), 20% HS, 10% NBCS, 150 μg/mL ECGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin. The cells were cultured on fibronectin-coated dishes under humidified 5% CO_2 / 95% air atmosphere. VEGF-A (5 ng/mL) was added to the culture medium of the primary isolates to facilitate the initial growth of the endothelial cells. Endometrial tissues were obtained from all phases of the menstrual cycle, as determined by histological dating according to Noyes *et al*[48], and hEMVEC from different stages showed comparable functions *in vitro*.

Human foreskin microvascular endothelial cells (hFMVEC) were isolated, characterized and cultured as previously described[10,49].

In vitro capillary-like tube formation assay

Human fibrin matrices were prepared as described before[9]. For the collagen gels, 7 volumes of rat tail collagen type-I (3 mg/mL) were mixed with 1 volume of 10×M199 with phenol red and 2 volumes of 2% (w/v) Na_2CO_3 (final pH 7.4). 300μl Aliquots were added to each well of a 48-wells plate and allowed to gelate at 37°C in the absence of CO_2.

Confluent hEMVEC were detached and seeded at a split ratio of 2:1 on top of the fibrin and/or collagen matrices and cultured for 24 h hEMVEC culture medium without ECGF and heparin. Subsequently, the endothelial cells were cultured with the mediators indicated for 2 - 5 days. Invading cells and the formation of capillary-like structures of endothelial cells in the three-dimensional fibrin and/or collagen matrix were analyzed by phase contrast microscopy. The total length of the structures formed was measured in 6 randomly chosen microscopic fields (7.3 mm^2/field) by computer-equipped Optimas image analysis software (Bioscan, Demons, WA) connected to a monochrome CCD camera (MX5) and expressed as mm/cm^2 [7,24].

Gelatin zymography

Gelatinolytic activities of MMPs secreted by hEMVEC were analyzed by zymography on gelatin-containing polyacrylamide gels as described[50]. Using this technique both active and latent species can be visualized. Samples were applied to a 10% (w/v) acrylamide gel co-polymerized with 0.2% (w/v) gelatin. After electrophoresis the gels were washed three times for 10 min in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, 1μmol/L $ZnCl_2$ and 2.5% (w/v) Triton X-100 to remove the SDS, followed by three washes of 5 min in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, 1μmol/L $ZnCl_2$ and incubated overnight at 37ºC. The gels were stained with Coomassie Brilliant Blue R-250.

Immunohistochemistry

Immunohistochemical staining of MT3-MMP was performed in paraffin-embedded sections of human endometrium. Sections were deparaffinized and endogenous peroxidase was quenched with 3% H_2O_2 in 100% methanol. To prevent aspecific binding, sections were incubated with 5% bovine serum albumin for 15 minutes. The primary monoclonal mouse anti-MT3-MMP antibody (1 μg/ml in 1% bovine serum albumin in phosphate buffered saline [BSA/PBS]) was applied overnight at 4ºC, followed by a one hour incubation with a biotinylated secondary horse anti mouse antibody (5 μg/ml in 1% BSA/PBS). Streptavidin-horseradish peroxidase conjugate was used to obtain red staining of the antigens. Specificity of the immunohistochemical reaction was verified by omission of the first antibody as wells as using normal mouse serum in stead of the first antibody. Sections were counterstained with Mauer hematoxylin.

Western blotting

Total cellular extracts were prepared in the presence of protease inhibitors and applied to SDS-PAGE electrophoresis essentially as described[51]. After proteins were blotted onto Immobilon-P polyvinylidene fluoride transfer membranes, the blots were blocked with 5% (w/v) skim milk powder diluted in 20 mM Tris (pH 7.4), 55 mM NaCl, and 0.1% (v/v) Tween-20. Then, blots were incubated with a mouse anti-MT3-MMP antibody or a goat anti-β-actin antibody followed by horseradish peroxidase-conjugated secondary antibodies. All antibodies were diluted in 20 mM Tris (pH 7.4), 55 mM NaCl, 0.1% (v/v) Tween-20, and 5% (w/w) bovine serum. The Super Signal West Dura Extended Duration Substrate and the luminescent image workstation were used for visualization.

RNA Isolation and real-time RT-PCR

Total RNA from hEMVEC and hFMVEC was isolated as described by Chomczynski and Sacchi[52]. RNA was quantified by measuring its absorbance using a spectrophotometer and considered of good quality when the OD_{260}/OD_{280} ratio ranged between 1-8-2.0. Reverse transcription (RT) was carried out in 20 μl volumes using random primers and a cDNA synthesis kit purchased from Promega. MMP and MT-MMP expression was quantified using real-time PCR according to the Taqman method of Applied Biosystems (Perkin Elmer) using a forward and reverse primer combined with a specific (6-carboxy-fluorescein/6-carboxy-tetramethyl-rhodamine [FAM/TAMRA]) double-labeled probe. The following sequences were used for MT3-MMP (MMP-16): forward primer, 5'-GGC TCG TGT GGG AAA TGG TA-3'; reverse primer, 5'-AGA ACT CTT CCC CCT CAA GTG-3'; and probe, 5'-ACA GCT GGC TCT ACT TCC CCA TGG C-3'. Primers and probes for MT1-MMP were described previously (16). All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene GAPDH (VIC-labeled) as follows. For each RNA sample, a difference in Ct values (dCT) was calculated for each mRNA by taking the mean Ct of duplicate wells and subtracting the mean Ct of the duplicate wells for the reference RNA GAPDH measured in the same RT reaction. All RT reactions were carried out in quadruplicate. As positive controls were used: cDNA of human endometrial stromal cells for MMP-12, cDNA of HT1080 cells for MMP-13 and ds cDNA encoding for MMP-3, MMP-7 and MMP-8.

Adenoviral gene transfer of TIMP-1 and TIMP-3 to hEMVEC and hFMVEC

Replication-deficient adenoviral vectors (E1-deleted, transcriptional control *via* the CMV promoter) encoding human TIMP-1 (AdTIMP-1), human TIMP-3 (AdTIMP-3) and a β-galactosidase-encoding adenoviral vector (AdLacZ), as a control, were used for the experiments[43]. Confluent hEMVEC and hFMVEC were washed twice with M199 supplemented with 0.1% HSA to remove human serum components, subsequently the hEMVEC were incubated with the adenoviral constructs in M199 containing 0.1% HSA for 2 hours. After transduction the medium was replaced with hEMVEC culture medium without VEGF-A. 24 h later the cells were seeded on top of a three-dimensional fibrin/fibrin-collagen matrix and stimulation was started 6 h after seeding.

TIMP-1 ELISA and MMP Bioactivity Assays

TIMP-1 antigen was assayed by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Oxon, United Kingdom). MT1-MMP and MMP-9 activity were determined by MMP

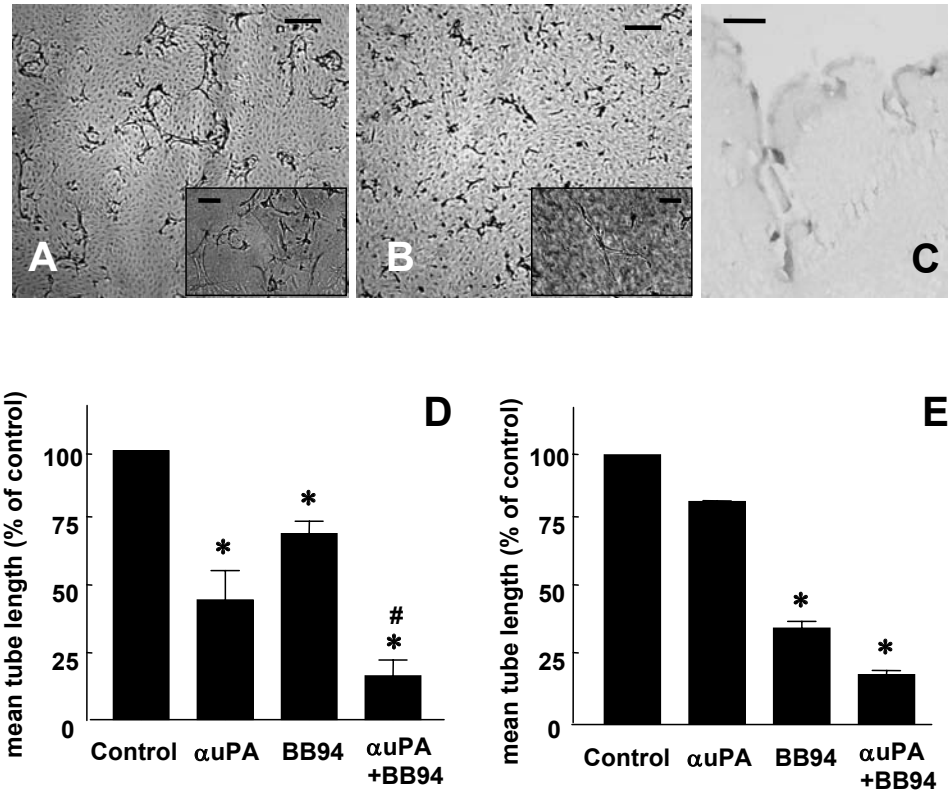


Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.

HEMVEC were cultured on top of a three-dimensional fibrin matrix (A,C,D) or 50-50% fibrin/collagen-type-1 matrix (B,E) and stimulated with VEGF-A (10 ng/mL). A and B: Micrographs taken after 4 days of culturing; *insets* in A and B show details of capillary-like structures. *Bar* = 300 μm, *Bar insets* = 100 μm. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (*bar* = 50 μm). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (αuPA, 100 μg/mL), BB94 (5 μg/mL) or a combination of BB94 and anti-u-PA. After 3-5 days of culturing, mean tube length was measured by image analysis. The data in panel D are expressed as a percentage of VEGF-A-induced tube formation ±SEM of 6 independent experiments of duplicate wells performed with 3 different hEMVEC isolations. Panel E represents 3 experiments. *: $p<0.05$ *vs*. control, #: $p<0.05$ *vs*. αuPA. *[See appendix: color figures]*

activity assays (Biotrak; Amersham, Biosciences, Buckingshamshire, UK) as previously indicated[16,46]. Selective TIMP-3 activity over that of TIMP-1 was assayed by determination of active MT1-MMP in extracts of hEMVEC transduced with AdLacZ, AdTIMP-1, and AdTIMP-3. Inhibition of MMP-9 activity by TIMP-1 and TIMP-3 was determined by addition of serial dilutions of 48-hour conditioned media of hEMVEC transduced with AdLacZ, AdTIMP-1, and AdTIMP-3 to APMA activated recombinant pro-MMP-9.

Statistics

Experiments were performed with duplicate wells and expressed as mean ± SEM. For statistical evaluation the analysis of variance (ANOVA) was used, followed by a modified t-test according to Bonferroni. Statistical significance was accepted at $p < 0.05$.

Results

Capillary-like tube formation by hEMVEC is inhibited by collagen type-I

Three-dimensional matrices were prepared consisting of pure fibrin, collagen or mixtures of fibrin and collagen. As previously reported[9], hEMVEC form spontaneously capillary-like tubular structures in a fibrin matrix, a process that is markedly enhanced by VEGF-A (Fig. 1A, C). When hEMVEC were seeded on top of matrices containing 0-50% type-I collagen homogeneously mixed with fibrin, a concentration-dependent decrease in the extent of tube formation was seen. In a mixed collagen-fibrin matrix (50/50), the decrease was 55±4% under basal conditions (n = 3, not shown) and 53±2% in the presence of VEGF-A (Fig. 1B) as compared to the tube formation in a pure fibrin matrix (Fig. 1A). In a pure collagen type-I matrix, capillary-like structure formation by hEMVEC was hardly detectable, even after stimulation with VEGF-A (data not shown).

U-PA/plasmin and MMPs are involved in tube formation by hEMVEC in matrices composed of fibrin and/or collagen

To establish the involvement of u-PA/plasmin and MMPs in the formation of capillary-like structures by hEMVEC, u-PA-blocking antibodies, the plasmin inhibitor aprotinin, or the broadly acting metalloproteinase inhibitor BB94 were used (Fig. 1C and D). The VEGF-A-enhanced tube formation in a fibrin matrix was reduced by 55±11% by u-PA-blocking antibodies (Fig. 1D), and by 54±7% by the plasmin inhibitor aprotinin (data not shown). In a matrix consisting of an equal mixture of fibrin and collagen anti-u-PA

Table 1. Analysis of MT-MMP mRNA expression in VEGF-A-stimulated hEMVEC and hFMVEC.

		Human Endometrial MVEC		Human Foreskin MVEC	
		CT	dCT	CT	dCT
Transmembrane MT-MMPs	MT1-MMP	27.8 ± 0.4	9.0 ± 0.4	27.3 ± 0.9	8.4 ± 0.4
	MT2-MMP	33.7 ± 1.2	14.1 ± 1.2	35.9 ± 1.6	16.6 ± 1.3
	MT3-MMP	26.4 ± 0.2	7.4 ± 0.4*	27.8 ± 0.8	9.1 ± 0.4
	MT6-MMP	36.3 ± 1.0	17.7 ± 0.8	34.5 ± 1.1	15.6 ± 1.8
GPI-anchored MT-MMPs	MT4-MMP	26.9 ± 0.2	7.3 ± 0.3*	30.2 ± 1.2	10.6 ± 0.6
	MT5-MMP	31.6 ± 0.3	12.8 ± 0.3	33.8 ± 2.1	14.6 ± 1.4

Confluent hEMVEC and hFMVEC were stimulated with 10 ng/ml VEGF-A for 24 hours. After stimulation, RNA was isolated and cDNA was synthesized as described. Real-time RT-PCR for MT-MMP/GAPDH pairs were performed as described and expressed as the number of cycles (CT ± SEM). The housekeeping gene GAPDH was used to correct for the total mRNA content of the samples. The dCT values were calculated as the difference in number of cycles required for the PCR reaction to enter logarithmic phase and expressed as dCT ± SEM. The gene expression of MT3-MMP and MT4-MMP mRNA was significantly higher in hEMVEC compared to the expression in hFMVEC (*: $p<0.01$). The gene expression of the other MT-MMPs was comparable between the two cell types.

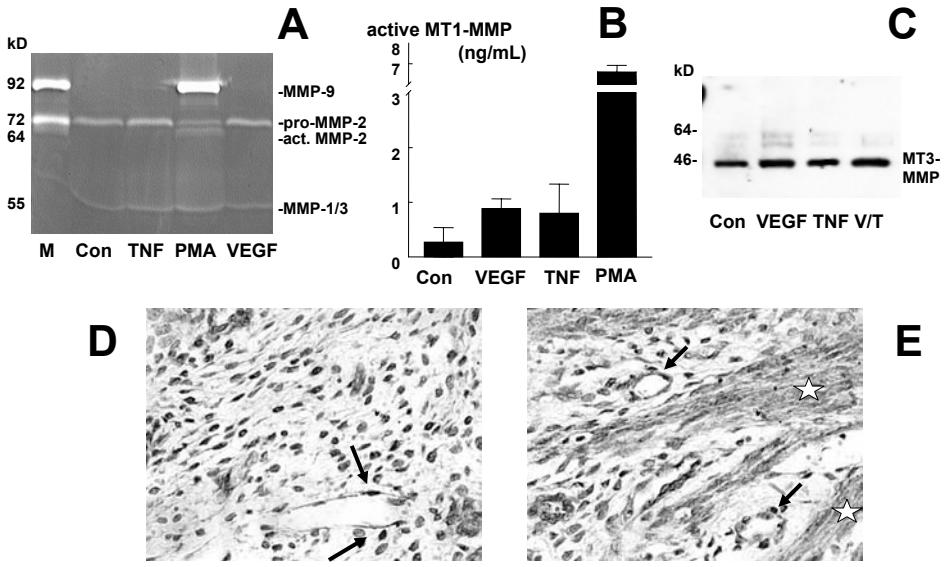


Figure 2. **HEMVEC express various MMPs and MT-MMPs.**
HEMVEC were cultured for 24 h in M199 supplemented with 0.5% HSA (A) or 20% HS (B,C) and were not stimulated (control) or stimulated with TNFα (2.5 ng/mL), VEGF-A (10 ng/mL) or PMA (10^{-8} M), as indicated. A: Gelatin zymography of 24 h conditioned medium. (M = ladder) B: MT1-MMP activity in cell lysates (mean ± range of two experiments performed in duplicate wells with two different isolations; detection limit of the assay 0.2 ng/mL). C: Western blot of MT3-MMP in 24 h conditioned medium. D and E: Immunohistochemical analysis of MT3-MMP in endometrial tissue shows the presence of MT3-MMP in endothelial cells (D, *arrows*) and myometrium (E, *stars*). Similar results were obtained in the tissue of three other donors. *[See appendix: color figures]*

antibodies reduced tube formation only by 17±0% (Fig. 1E). The inhibiting effect of BB94 was increased by adding collagen, since tube formation in pure fibrin was inhibited by 31±5% and in collagen-fibrin matrices by 64±3%. An almost complete inhibition (84±6% and 82±2%, respectively) of capillary-like structure formation was seen after the simultaneous addition of BB94 and anti-u-PA antibodies (Fig. 1D and E).

HEMVEC express various MMPs and MT-MMPs

To study which MMPs are expressed by hEMVEC, real-time RT-PCR was used to assess the expression and regulation of MMP mRNA levels in hEMVEC. Real-time RT-PCR revealed that hEMVEC expressed considerable amounts of MMP-1, MMP-2, MT1-MMP, MT3-MMP and MT4-MMP mRNAs (i.e. less than 30 cycles and dCT< 9) under basal as well as VEGF-A-stimulated conditions. The data for the MT-MMPs are given in Table 1. HFMVEC had a similar expression pattern as hEMVEC, except for MMP-1, which was poorly expressed by hFMVEC under basal conditions (not shown), and MT3-MMP and MT4-MMP, which were expressed to a higher degree in hEMVEC (Table 1). Under basal and VEGF-A-stimulated conditions hEMVEC expressed relatively small amounts of MMP-9 (mean CT = 35.3 ± 1.5 cycles; mean dCT 14.4 ± 1.3 (± SEM)) and MT2-, MT5- and MT6-MMP (Table 1). The MMP-9 mRNA expression increased markedly when the cells were stimulated with 10^{-8} M phorbol ester PMA (mean CT 27.4 ± 1.0; dCT 8.4 ± 0.7 (± SEM)). No mRNA of MMP-3, MMP-7, MMP-8, MMP-12 and MMP-13 was detected in hEMVEC. Positive controls resulted in abundant signals: ds cDNA encoding for MMP-3, MMP-7 and MMP-8, cDNA of human endometrial stromal cells for MMP-12, and cDNA of HT1080 cells for MMP-13.

The expression of active MMPs was confirmed by gelatin zymography and activity assays. Gelatin zymography of serum-free hEMVEC-conditioned media (24 h) showed expression of latent MMP-2 (72 kDa) and a 55kDa band that represents MMP-1 or MMP-3. From the mRNA data we assume that the 55kDa band represents MMP-1 rather than MMP-3. Stimulation with 10^{-8} M PMA induced MMP-9 (92kDa) protein synthesis and activation of MMP-2 (64 kDa, Fig. 2A). The presence of MT1-MMP was demonstrated by activity assay (Fig. 2B). Both VEGF-A and TNFα exposure doubled the activity of MT1-MMP, while phorbol ester caused a dramatic increase in MT1-MMP activity in hEMVEC (Fig. 2B). The presence of MT3-MMP protein was confirmed by Western blotting. The production of MT3-MMP was not affected by TNFα and increased slightly after VEGF-A exposure (Fig. 2C). MT3-MMP was detectable in endothelial cells of proliferative human endometrial tissue, as well as in endometrial epithelial cells and myometrial cells (Fig. 2D and E).

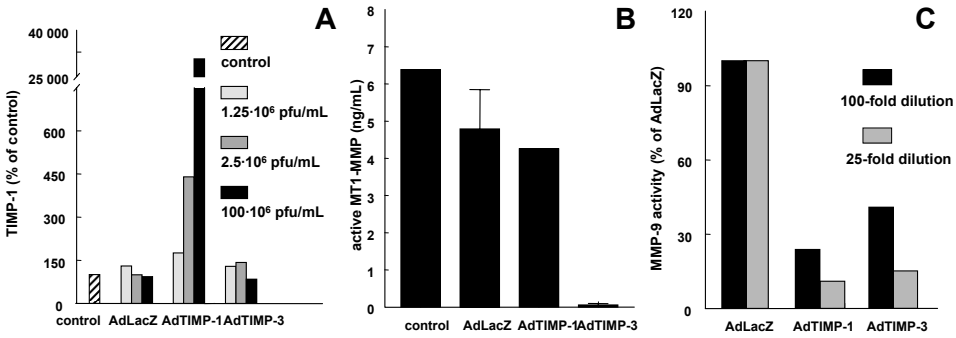


Figure 3. **HEMVEC overexpress active TIMP-1 and –3 antigen after transduction.**
Confluent hEMVEC were transduced with 1.25×10^6, 2.5×10^6 and 1.0×10^8 pfu/mL AdLacZ, AdTIMP-1 or AdTIMP-3 as described in the *Methods* section. After 2 h the medium was removed and the cells were incubated for 6 h with hEMVEC culture medium and incubated for 48 h in M199 supplemented with 0.5 % HSA, 10 ng/mL VEGF-A with or without PMA (10^{-8} M). A: TIMP-1 levels were determined in the hEMVEC-conditioned medium by ELISA following the manufacturers' descriptions. B: MT1-MMP activity in the lysates of PMA-stimulated transduced hEMVEC was inhibited by TIMP-3 and not by TIMP-1. MT1-MMP activity was analyzed as indicated in the *Methods* section. C: The ability of TIMP-1 and TIMP-3 to inhibit MMP-9 activity was measured in 100- (*black bars*) and 25-fold (*grey bars*) dilutions of the conditioned media of hEMVEC overexpressing TIMP-1 or TIMP-3.

Adenoviral gene transfer of both TIMP-1 and TIMP-3 impairs VEGF-A-induced tube formation by hEMVEC

As the general metalloproteinase inhibitor BB94 inhibited tube formation by hEMVEC, the effects of TIMP-1 and TIMP-3, two physiological tissue inhibitors of MMPs, on this process were studied. HEMVEC were infected for 2 h with replication-deficient adenoviruses expressing human TIMP-1 (AdTIMP-1), TIMP-3 (AdTIMP-3) or a control LacZ (AdLacZ). Transduction of hEMVEC with AdTIMP-1 caused a concentration-dependent increase in TIMP-1 antigen production, while AdLacZ or AdTIMP-3 did not affect TIMP-1 production (Fig. 3A). To verify whether the overexpressed TIMP-1 and -3 were functional and active, their effects on MT1-MMP and MMP-9 activity were analyzed. In contrast to cell extracts of AdLacZ- or AdTIMP-1-transduced-hEMVEC, in which MT1-MMP remained active, MT1-MMP activity was completely inhibited in cell extracts of AdTIMP-3-transduced-hEMVEC (Fig. 3B). AdTIMP-1- and AdTIMP-3-transduced hEMVEC inhibited exogenous active MMP-9 comparably (Fig. 3C).

Previous studies on HUVEC and hFMVEC have shown that TIMP-3 was a more potent inhibitor of capillary-tube formation than TIMP-1[15,16,53]. Unexpectedly, in hEMVEC both TIMP-1 and TIMP-3 overexpression inhibited VEGF-A-induced tube formation, to an ex-

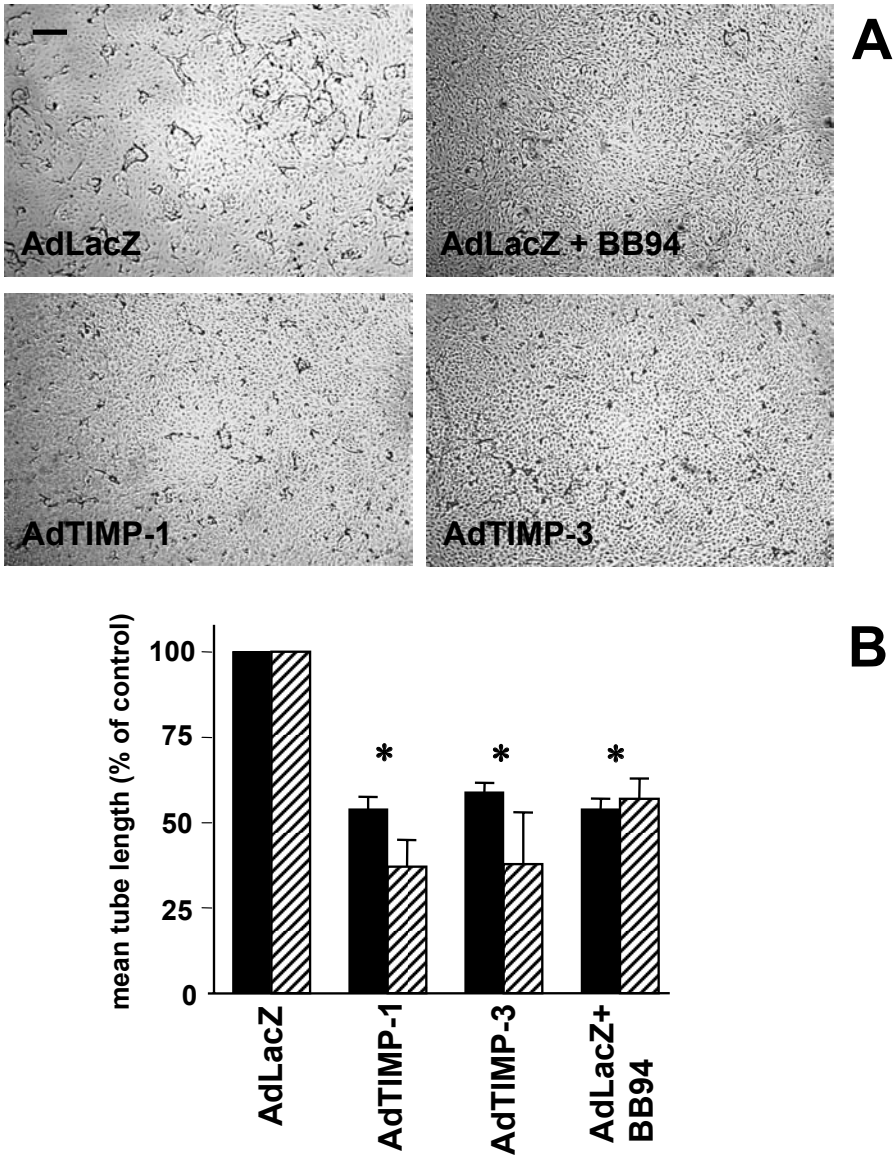


Figure 4. Both TIMP-1 and TIMP-3 inhibit capillary-like tube formation by hEMVEC.
HEMVEC were transduced with 2.5×10^6 pfu/mL AdLacZ, AdTIMP-1 and AdTIMP-3 and were cultured on top of a three-dimensional fibrin matrix or a fibrin-10% collagen matrix and stimulated with VEGF-A (10 ng/mL) with or without BB94 (5 μg/mL). A: Phase contrast micrographs after 3 days of culturing showing tube formation in the fibrin matrix, *Bar* = 300 μm. B: Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells ± SEM/range of 5 (fibrin matrix, *black bars*) and 2 (fibrin-collagen matrix, *striped bars*) independent experiments performed in duplicate wells. The mean tube length of the AdLacZ-transduced hEMVEC was 239±13 mm/cm^2 on the fibrin-collagen matrix. * $p<0.03$ *vs* LacZ transduced cells.

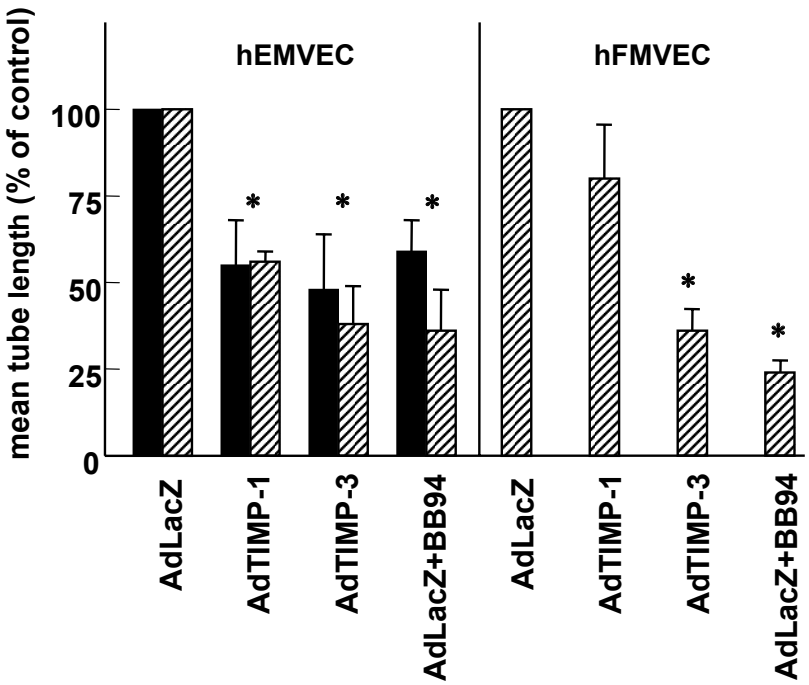


Figure 5. **TIMP-1 inhibits capillary-like tube formation by hEMVEC but not by hFMVEC.** HEMVEC and hFMVEC were transduced with 2.5×10^6 pfu/mL AdLacZ, AdTIMP-1 and AdTIMP-3. Subsequently the cells were cultured on top of a three-dimensional fibrin matrix or a fibrin-10% collagen matrix in M199 supplemented with 10% HS and 10% NBCS and stimulated with VEGF-A (10 ng/mL) and TNFα (10 ng/mL) with or without BB94 (5 μg/mL). Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells ± SEM/range of 2-3 independent experiments performed in duplicate or triplicate wells (fibrin matrix; *black bars*, fibrin-collagen matrix; *striped bars*). The mean tube length of the AdLacZ-transduced hEMVEC was 270±80 mm/cm^2 on the fibrin matrix and 266±83 mm/cm2 on the fibrin-collagen matrix, * $p<0.05$ *vs*. LacZ transduced cells.

tent similar as BB94 (Fig. 4A and B). This was found both in fibrin and in fibrin-collagen matrices (Fig. 4B). No apparent cell death or morphological changes were observed either in the AdTIMP-1- or AdTIMP-3-transduced hEMVEC.

Comparison of the effect of TIMP-1 and TIMP-3 overexpression on tube formation by hEMVEC and hFMVEC

Because of the lack of effect of TIMP-1 on tube formation in our previous experiments with VEGF/TNFα-stimulated hFMVEC[16], we compared the effects of TIMP-1 and TIMP-3 overexpression on capillary-like tube formation by hEMVEC and hFMVEC under identi-

cal culture conditions. Both cells types were grown on a fibrin-10% collagen matrix and stimulated by the simultaneous addition of VEGF and TNFα, which is required to induce tubules by hFMVEC[11]. Like in VEGF-stimulated hEMVEC, both TIMP-1 and TIMP-3 reduced capillary-like tube formation in VEGF/TNFα-stimulated hEMVEC to the same extent as BB94 (Fig. 5, *striped bars*). In contrast, only TIMP-3 inhibited tube formation by hFMVEC to a significant extent. Similar data were obtained with fibrin matrices (Fig. 5, *black bars*). No significant cell detachment was observed in the AdTIMP-1- or AdTIMP-3-transduced hFMVEC and hEMVEC grown on the fibrin matrix, neither under control conditions nor in cells stimulated with VEGF/TNFα or TNFα alone (data not shown). This indicates that the overexpression of TIMP-1 or TIMP-3 did not induce a visible degree of apoptosis or cell death under our experimental conditions.

Inhibition of MT3-MMP reduces tube formation by hEMVEC

The inhibition of tube formation by both TIMP-1 and TIMP-3 overexpression indicates that MMPs other than MT1-MMP play a role in the regulation of tube formation by hEMVEC. To obtain evidence for the involvement of MT3-MMP in the regulation of this process, tube formation by hEMVEC was induced in the presence of anti-MT3-MMP IgG. Inhibition of MT3-MMP significantly reduced the VEGF-A-enhanced capillary-like tube formation by hEMVEC, while non-specific anti-FITC IgG had no effect (Fig. 6). The inhibition of VEGF-enhanced tube formation by MT3-MMP IgG was 48.8% of the inhibition achieved by BB94, suggesting that other metalloproteinases may contribute additionally.

Discussion

The present study demonstrates that both the u-PA/plasmin system and MMPs contribute to the invasion and tubular structure formation by endothelial cells in a 3D-fibrin-collagen matrix. Since TIMP-1 and TIMP-3 overexpression reduced capillary-like tube formation by hEMVEC to the same extent, not primarily MT1-MMP, but other MMPs play a regulatory role in this process in hEMVEC. Major MMPs expressed by hEMVEC were MMP-1, MMP-2, MT1-MMP (MMP-14) MT3-MMP (MMP-16) and MT4-MMP (MMP-17) under basal and VEGF-A-stimulated conditions. Our data suggest that MT3-MMP is involved in the regulation of tube formation by hEMVEC, because tube formation by hEMVEC was inhibited of by anti-MT3-MMP IgG *in vitro* (Fig. 6), and MT3-MMP was encountered in endothelial cells of proliferative endometrium *in vivo* (Fig. 2).

Our data on the expression of MMPs by hEMVEC *in vitro* are in agreement with observations reported from immunohistochemical studies in endometrial tissue sections.

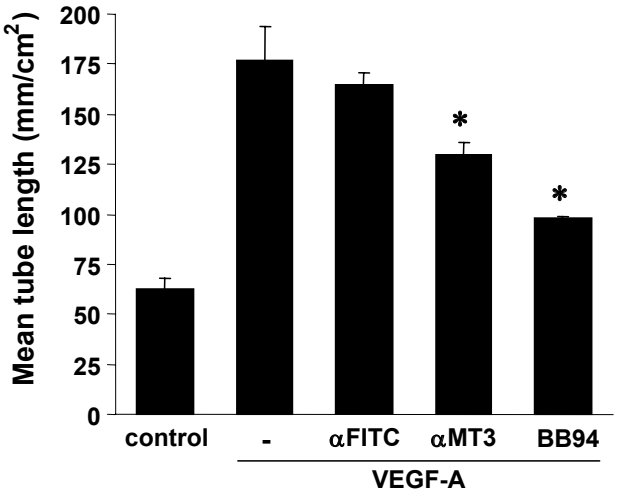


Figure 6. **Inhibition of MT3-MMP reduces tube formation by hEMVEC.**
HEMVEC were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS. Cells were cultured under control conditions in the presence of 0,5 ng/ml VEGF or stimulated with VEGF-A (10 ng/mL), VEGF-A (10 ng/mL) and anti-FITC IgG (25 μg/ml), VEGF-A (10 ng/mL) and anti MT3-MMP IgG (25 μg/ml) or VEGF-A (10 ng/mL) and BB94 (5 μg/mL). Mean tube length was measured and expressed as the mean tube length ± SEM/range of 2 experiments performed in duplicate wells. * $p<0.02$ *vs* VEGF stimulated hEMVEC; $p<0,05$ vs VEGF /anti FITC IgG treated hEMVEC.

Freitas *et al.*[54] found MMP-1, MMP-2, MMP-3 and MMP-9 in endometrial vascular structures, which might include endothelial cells. MMP-2 was demonstrated in newly formed capillary strands[54]. Skinner *et al.*[55] only found MMP-9 on endometrial endothelial cells after exposure to high progestagen levels. MT1-MMP was detected at low levels on endothelial cells in proliferative and secretory endometrium[36,37]. MT2-MMP was observed at a constant low level throughout the menstrual cycle[35,36]. In addition, TIMP-1, -2, and -3 were demonstrated in endometrial endothelial cells by in situ hybridization[26,37,56]. Recently Goffin *et al.* also reported the presence of MMP-19 mRNA in endometrial tissue throughout the cycle and the mRNAs of MMP-7, MMP-26 and MT3-MMP in this tissue during the proliferative phase of the cycle[35]. However, no information on their expression by specific cells is currently available.

Within the large group of MMPs the MT-MMPs attract specific attention, because of their membrane localization that enables them to regulate localized proteolytic activities directly at the cell-matrix interaction sites. Hotary *et al* showed that overexpression of the transmembrane MT1-MMP, MT2-MMP or MT3-MMP induced endothelial invasion and tube formation in fibrin, while the GPI-anchored MT4-MMP was unable to do so[28]. MT1-MMP and MT3-MMP are involved in the migration and invasion of various mesen-

chymal cells, such as fibroblasts and smooth muscle cells[57], while other cells, such as leukocytes and trophoblasts, use MT2-MMP[58,59]. Our data indicate that human endometrial endothelial cells *in vitro* largely express MT1-MMP, MT3-MMP and MT4-MMP while only tiny amounts of MT2- and MT5-MMP mRNA are present. Previous studies on HUVEC and hFMVEC[14-16,28,53] indicated that invasion and tube formation of endothelial cells was inhibited by TIMP-3 and not by TIMP-1, suggesting that MT1-MMP has a dominant role among the MMPs in regulating endothelial migration and invasion. The present data confirm our previous data for hFMVEC, but also show consistently that both TIMP-1 and TIMP-3 inhibited tube formation by endometrial endothelial cells. Although these data do not exclude the involvement of MT1-MMP, they strongly suggest that other MMPs than MT1-MMP may contribute more dominantly to endometrial angiogenesis.

The expression of MMP-1 differed markedly between hEMVEC and hFMVEC, however a role for MMP-1 is less likely since MMP-1 is only upregulated in the secretory phase of the menstrual cycle and not in the proliferative phase. However, data on cell-specific expression are required before definitive conclusions can be drawn. A second possible explanation of the comparable inhibition by TIMP-1 and TIMP-3 might be that MT1-MMP acts in concert with other MMPs, in particular MMP-2, and that inhibition of the other MMPs is rate-limiting. However, the comparable expressions of MMP-2 and MT1-MMP in hEMVEC and hFMVEC do not favor this suggestion. Finally, a more likely candidate may be MT3-MMP, which like MT1-MMP can contribute potently to angiogenesis in a fibrinous matrix[28]. The recent finding that the expression of MT3-MMP mRNA is elevated in endometrial tissue during the proliferative phase of the menstrual cycle suggests such a role[35]. Furthermore, our data on the relative expressions of MT3-MMP mRNAs in hEMVEC and hFMVEC, the presence of MT3-MMP protein on endometrial endothelial cells and the inhibition of capillary tube formation by inhibiting MT3-MMP are strongly in favor of a contribution of MT3-MMP in capillary-like tube formation by hEMVEC.

To summarize, MMPs contribute to *in vitro* capillary tube formation by human endometrial endothelial cells. Whereas capillary tube formation by hFMVEC depends largely on MT1-MMP, the described data for hEMVEC suggest that other MMPs than MT1–MMP, in particular MT3-MMP, play an important role in tube formation by human endometrial endothelial cells.

Acknowledgements

We would like to thank Dr. R. M. F. van der Weiden (St. Franciscus Gasthuis, Rotterdam, The Netherlands) and Dr. R.A. Verwey and colleagues (Bronovo Hospital, The Hague, The Netherlands), who provided us with endometrial tissue. We would like to thank Dr. R. Kleemann for his advice and help regarding Western blotting.

References

1. Folkman J 1995 Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1: 27-31.
2. Carmeliet P 2003 Angiogenesis in health and disease. Nat Med 9:653-60.
3. Smith SK 1998 Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 4: 509-519.
4. Rogers PA, Gargett CE 1998 Endometrial angiogenesis. Angiogenesis 2: 287-294.
5. Bacharach E, Itin A, Keshet E 1992 *In vivo* patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc Natl Acad Sci U S A 89:10686-10690.
6. Hanahan D, Folkman J 1996 Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86: 353-64.
7. Bergers G, Benjamin LE 2003 Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3:401-10.
8. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein N, Brown PO 2003 Endothelial cell diversity revealed by global expression profiling. PNAS 100:10623-10628.
9. Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van Der V, Helmerhorst FM, van Hinsbergh VWM 2001 Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. J Clin Endocrinol Metab 86:3359-3367.
10. Defilippi P, van Hinsbergh VWM, Bertolotto A, Rossino P, Silengo L, Tarone G 1991 Differential distribution and modulation of expression of alpha 1/beta 1 integrin on human endothelial cells. J Cell Biol 114:855-863.
11. Koolwijk P, van Erck MG, de Vree WJA, Vermeer MA, Weich HA, Hanemaaijer R, Van Hinsbergh VWM1996 Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol 132:1177-1188.
12. Kroon ME, Koolwijk P, van Goor H, Weidle UH, Collen A, van der Pluijm G, van Hinsbergh VWM 1999 Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. Am J Pathol 154:1731-1742.
13. Pepper MS 2001 Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 21:1104-17.
14. Galvez BG, Matias-Roman S, Albar JP, Sanchez-Madrid F, Arroyo AG 2001 Membrane type 1-matrix metalloproteinase is activated during migration of human endothelial cells and modulates endothelial motility and matrix remodeling. J Biol Chem 276:37491-37500.
15. Lafleur MA, Handsley MM, Knauper V, Murphy G, Edwards DR 2002 Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). J Cell Sci 115:3427-3438.
16. Collen A, Hanemaaijer R, Lupu F, Quax PH, van Lent N, Grimbergen J, Peters E, Koolwijk P, van Hinsbergh VWM 2003 Membrane-type matrix metalloproteinase-mediated angiogenesis in a fibrin-collagen matrix. Blood 101:1810-1817.
17. Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ 2000 Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. J Cell Biol 149:1309-1323.
18. Pepper MS, Belin D, Montesano R, Orci L, Vassalli JD. 1990 Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells *in vitro*. J Cell Biol 111:743-755.
19. Pepper MS 1997 Manipulating angiogenesis. From basic science to the bedside. Arterioscler Thromb Vasc Biol 17:605-619.
20. Werb Z 1997 ECM and cell surface proteolysis: regulating cellular ecology. Cell 91:439-442.
21. Visse R, Nagase H 2003 Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92:827-839.
22. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ Carmeliet P 1999 Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med 5:1135-1142.
23. Stetler-Stevenson WG 1999 Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest 103:1237-1241.
24. Salamonsen LA., Woolley DE 1996 Matrix metalloproteinases in normal menstruation. Hum Reprod 11 S2:124-133.

25. Tabibzadeh S, Babaknia A 1996 The signals and molecular pathways involved in human menstruation, a unique process of tissue destruction and remodelling. Mol Hum Reprod 2:77-92.
26. Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F, Osteen KG 1994 Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest 94: 946-953.
27. Smith SK 2001 Regulation of angiogenesis in the endometrium. Trends Endocrinol Metab 12:147-151.
28. Hotary KB, Yana I, Sabeh F, Li XY, Holmbeck K, Birkedal-Hansen H, Allen ED, Hiraoka N, Weiss SJ 2002 Matrix metalloproteinases (MMPs) regulate fibrin-invasive activity *via* MT1-MMP-dependent and -independent processes. J Exp Med 195:295-308.
29. Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K 2000 Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. Proc Natl Acad Sci U S A 97:4052-4057.
30. Sounni NE, Devy L, Hajitou A, Frankenne F, Munaut C, Gilles C, Deroanne C, Thompson EW, Foidart JM, Noel A 2002 MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. FASEB J 16:555-564.
31. Deryugina EI, Bourdon MA, Jungwirth K, Smith JW, Strongin AY 2000 Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int J Cancer 86:15-23.
32. Taraboletti G, D'Ascenzo S, Borsotti P Giavazzi R, Pavan A, Dolo V 2002 Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. Am J Pathol 160:673-680.
33. Vagnoni KE, Zheng J, Magness RR 1998 Matrix metalloproteinases-2 and -9, and tissue inhibitor of metalloproteinases-1 of the sheep placenta during the last third of gestation. Placenta 19:447-455.
34. Shofuda KI, Hasenstab D, Kenagy R, Shofuda T, Li ZY, Lieber A, Clowes AW 2001 Membrane-type matrix metalloproteinase-1 and -3 activity in primate smooth muscle cells. FASEB J15:2010-2012.
35. Goffin F, Munaut C, Frankenne F, Perrier D'Hauterive S, Beliard A, Fridman V, Nervo P, Colige A, Foidart JM 2003 Expression Pattern of Metalloproteinases and Tissue Inhibitors of Matrix-Metalloproteinases in Cycling Human Endometrium. Biol Reprod 69: 976-84.
36. Zhang J, Hampton AL, Nie G, Salamonsen LA 2000 Progesterone inhibits activation of latent matrix metalloproteinase (MMP)-2 by membrane-type 1 MMP: enzymes coordinately expressed in human endometrium. Biol Reprod 62:85-94.
37. Maatta M, Soini Y, Liakka A, Autio-Harmainen H 2000Localization of MT1-MMP, TIMP-1, TIMP-2, and TIMP-3 messenger RNA in normal, hyperplastic, and neoplastic endometrium. Enhanced expression by endometrial adenocarcinomas is associated with low differentiation. Am J Clin Pathol 114:402-411.
38. Chung HW, Lee JY, Moon HS, Hur SE, Park MH, Wen Y. Polan ML 2002 Matrix metalloproteinase-2, membranous type 1 matrix metalloproteinase, and tissue inhibitor of metalloproteinase-2 expression in ectopic and eutopic endometrium. Fertil Steril 78: 787-795.
39. Salamonsen LA 1994 Matrix metalloproteinases and endometrial remodelling. Matrix Cell Biol Int 18:1139-1144.
40. Woessner JFJ 2001 That impish TIMP: the tissue inhibitor of metalloproteinases-3. J Clin Invest 108:799-800.
41. Li H, Lindenmeyer F, Grenet C, Opolon P, Menashi S, Soria C, Yeh P, Perricaudet M, Lu H 2001 AdTIMP-2 inhibits tumor growth, angiogenesis, and metastasis, and prolongs survival in mice. Hum Gene Ther 12:515-526.
42. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R 1979 An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc Natl Acad Sci U S A 76:5674-5678.
43. Quax PH, Lamfers ML, Lardenoye JH, Grimbergen JM, de Vries MR, Slomp J, de Ruiter MC, Kockx M M, Verheijen JH, van Hinsbergh VWM 2001 Adenoviral expression of a urokinase receptor-targeted protease inhibitor inhibits neointima formation in murine and human blood vessels. Circulation 103:562 -569.
44. Lamfers ML, Grimbergen JM, Aalders MC, Havenga MJ, de Vries MR, Huisman LG, van Hinsbergh VW, Quax PH 2002 Gene transfer of the urokinase-type plasminogen activator receptor-targeted matrix metalloproteinase inhibitor TIMP-1.ATF suppresses neointima formation more efficiently than tissue inhibitor of metalloproteinase-1. Circ Res 91:945-952.
45. Van der Laan WH, Quax PH, Seemayer CA, Huisman LG, Pieterman EJ, Grimbergen JM, Verheijen JH, Breedveld FC, Gay RE, Gay S, Huizinga TW, Pap T 2003 Cartilage degradation and invasion by rheumatoid syno*via*l fibroblasts is inhibited by gene transfer of TIMP-1 and TIMP-3. Gene Ther 10:234-242.
46. Hanemaaijer R, Visser H, Konttinen YT, Koolwijk P, Verheijen JH 1998 A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjogren's syndrome contain increased latent and active gelatinase-B levels. Matrix Biol 17:657-665.
47. Van Boheemen PA, Van den Hoogen CM, Koolwijk P 1995 Comparison of the inhibition of urokinase-type plasminogen activator (u-PA) activity by monoclonal antibodies specific for u- PA as assessed by different assays. Fibrinolysis 9:343-349.

48. Noyes RW, Hertig AT, Rock J 1975 Dating the endometrial biopsy. Am J Obstet Gynecol 122:262-263.
49. Van Hinsbergh VWM, Sprengers ED, Kooistra T 1987 Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. Thromb Haemost 57:148-153.
50. Birkedal-Hansen H, Taylor RE 1982 Detergent-activation of latent collagenase and resolution of its component molecules. Biochem Biophys Res Commun 107:1173-1178.
51. Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HM, Kooistra T 2003 Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. Blood 101: 545-551.
52. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159.
53. Anand-Apte B, Pepper MS, Voest E, Montesano R, Olsen B, Murphy G, Apte SS, Zetter B 1997 Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. Invest Ophthalmol Vis Sci 38:817-823.
54. Freitas S, Meduri G, Le Nestour E, Bausero P, Perrot-Applanat M 1999 Expression of metalloproteinases and their inhibitors in blood vessels in human endometrium. Biol Reprod 61:1070-1082.
55. Skinner JL, Riley SC, Gebbie AE, Glasier AF, Critchley HO 1999 Regulation of matrix metalloproteinase-9 in endometrium during the menstrual cycle and following administration of intrauterine levonorgestrel. Hum Reprod 14:793-799.
56. Chegini N, Rhoton-Vlasak A, Williams RS 2003 Expression of matrix metalloproteinase-26 and tissue inhibitor of matrix metalloproteinase-3 and -4 in endometrium throughout the normal menstrual cycle and alteration in users of levonorgestrel implants who experience irregular uterine bleeding. Fertil. Steril 80: 564-570.
57. Vernon RB, Lara SL, Drake CJ, Angello JC, Little CD, Wight TN, Sage EH 1995 Organized type I collagen influences endothelial patterns during "spontaneous angiogenesis *in vitro*": planar cultures as models of vascular development. *In vitro* Cell Dev Biol Anim 31:120-131.
58. Eeckhout Y, Vaes G 1977 Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Effects of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. Biochem J 166:21-31.
59. Giavazzi R, Albini A, Bussolino F, DeBraud F, Presta M, Ziche M, Costa A 2000 The biological basis for antiangiogenic therapy. Eur J Cancer 36:1913-1918.

6

STEROIDS AND CYTOKINES IN ENDOMETRIAL ANGIOGENESIS
- review -

K. Kapiteijn
P. Koolwijk
R.M.F. van der Weiden
F.M. Helmerhorst
T. Kooistra
V.W.M. van Hinsbergh

Anticancer Research 2001;21:4231-4242

Cyclic regulation of angiogenesis in the endometrium

The cyclic remodeling of the endometrium results in a receptive endometrium, essential for successful implantation and placentation. Angiogenesis (Fig. 1) is needed to support the proliferation and differentiation of endometrial cells after menstruation[9]. The menstrual cycle consists of three consecutive phases: the proliferative phase, the secretory phase and the menstruation (Fig. 2). Angiogenesis occurs during post-menstrual repair and the subsequent thickening of the endometrial tissue. The post-menstrual repair process occurs during the early proliferative phase. The subsequent episode of angiogenesis takes place during the mid-proliferative phase and contributes, in interaction with other tissue cells, to further thickening of the endometrium under the influence of increasing estrogen concentrations. Further adaptation of the newly-formed vessels, including enlargement of the vascular structures, proceeds during the early secretory phase[10]. As a result, spiral (coiled) arteries and a subepithelial capillary complex are developed in the endometrium[11]. During the late secretory and menstrual phase little angiogenic activity is observed[12].

The spiral arteries, which are highly sensitive to ovarian steroids, maintain the blood supply of the functional layer of the endometrium (Fig. 3)[13]. The functional endometrium is shed during menstruation due to the fall in steroid level[14]. The blood supply to the basal layer of the endometrium is *via* the basal (straight) arteries, which are considered to be insensitive to steroidal stimulation[15]. The basal layer gives rise to a new functional layer: new arteries form from arterial stumps left over after menstruation. Endometrial cellular growth and differentiation combined with changes in vascular permeability transform a dense, thin endometrium into a highly edematous, thick endometrium[16].

Markee[13] was the first to demonstrate the steroidal regulation of endometrial angiogenesis. Endometrium was transplanted into the anterior chamber of a rhesus monkey eye and endometrial angiogenic activity was found to parallel the changes in the uterus. Later, Abel[17] observed rapid cyclic vascularisation of human endometrial explants that were transplanted to the hamster cheek pouch. In both models, the extent of vascularisation and incidence of bleeding were influenced by ovarian steroids.

Endothelial cells of the endometrium appear to be highly angiogenic, as can be concluded from studies in our own laboratory and from studies by others which showed that endometrial tissue induced an angiogenic response in the chorioallantoic membrane (CAM) assay. In contrast, endothelial cells from most other benign tissues did not easily show angiogenic activity[18]. Therefore the endometrium is pre-eminently suited for studying the effects of steroids on the process of angiogenesis.

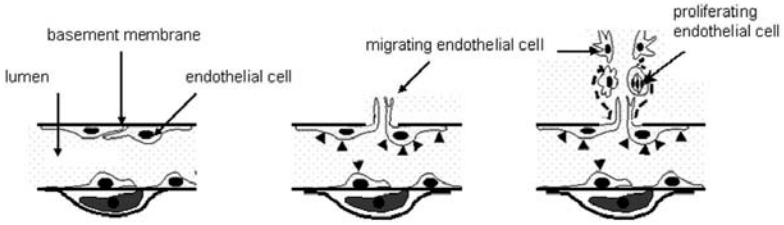


Figure 1. In mature (non-growing) capillaries the vessel wall is composed of an endothelial cell lining and a basement membrane, in which pericytes (blue) usually are present. Angiogenic factors (▲) bind to endothelial cell receptors and initiate angiogenesis. When the endothelial cells are stimulated by angiogenic growth factors, they secrete proteolytic enzymes like metallo proteinases (MMPs) and enzymes of the plasminogen activator (PA) system, which degrade the basement membrane surrounding the vessel. The junctions between endothelial cells are loosened, the cells migrate through the space created, and the newly formed sprouts migrate and proliferate. *[See appendix: color figures]*

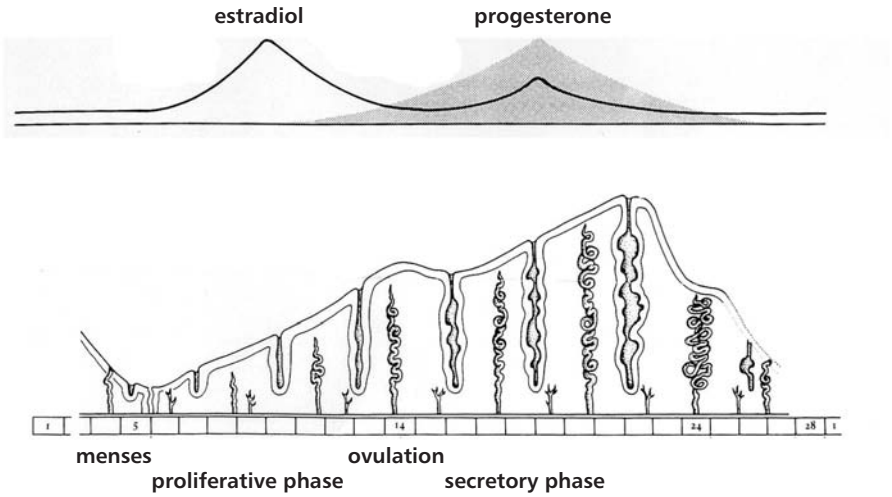


Figure 2. The endometrium undergoes cycles of rapid growth, remodeling, differentiation and angiogenesis, directly or indirectly in response to changes in ovarian steroids. After menstruation increased angiogenesis takes place as a process of post-menstrual repair. During the mid-proliferative phase and early secretory phase further thickening of the endometrium is supported by angiogenesis. As a result elongated, coiled spiral arteries and a subepithelial capillary complex can be seen at the end of the early secretory phase. In the absence of pregnancy, progesterone declines, coincident with breakdown of the functionalis layer, which is expelled with the menstrual flow. Estradiol rises at the end of menstruation, coincident with a new cycle of tissue renewal originating from the intact basal layer.

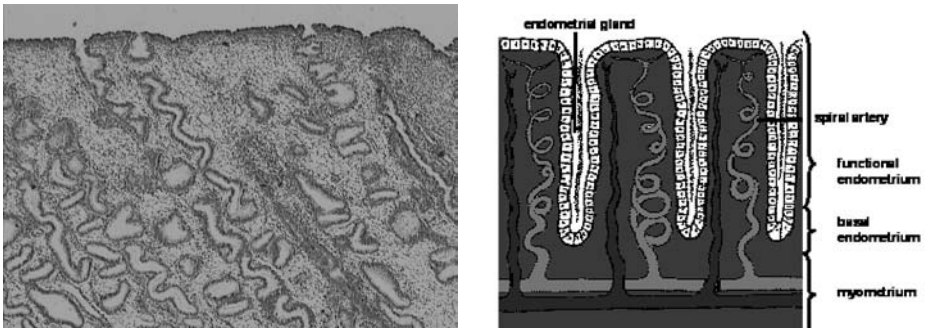


Figure 3. After menstruation from the basal endometrium a new functional endometrium grows. The basal arteries give rise to new blood vessels, which will form spiral arteries and a subepithelial capillary complex. Together with stromal and epithelial growth and differentiation, and increased vascular permeability, an edematous, thick receptive endometrium is prepared for implantation. *[See appendix: color figures]*

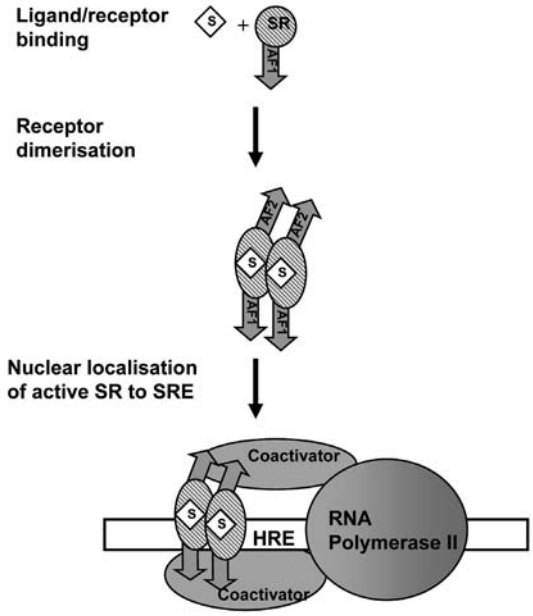


Figure 4. Steroid (S) binding to steroid receptor (SR) initiates a sequence of events including dimerization and binding to DNA sequences, termed hormone response elements (HRE), in the regulatory regions of target genes. The ligand-receptor complex recruits other proteins to the transcriptional complex which act with SR as co-activators or co-repressors of transcription. It is likely that ligand induced conformational changes in the SR influence protein-protein interactions in the transcriptional complex by altering the relative orientation of the independent transcriptional activations domain (AF).

Steroids

Steroid hormones are small lipid-soluble molecules with a structure derived from cholesterol. The steroid hormones include the sex steroids (estrogens, progestagens and androgens; Table 1), glucocorticoids, mineralocorticoids, cholecalciferol, and derivatives. The sex steroids promote sexual function (reproduction) and are responsible for the development of the male and female sexual characteristics. The male sex steroids (androgens, especially testosterone) are produced in the Leydig interstitial cells of the testes and in small amounts also in the ovary and the adrenal cortex. The female sex-hormones estradiol and progesterone are produced in the ovary, the placenta and in small amounts also in the adrenal cortex. Estradiol and progesterone have an important role in the cyclic alterations of the endometrium.

Steroid receptors

Steroids can pass through the cell membrane and bind to specific (nuclear) receptors. The steroid hormone receptors belong to a large superfamily of ligand-regulated nuclear receptors, which share a common structural and functional organization with distinct domains[19]. Nuclear receptors can control the activity of target genes through direct association with specific DNA sequences known as hormone response elements (HREs)[20,21]. The nuclear receptors bind mainly as dimers to the HRE and each monomer interacts with a half-site sequence within the HRE. Receptor-binding specificity is determined by the primary nucleotide sequence, but also by orientation (palindromic or direct repeats) and spacing between the two half sites of the HRE. After binding to DNA, the receptor is thought to interact with co-activators, co-repressors and other transcription factors that link the receptor to components of the basal transcriptional machinery, including RNA polymerase II (Fig. 4).

Two estrogen receptors (ERs) are known, ERα and ERβ. The two ERs are derived from different genes, and appear to have unique tissue distribution and their own sets of specific functions[22,23]. The human ERβ shows homology to ERα especially in the central DNA-binding domain, less in the ligand-binding domain, with only minimum homology in the amino-terminal domain, which has a transactivating function.

It is becoming increasingly clear that the classical model of ER action, *i.e.* ligand-activated ERs interact as homodimers with high-affinity estrogen response elements (EREs) within target gene promoters, is too simple. ERα and ERβ can also interact with DNA sites that do not contain EREs, for example the activator protein-1 site[24]. Also, ERα can modulate gene expression *via* complexing of the transcription factors NF-IL6 and NFκB[25]. Furthermore, estrogens may also act *via* receptors on the cell surface to achieve rapid, non-genomic effects[26,27]. These recent discoveries of several new regulatory mechanisms

Table 1. Sex steroids.

Androgens	Progestagens	Estrogens
5α-Dihydrotestosterone*	Progesterone*	17β-Estradiol*
Testosterone	17α-Hydroxyprogesterone	Estradiol
Androstenedione	20α-Dihydroprogesterone	Estrone
Dehydroepiandrosterone		

*Most effective endogenous steroid at binding and activating its receptor.

Table 2. Relative antiangiogenic potency of steroids.

Compound	Percent potency
Tetrahydrocortisol	100
17α-Hydroxyprogesterone	56
Hydrocortisone	51
11α-Epihydrocortisol	14
Cotexolone	12
Corticosterone	12
Desoxycorticosterone	8
Testosterone	3
Estrone	2
Progesterone	0
Pregnelone	0
Cholesterol	0

Relative antiangiogenic potency of steroids and related compounds in the CAM-assay (J. Folkman and D.E. Ingber 1987)

are probably only a fraction of the many different ways in which ERs can alter cellular functioning.

Progesterone receptor isoforms A and B (PRA, PRB) are the two principal mediators of the biological activities of progesterone in humans and many other vertebrate species. The A- and B- isoforms arise from a single gene and are identical except for the extended N-terminus of B-receptors. Evidence is accumulating that the two isoforms differ extensively in function, suggesting that their ratio of expression may control progesterone responsiveness in target cells[21,28].

Only in recent literature has a distinction been made between ERα and ERβ and PRA and PRB. Consequently, in discussing the older literature we will use the terms ER and PR without specifying which isoform(s) is (are) involved.

In the endometrium epithelial and stromal cells express ERs and PRs[29-33]. The higher uterine expression of ERα as compared to ERβ may suggest that ERα is responsible for mediating the uterotropic response upon estrogen exposure[34]. ERα knockout mice have a uterus that shows a lack of cell proliferation[35] while ERβ knockout mice show diminished reproductive capacity (small litter size, multiple resorbed fetuses)[36]. ERβ seems to act as a modulator of ERα-mediated gene transcription in the uterus (anti-uterothropic); furthermore ERβ is responsible for the down-regulation of PR in the luminal epithelium[36]. PR knockout mice show an inflammatory response to estradiol in the uterus, with no specific differentiation of the endometrial cells (decidual response)[35]. The expression of the ERs and PRs within stromal and epithelial cells varies during the course of the menstrual cycle[30,37]. Estrogen induces ER and PR during the proliferative phase; progesterone has therefore mainly an effect on an estrogen-primed endometrium[35]. In addition, progesterone by itself and steroid withdrawal downregulate the PR and ER expression[31,38]. PR reaches highest concentrations around mid-cycle, and ER around the mid-proliferative phase, correlating with the plasma peak of estradiol and the maximum mitotic rate of the endometrial cells[38,39]. The receptors decrease during the secretory phase[40]. During the late secretory phase, PR disappears from the glandular epithelial cells, but not from the stromal cells[37,38].

Contradictory results have been reported on the expression of ER and PR in endometrial endothelial cells: Iruela-Arispe *et al.*[41] were able to detect ER and PR in endothelial cells, whereas Kohnen *et al.*[32] were not. Therefore, it remains to be established whether the effects of estrogens and progestagens on angiogenesis are directly on endothelial cells or indirectly *via* cytokines derived from estrogen- or progestagen-activated stroma or epithelial cells.

The role of angiogenic growth factors in the endometrium and their regulation by steroids

Polypeptide growth factors are recognized as key regulators of cell proliferation, differentiation and angiogenesis. Several angiogenic factors are synthesized in the endometrium[42,43]. Only those angiogenic factors that have been found (or suggested) to respond to ovarian steroids in the endometrium are discussed. These factors include vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), epidermal growth factor (EGF), insulin-like growth factors (IGFs), transforming growth factor α (TGFα), transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), thymidine phosphorylase, adrenomedullin and erythropoietin (Epo).

Endometrial angiogenesis depends on locally produced growth factors and cytokines, which act in a paracrine way on endothelial cells. It has been generally assumed that

these angiogenic growth factors are under steroidal control on the basis of the cyclic variation of these factors, their receptors and regulatory proteins[18,44] and the *in vivo* modulation of these factors by steroids in endometrial tissue[45]. The angiogenic growth factors exert their action by affecting endothelial proliferation and migration/invasion. The latter process depends on the interaction of endothelial cells with their extracellelular matrix, which is controlled by the expression of integrins and matrix-degrading proteases, in particular the plasminogen activator/plasmin system and matrix-degrading metalloproteinases (MMPs). Ample evidence has been provided that the expression of these matrix degrading proteases is under the control of steroids in the endometrium[42,46-51].

Steroids stimulate paracrine VEGF production

A major angiogenic stimulus is the endothelial cell mitogen VEGF. In addition to inducing endothelial proliferation, it modulates the expression of many genes including proteases, it affects endothelial permeability, and it is involved in the maintenance of immature blood vessels[9,52]. Various cell types in the endometrium express VEGF, while estradiol and progesterone appear to regulate its expression. VEGF expression is an important target for estrogens and progestagens in the regulation of angiogenesis in the endometrium.

In the human endometrium, the glandular epithelium and stromal cells produce VEGF, with a higher expression in the glands than in the stroma[53-56]. Stromal macrophages and leucocytes may also be an important source of VEGF[57,58]. VEGF diffuses into the interstitial tissue and binds to capillaries and spiral arteries. The predominant endometrial isoforms of VEGF are the diffusible $VEGF_{121}$ and $VEGF_{165}$, whereas the $VEGF_{189}$ and a $VEGF_{145}$ isoforms are only weakly detectable[16,54,56,59]. Several studies have reported a cyclic or a steroid-dependent variation in the expression of VEGF and VEGF receptors in the endometrium and in isolated endometrial stromal cells[16,29,55,60-65]. This expression pattern, mainly seen in the endometrial stroma, appears to run parallel to the proliferation of blood vessels, *i.e.* the highest in the proliferative and early secretory phase[54,65], although contradictory results has been described[66]. VEGF expression by epithelial cells increased in the secretory and menstrual phase[54,66]. However, it is uncertain whether this VEGF is available for endometrial microvessels, or excreted *via* the glands into the uterine lumen.

VEGF may be an early response gene for estradiol since two sequences have been identified as being homologous to the estrogen response element. These two elements bind both ERα and ERβ specifically, mutations abolish receptor binding, ERα- and ERβ-specific antibodies interact with complexes formed with the corresponding receptor subtypes and transcriptional activity is blocked by the anti-estrogen ICI 182,780. There may be also non-genomic steroid effects, explaining the rapid effects observed[67-69]. Progesterone response elements have not been described[64].

Direct proof of the regulation of VEGF production by estradiol and progesterone was given in a number of *in vivo* and *in vitro* experiments. Both estradiol and progesterone stimulated VEGF expression in the rat and ewe uterus[32,62-64,70-72], at least partly due to an increased VEGF transcription[64,71]. The addition of estradiol and/or a progestagen (methoxyprogesterone acetate (MPA) or progesterone) to isolated stromal or epithelial cells from the human endometrium also increased VEGF mRNA expression significantly[29,59,61,62;66]. In human stromal cells, the exposure to estradiol was accompanied by an increased VEGF secretion into the conditioned medium[59]. An increase in VEGF mRNA was further seen in an endometrial carcinoma cell line after estradiol treatment[54]. The anti-estrogens tamoxifen and nafoxidine have been reported to induce VEGF-A mRNA expression in rodents[71]. These anti-estrogens act in a tissue-specific manner and act as estrogen agonists in the endometrial cells studied. ICI 182,780, thought to be a more general anti-estrogen[16,73], and mifepristone, a progesterone receptor antagonist[66], both blocked the VEGF-A expression.

The high-affinity receptors VEGFR-1 and VEGFR-2 were mainly found on endothelial cells in the endometrium[74]. Endothelial strands, which had not formed a lumen, were strongly stained for both receptors. Rogers *et al.*[55] found the receptors expressed at low levels throughout the cycle with an increase in the menstrual phase. Inhibition of VEGF activity using soluble-VEGFR-1 prevents endometrial maturation[75]. No information is presently available on the steroid regulation of VEGF receptors.

The overall picture that emerges from the VEGF studies is that VEGF expression, especially by the stromal cells, is likely to be under the control of both estrogens and progestagens. It seems that VEGF takes care of the formation of the subepithelial capillary complex during the proliferative phase and the further growth of the spiral arteries in the secretory phase as can be concluded from the immunostaining on these vessels.

Effect of steroids on bFGF synthesis

Fibroblast growth factor-2 (basic FGF, bFGF) regulates the proliferation and differentiation of many cell types[76,77], and is known to stimulate different steps of the angiogenesis process, including endothelial migration, proliferation and invasion, the production of plasminogen activation factors (u-PA and u-PAR) and the induction of $\alpha_2\beta_1$, an integrin with adherent potencies for extra-cellular matrix components[76,78-81]. In the human endometrium bFGF was found to be present at levels higher than those found in other tissues[82,83]. Staining for bFGF was seen in epithelial cells and stromal cells, in basement membranes and smooth muscle cells of medium-sized blood vessels and in endothelial cells and the basal lamina of capillaries[77,84,85]. Its homoloque FGF-1 (acidic FGF) was also found in the human endometrium[84]. bFGF and its receptor increased specifically in the stromal compartment in the proliferative phase and decreased in

the secretory phase[85,86]. FGF receptor-1 was detected on endothelial cells throughout the cycle[85].

Estradiol has been found to stimulate bFGF production and excretion by isolated human endometrial stromal cells (fibroblasts) and by endometrial adenocarcinoma cells, whereas progesterone inhibited the estradiol-induced increase in bFGF synthesis but did not affect basal bFGF synthesis[87,88]. Furthermore, estradiol up-regulated bFGF expression in the human, rat and ewe uterus[89] as reviewed elsewhere[55,72]. In addition, the timing of the increased expression of bFGF, as well as that of VEGF, preceded the microvascular growth in ewe uteri[90].

These data suggest that bFGF may be involved in the physiological and tumor angiogenesis of the endometrium and that, similarly to VEGF, with which bFGF acts synergistically[4], the endometrial stroma seems most sensitive to steroidal regulation of bFGF expression. However, the importance of bFGF in endometrial angiogenesis has been disputed by others[83,84].

Tumor necrosis factor α (TNFα)

TNFα is a pleiotropic cytokine that exerts a variety of effects in the endometrium, one of which might be angiogenesis. *In vivo*, TNFα induced capillary blood vessel formation in the rat cornea and the developing chick chorioallantoic membrane at very low doses[91]. *In vitro*, TNFα induced capillary-like structures by bovine adrenal capillary endothelial cells grown on type-1 collagen gels[91]. In human endothelial cells TNFα acted in concert with angiogenic growth factors, such as VEGF and bFGF, and stimulated their angiogenic effect. TNFα had an important stimulatory effect on angiogenesis in this way[4]. However, TNFα alone did not induce the formation of capillary-like structures.

The expression of TNFα and its receptors in the endometrium was markedly affected by the menstrual cycle[92-96]. TNFα was expressed in the endometrium mainly in epithelial cells and further in stromal cells, immunocompetent cells and vascular cells[94,95]. The menstrual cycle-dependency of TNFα in the endometrium, although not always confirmed by all the different studies, suggests that this cytokine is subject to regulation by steroids. Consistently with this suggestion, Tabibzadeh[93] found three half-palindromic estrogen response elements in the promoter region of the TNFα gene. Studies on ovariectomized and hormone-substituted mice showed that uterine TNFα mRNA expression is stimulated by estradiol and progesterone[96]. Progesterone and estradiol caused an increase in TNFα production in cells prepared from the proliferative endometrium[97]. In addition, its peak expression in the proliferative phase and in the early- to mid-secretory phase suggests that TNFα may contribute to endometrial angiogenesis.

Other angiogenic growth factors in the endometrium

Yasuda *et al*.[98] showed estradiol-dependent Erythropoietin (Epo) production in the mouse uterus both *in vitro* and *in vivo*, suggesting cycle-dependent fluctuation of Epo concentrations in the uterine tissue. The induction of the expression of Epo was rapid (similar to that seen with VEGF) after administration of estradiol. The uterine endothelial cells were positive for the Epo-receptor[98,99], which had a low ligand affinity compared with the receptors on erythroid precursor cells. Epo stimulated migration, proliferation and angiogenesis of endothelial cells *in vitro*[99,100]. The fact that Epo plays a role in endometrial angiogenesis was underpinned by the finding that intra-uterine injection of Epo stimulated angiogenesis in the mouse endometrium[98].

Other growth factors that induce angiogenesis in classical angiogenesis models are encountered in the endometrium and are under cyclic control. As such it is likely that they are directly or indirectly affected by sex hormones, but this relationship remains to be elucidated. Thymidine phosphorylase (PD-ECGF) and adrenomedullin have recently been reviewed by Oehler[30]. In addition, epidermal growth factor (EGF)[35,101,102], transforming growth factors-α and -β (TGFα, TGFβ)[61,103-105], and insulin-like growth factors (IGF-I, IGF-II)[106,107] are found in the endometrium, where they probably act as paracrine factors.

Cell-matrix interactions and pericellular proteolysis

An important function of the angiogenic growth factors is to induce endothelial proliferation and thus to increase the mass of available endothelial cells. In addition, the process of angiogenesis involves a number of subsequent and mutually interacting steps. First, the endothelial cells have to penetrate their own basal membrane, to migrate, and to invade in the underlying interstitial tissue. This migration is accompanied by endothelial proliferation. Subsequently, the endothelial cells reorganize into tubular structures and acquire a new basal membrane. Finally the vascular structures become connected with the flowing blood and the new vascular structures become stabilized by interaction with pericytes and paracrine factors (see Fig. 1). Cell-matrix interaction is an important factor in the maintenance of healthy blood vessels and is subject to alterations during migration and invasion of cells. Cell receptors, in particular integrins, and cell-bound matrix remodeling proteases are involved in this process. In addition to their effects on cell migration and invasion, these matrix-degrading proteases are also involved in the degradation of the basal membrane and in lumen formation. These proteolytic activities are mainly exerted by the plasminogen activator (PA)/ plasmin system and the family of matrix-degrading metalloproteinases (MMPs). They are regulated by their expression and the expression of their inhibitors and cellular recep-

tors. It is of interest to note that the expression of many of these proteins is under the control of estrogens and progestagens. It is likely that these proteases show partial redundancy with respect to their function in cell migration and tissue remodeling. This explains why deletion of a single protease, such as urokinase-type plasminogen activator (u-PA)[108], plasminogen[109] or PA inhibitor type-1 (PAI-1)[110] does not gravely interfere with fertility in mice.

Plasminogen activator/plasmin system

The plasminogen activator/plasmin system is a protease cascade, in which plasminogen activators (PAs), *viz.* tissue-type PA (t-PA) and urokinase-type PA (u-PA), activate the plasma protein plasminogen. The subsequently generated plasmin is a broadly acting protease involved primarily in fibrin degradation (fibrinolysis) but also able to degrade matrix proteins directly and to activate several pro-MMPs. Studies *in vitro* and in specific *in vivo* models have shown the importance of the PA/plasmin system in angiogenesis[111,112]. In particular, in a fibrin matrix the formation of capillary-like tubular structures is completely dependent on the cell-bound u-PA and plasmin activities[5]. The ingrowth of such tubular structures was strongly inhibited by testosterone, partly by estradiol and 2-methoxyestradiol but not by progesterone[7]. The effect of testosterone appeared to be related to the expression of u-PA by microvascular endothelial cells[7]. Because these experiments were done with male (foreskin) microvascular endothelial cells, it is possible that the effects of estradiol and progesterone have been underestimated.

The endometrium releases t-PA and u-PA as well as their inhibitor PAI-1[46,48,113]. U-PA and u-PA receptor (protein and mRNA) are found in endometrial stromal cells[114] and endothelial cells[114,141]. Casslen *et al*. however found that epithelial cells also produce u-PA[46]. The maximum release of u-PA from endometrial tissue was during the late proliferative phase[115]. While estradiol did not affect u-PA expression in endometrial tissue cultures and in isolated stromal cells, addition of progesterone, after priming with estrogen, resulted in a reduced secretion of u-PA[46,47]. The release of u-PA by cultured endometrial epithelial cells was not influenced by these steroids[46]. In the secretory phase a lower u-PA activity was seen[47], which could be related to a reduced u-PA activity in endometrial tissue and stromal cell cultures after stimulation with progesterone. The reduced u-PA activity may be caused by an increased expression of PAI-1 in both tissue and stromal cell culture, after the addition of progesterone[48,49]. The increased number of u-PA receptors in the secretory phase[116], which was demonstrated in the cultured stromal cells after adding progesterone, may facilitate the removal of u-PA:PAI-1 complexes and thus keep new receptors available for u-PA interaction[5].

Despite an interesting co-expression of PAs and PAI-1 with early developmental processes, mice that lack u-PA[108], plasminogen[109] or PAI-1[110] have a normal development and are fertile. Therefore, the cell-bound u-PA and plasmin-dependency of angiogenesis is probably conditional, *i.e.* it occurs only under specific conditions, such as in fibrin-rich wound matrices. Even in such conditions a rescue system exists. Hiraoka *et al*.[117] recently demonstrated that in plasminogen-deficient conditions, MMP activity could serve as a fibinolysin and substitute for plasmin activity in fibrinolysis and angiogenesis in the mouse.

MMPs

MMps are a highly regulated family of enzymes, which together can degrade most components of the extracellular matrix. They play a role in the menstrual bleeding, tissue degradation, and reorganization and repair within the endometrium.

MMPs are expressed in the endometrium in cell-type and cycle-specific patterns, consistent with regulation by steroid hormones[118]. One may suggest that the MMPs, which are present in the late secretory phase and during menstruation, play a role in the tissue degradation leading to the breakdown of the endometrium[119,120]. The MMPs, which are abundant in the proliferative and early secretory phase, might play a role in the endometrial growth, remodeling and angiogenesis. However, no direct proof has been given yet regarding their involvement in endometrial angiogenesis and little is known about their regulation by steroids in the proliferative and early secretory phase.

MMP-1, MMP-2, MMP-7 and MMP-11 were expressed in the proliferative phase, while MMP-3 and MMP-9 were occasionally present[118,121]. MMP-2 showed staining in the stromal cells and vessels[121], MMP-9 expression was seen in leucocytes and spiral arteries and in the early secretory phase in the epithelium[121], while MMP-1 and MMP-3 were present in vascular structures[121]. MMP-7 and MMP-11 appeared restricted to the epithelium and stromal cells, respectively[118]. In isolated stromal cells progesterone inhibited the expression of pro-MMP-7, pro-MMP-11 and pro-MMP-3[51]. There is relatively little cyclical variability of the specific tissue inhibitors of metalloproteinases (TIMPs) TIMP-1 and TIMP-2: both were strongly expressed in endometrial vessels throughout the cycle[121].

Inhibitors of angiogenesis

Because angiogenesis plays an important role in physiological and pathological processes, stimulation and inhibition of angiogenesis could hold a potential therapeutic application. While angiogenesis is stimulated by the application of angiogenic growth factors, inhibition of angiogenesis can be achieved both by naturally occurring angio-

genesis inhibitors and by exogenously applied compounds. Among naturally occurring anti-angiogenic factors are thrombospondin-1[33], platelet factor-4, interferons, vascular endothelial growth inhibitor and 2-methoxyestradiol[122]. In addition, anti-angiogenic products can be formed by proteolysis of natural proteins, such as angiostatin (peptide derived from plasminogen)[123] and endostatin (fragment of type XVIII collagen)[124]. Pharmacological inhibitors comprise amongst others thalidomide[125], AGM-1470, a fumagillin derivative[126], VEGF receptor antagonists, inhibitors of MMPs[127] and the amino terminal fragment of u-PA[128].

Thrombospondin-1 (TSP-1)

TSP-1 has been reported to inhibit angiogenesis *in vivo* and *in vitro*[129,130]. TSP-1 was predominantly found in the basement membranes of glands, in small blood vessels and capillaries and diffusely in the stroma of the functional, secretory endometrium[33]. Its expression seems to coincide with the suppression of angiogenesis.

TSP-1 expression and secretion appeared to be stimulated in isolated endometrial stromal cells by progesterone and not by estradiol, with the effect being blocked by anti-progestin[33]. In the human TSP-1 gene two progesterone-responsive elements were found in the promoter[33,131]. It remains to be seen whether these sites are functional and responsible for progesterone-induced effects.

Angiostatic steroids

Angiostatic behavior has been reported for some steroids, whether or not in the presence of heparin or heparin derivatives[7,8,132-135]. Angiogenesis inhibition by steroids was said to be caused by the dissolution of the basement membrane in regressing capillaries, by decreasing endothelial cell proteolytic capacity and/or by disrupting microtubules in endothelial cells[7,8,122,136]. When combined with heparin the inhibition was independent of the anticoagulant activity of heparin. Also, the glucocorticoid and mineralocorticoid activity of the steroids did not play a role[132].

Table 2 shows several steroids that inhibit angiogenesis in combination with heparin, together with their anti-angiogenic potency[8]. Testosterone, dexamethasone, methoxyestradiol, cortisol, 17α-hydroxyprogesterone, medroxyprogesterone acetate, androstenedione and tetrahydro S also exert inhibitory effects on angiogenesis *in vitro* in the absence of heparin [7,122,135,137,138]. The effect of methoxyestradiol was also demonstrated *in vivo*[122,135,138]. Jaggers *et al*.[137], using very high concentrations of estradiol, found a complete inhibition of angiogenesis in a rat aorta explant assay. Reports on effects of estradiol and progesterone on angiogenesis are conflicting, but most studies agree that neither estradiol nor progesterone appears to have intrinsic angiogenic activity[8,132,139].

Conclusion & perspectives

From the preceding data pictures emerges in which estrogens and progestagens act on endometrial cells, in particular stromal and epithelial cells, and induce paracrine factors that stimulate angiogenesis in the endometrial vessels. Whether these steroids also act directly on endometrial endothelial cells remains uncertain. The data provide insight into the molecular mechanisms underlying the original finding of Markee of steroidal regulation of endometrial angiogenesis. The paracrine interaction between endometrial cells may also explain why it has been difficult to demonstrate in cultured cells *in vitro* the obvious effects of steroids on the endometrium seen *in vivo*. The interaction between different tissue cell types appears to be essential for observing the effect of the steroids. In addition to the paracine regulation or the production of angiogenic growth factors by steroids, the endothelial response is also influenced by other, non-steroid regulators, such as growth factors/cytokines, prostaglandins, and hypoxia. The elucidation of the various interactions between endometrial cells upon challenge by steroids and other factors will clarify how steroids act on endometrial angiogenesis.

Insight into the regulation of angiogenesis in the endometrium by steroids might contribute to the understanding of the effects of steroids and their derivatives on angiogenesis in tumors. Many of the angiogenic factors discussed have also been shown to play an important role in tumor angiogenesis. In tumors that are affected by steroids, such as breast cancer, angiogenesis might very well be regulated by steroidally-induced angiogenic factors. These factors may act in addition to the direct effect of the steroids on the tumor cells themselves. Knowledge of the factors important in regulating tumor angiogenesis by steroids, and understanding in greater detail the cell-specific expression of steroid receptors and coactivator proteins involved in the induction of gene expression by these steroids may lead to a more effective treatment of cancer with fewer side-effects. In this respect one not only has to take into account tumor-specific expression of receptors and related proteins, but also organ-specific characteristics of endothelial cells[140]. Understanding the mechanisms involved in the local action of specific steroids may provide a rational approach for inducing or inhibiting angiogenesis by steroids or (anti-)steroid derivatives.

References

1. Folkman J, Shing Y: Angiogenesis. J Biol Chem 267: 10931 -10934, 1992.
2. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N.Engl.J.Med. 331: 1480-1487, 1994.
3. Weinberg JB, Pippen AMM, Greenberg CS: Extravascular fibrin formation and dissolution in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum. 34: 996-1005, 1991.
4. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, van Hinsbergh, VWM: Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol 132: 1177-1188, 1996.
5. Kroon ME, Koolwijk P, van Goor H, Weidle UH, Collen A, van der Pluijm G, van Hinsbergh , VWM: Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. Am J Pathol 154: 1731-1742, 1999.
6. Sharkey AM, Day K, McPherson A, Malik S, Licence D, Smith SK, Charnock-Jones DS: Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. J Clin Endocrinol Metab 85: 402-409, 2000.
7. Lansink M, Koolwijk P, van Hinsbergh , VWM, Kooistra T: Effect of steroid hormones and retinoids on the formation of capillary- like tubular structures of human microvascular endothelial cells in fibrin matrices is related to urokinase expression. Blood 92: 927-938, 1998.
8. Folkman J, Ingber DE: Angiostatic steroids. Method of discovery and mechanism of action. Ann Surg 206: 374-383 , 1987.
9. Smith SK: Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update. 4: 509-519, 1998.
10. Rogers PA, Abberton KM, Susil B: Endothelial cell migratory signal produced by human endometrium during the menstrual cycle. Hum Reprod 7: 1061-1066, 1992.
11. Noyes RW, Hertig AT, Rock J: Dating the endometrial biopsy. Am J Obstet Gynecol 122: 262-263, 1975.
12. Au CL, Rogers PA: Immunohistochemical staining of von Willebrand factor in human endometrium during normal menstrual cycle. Hum Reprod 8: 17-23, 1993.
13. Markee, J. E.: Menstruation in intraocular endometrial transplants in the rhesus monkey. Contrib Embryol 177: 221-308, 1940.
14. Kaiserman-Abramof IR, Padykula HA: Angiogenesis in the postovulatory primate endometrium: the coiled arteriolar system. Anat Rec 224: 479-489, 1989.
15. Kaiserman-Abramof IR, Padykula HA: Ultrastructural epithelial zonation of the primate endometrium (rhesus monkey). Am J Anat. 184: 13-30, 1989.
16. Bausero, P., Cavaille, F., Meduri, G., Freitas, S., and Perrot-Applanat, M.: Paracrine action of vascular endothelial growth factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis 2: 167-182, 1998.
17. Abel MH: Prostanoids and menstruation. Mechanisms of menstrual bleeding 25: 139-156, 1985.
18. Reynolds LP, Killilea SD, Redmer DA: Angiogenesis in the female reproductive system. FASEB J 6: 886-892, 1992.
19. Beato M, Truss M, Chavez S: Control of transcription by steroid hormones. Ann N Y Acad Sci 784:93-123: 93-123, 1996.
20. Berg JM: DNA binding specificity of steroid receptors. Cell 57: 1065-1068, 1989.
21. Beato M, Klug J: Steroid hormone receptors: an update. Hum Reprod Update 6: 225-236, 2000.
22. Nilsson S, Kuiper GGJM, Gustafsson J-A: ERb: a novel estrogen receptor offers the potential for new drug development. Trends in Endocrinological Metabolism 9: 387-395 , 1998.
23. Mosselman S, Polman J, Dijkema R: ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392: 49-53, 1996.
24. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS: Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277: 1508-1510, 1997.
25. Ray P, Ghosh SK, Zhang DH, Ray A: Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor. FEBS Lett 409: 79-85, 1997.
26. Revelli A, Massobrio M, Tesarik J: Nongenomic actions of steroid hormones in reproductive tissues. Endocr. Rev 19: 3-17, 1998.
27. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR: Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci U S A 97: 5930-5935, 2000.

28. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM: Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science 289: 1751-1754, 2000.
29. Rogers PA, Lederman F, Taylor N: Endometrial microvascular growth in normal and dysfunctional states. Hum Reprod Update. 4: 503-508, 1998.
30. Oehler MK, Bicknell R, Rees MC: Steroids and the Endometrium. Curr Med Chem 7: 543-560 , 2000.
31. Classen-Linke I, Kusche M, Knauthe R, Beier HM: Establishment of a human endometrial cell culture system and characterization of its polarized hormone responsive epithelial cells. Cell Tissue Res 287: 171-185, 1997.
32. Kohnen G, Campbell S, Jeffers MD, Cameron IT: Spatially regulated differentiation of endometrial vascular smooth muscle cells. Hum Reprod 15: 284-292, 2000.
33. Iruela-Arispe ML, Porter P, Bornstein P, Sage EH: Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium. J Clin Invest 97: 403-412, 1996.
34. Dechering K, Boersma C, Mosselman S: Estrogen Receptors alpha and beta: Two Receptors of a Kind? Curr Med Chem 7: 561-574, 2000.
35. Curtis SH, Korach KS: Steroid receptor knockout models: phenotypes and responses illustrate interactions between receptor signaling pathways *in vivo*. Adv Pharmacol 47:357-80: 357-380, 2000.
36. Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA: Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A 97: 5936-5941, 2000.
37. Kokorine I, Marbaix E, Henriet P, Okada Y, Donnez J, Eeckhout Y, Courtoy PJ: Focal cellular origin and regulation of interstitial collagenase (matrix metalloproteinase-1) are related to menstrual breakdown in the human endometrium. J Cell Sci 109: 2151-2160, 1996.
38. Classen-Linke I, Alfer J, Hey S, Krusche CA, Kusche M, Beier HM: Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. Hum Reprod Update. 4: 539-549, 1998.
39. Ferenczy A, Bertrand G, Gelfand MM: Proliferation kinetics of human endometrium during the normal menstrual cycle. Am J Obstet Gynecol 133: 859-867, 1979.
40. Nisolle M, Casanas-Roux F, Wyns C, de Menten Y, Mathieu PE, Donnez J: Immunohistochemical analysis of estrogen and progesterone receptors in endometrium and peritoneal endometriosis: a new quantitative method. Fertil Steril 62: 751-759, 1994.
41. Iruela-Arispe ML, Rodriguezmanzaneque JC, Abujawdeh G: Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue response to angiogenic growth factors. Microcirculation 6: 127-140, 1999.
42. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. Annu.Rev Physiol. 53:217-39: 217-239, 1991.
43. Smith SK: Growth factors in the human endometrium. Hum Reprod 9: 936-946, 1994.
44. Giudice LC: Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. Fertil Steril 61: 1-17, 1994.
45. Murphy LJ, Ghahary A: Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. Endocr.Rev 11: 443-453, 1990.
46. Casslen B, Urano S, Lecander I, Ny T: Plasminogen activators in the human endometrium, cellular origin and hormonal regulation. Blood Coagul Fibrinolysis 3: 133-138, 1992.
47. Casslen B, Andersson A, Nilsson IM, Astedt B: Hormonal regulation of the release of plasminogen activators and of a specific activator inhibitor from endometrial tissue in culture. Proc Soc Exp Biol Med 182: 419-424, 1986.
48. Casslen B, Urano S, Ny T: Progesterone regulation of plasminogen activator inhibitor 1 (PAI-1) antigen and mRNA levels in human endometrial stromal cells. Thromb Res 66: 75-87, 1992.
49. Sandberg T, Eriksson P, Gustavsson B, Casslen B: Differential regulation of the plasminogen activator inhibitor-1 (PAI-1) gene expression by growth factors and progesterone in human endometrial stromal cells. Mol Hum Reprod 3: 781- 787, 1997.
50. Finlay TH, Katz J, Kirsch L, Levitz M, Nathoo SA, Seiler S: Estrogen-stimulated uptake of plasminogen by the mouse uterus. Endocrinology 112: 856-861, 1983.
51. Osteen KG, Rodgers WH, Gaire M, Hargrove JT, Gorstein F, Matrisian LM: Stromal-epithelial interaction mediates steroidal regulation of metalloproteinase expression in human endometrium. Proc Natl Acad Sci U S A 91: 10129-10133, 1994.
52. Benjamin LE, Golijanin D, Itin A, Pode D , Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103: 159-165, 1999.
53. Gargett CE, Lederman FL, Lau TM, Taylor NH, Rogers PA: Lack of correlation between vascular endothelial growth factor production and endothelial cell proliferation in the human endometrium. Hum Reprod 14: 2080-2088, 1999.
54. Charnock-Jones DS, Sharkey AM, Rajput-Williams J, Burch D, Schofield JP, Fountain SA, Boocock CA, Smith

SK: Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biol Reprod 48: 1120-1128, 1993.
55. Rogers PA, Gargett CE: Endometrial angiogenesis. Angiogenesis 2: 287-294, 1998.
56. Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR, Torry RJ: Vascular endothelial growth factor expression in cycling human endometrium. Fertil Steril 66: 72-80, 1996.
57. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK: Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 98: 482-489, 1996.
58. Mueller MD, Lebovic DI, Garrett E, Taylor RN: Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. Fertil Steril 74: 107-112, 2000.
59. Huang JC, Liu DY, Dawood MY: The expression of vascular endothelial growth factor isoforms in cultured human endometrial stromal cells and its regulation by 17beta- oestradiol. Mol Hum Reprod 4: 603-607, 1998.
60. Wheeler T, Evans PW, Anthony FW, Godfrey KM, Howe DT, Osmond C: Relationship between maternal serum vascular endothelial growth factor concentration in early pregnancy and fetal and placental growth. Hum Reprod 14: 1619-1623, 1999.
61. Zhang L, Rees MC, Bicknell R: The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on estragen and progesterone challenges. J Cell Sci 108: 323-331, 1995.
62. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN: Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol.Metab. 81: 3112-3118, 1996.
63. Popovici RM, Irwin JC, Giaccia AJ, Giudice LC: Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration. J Clin Endocrinol Metab. 84: 2245-2248, 1999.
64. Cullinan-Bove K, Koos RD: Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. Endocrinology 133: 829-837, 1993.
65. Li XF, Gregory J, Ahmed A: Immunolocalisation of vascular endothelial growth factor in human endometrium. Growth Factors 11: 277-282, 1994.
66. Greb RR, Heikinheimo O, Williams RF, Hodgen GD, Goodman AL: Vascular endothelial growth factor in primate endometrium is regulated by estragen-receptor and progesterone-receptor ligands *in vivo*. Hum Reprod 12: 1280-1292, 1997.
67. Weisz A, Bresciani F: Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. Mol Endocrinol 2: 816- 824, 1988.
68. Morley P, Whitfield JF, Vanderhyden BC, Tsang BK, Schwartz JL: A new, nongenomic estrogen action: the rapid release of intracellular calcium. Endocrinology 131: 1305-1312, 1992.
69. Aronica SM, Kraus WL, Katzenellenbogen BS: Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci U S A 91: 8517-8521, 1994.
70. Vassiliadou N, Bulmer JN: Expression of CD69 activation marker by endometrial granulated lymphocytes throughout the menstrual cycle and in early pregnancy. Immunology 94: 368-375, 1998.
71. Hyder SM, Stancel GM, Chiappetta C, Murthy L, Boettger-Tong HL, Makela S: Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Res 56: 3954-3960, 1996.
72. Reynolds LP, Kirsch JD, Kraft KC, Redmer DA: Time-course of the uterine response to estradiol-17beta in ovariectomized ewes: expression of angiogenic factors. Biol Reprod 59: 613-620, 1998.
73. Hyder SM, Chiappetta C, Murthy L, Stancel GM: Selective inhibition of estrogen-regulated gene expression *in vivo* by the pure antiestrogen ICI 182,780. Cancer Res 57: 2547-2549, 1997.
74. Meduri G, Bausero P, Perrot-Applanat M: Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. Biol Reprod 62: 439-447, 2000.
75. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH: Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 4: 336-340, 1998.
76. Gospodarowicz D, Neufeld G, Schweigerer L: Fibroblast growth factor: structural and biological properties. J Cell Physiol Suppl Suppl 5:15-26, 1987.
77. Cordon-Cardo C, Vlodavsky I, Haimovitz-Friedman A, Hicklin D, Fuks Z: Expression of basic fibroblast growth factor in normal human tissues. Lab Invest 63: 832-840, 1990.
78. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G: Structural characterization and biological functions of fibroblast growth factor. Endocr.Rev 8: 95-114, 1987.
79. Frederick JL, Shimanuki T, diZerega GS: Initiation of angiogenesis by human follicular fluid. Science 224: 389-390, 1984.

80. Folkman J, Klagsbrun M: Angiogenic factors. Science 235: 442-447, 1987.
81. Broadley KN, Aquino AM, Woodward SC, Buckley-Sturrock A, Sato Y, Rifkin DB, Davidson JM: Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. Lab Invest 61: 571-575, 1989.
82. Rusnati M, Casarotti G, Pecorelli S, Ragnotti G, Presta M: Estro-progestinic replacement therapy modulates the levels of basic fibroblast growth factor (bFGF) in postmenopausal endometrium. Gynecol Oncol 48: 88-93, 1993.
83. Rusnati M, Casarotti G, Pecorelli S, Ragnotti G, Presta M: Basic fibroblast growth factor in ovulatory cycle and postmenopausal human endometrium. Growth Factors. 3: 299-307, 1990.
84. Ferriani RA, Charnock-Jones DS, Prentice A, Thomas EJ, Smith SK: Immunohistochemical localization of acidic and basic fibroblast growth factors in normal human endometrium and endometriosis and the detection of their mRNA by polymerase chain reaction. Hum Reprod 8: 11-16, 1993.
85. Sangha RK, Li XF, Shams M, Ahmed A: Fibroblast growth factor receptor-1 is a critical component for endometrial remodeling: localization and expression of basic fibroblast growth factor and FGF-R1 in human endometrium during the menstrual cycle and decreased FGF-R1 expression in menorrhagia. Lab Invest 77: 389-402, 1997.
86. Fujimoto J, Hori M, Ichigo S, Tamaya T: Expression of basic fibroblast growth factor and its mRNA in uterine endometrium during the menstrual cycle. Gynecol Endocrinol 10: 193-197, 1996.
87. Presta M: Sex hormones modulate the synthesis of basic fibroblast growth factor in human endometrial adenocarcinoma cells: implications for the neovascularization of normal and neoplastic endometrium. J Cell Physiol 137: 593-597, 1988.
88. Fujimoto J, Hori M, Ichigo S, Tamaya T: Ovarian steroids regulate the expression of basic fibroblast growth factor and its mRNA in fibroblasts derived from uterine endometrium . Ann Clin Biochem 34: 91-96, 1997.
89. Cullinan, K and Koos, R. D.: Expression of basic fibroblast growth factor (bFGF), vascular endothelial growth (VEGF), and keratinocyte growth factor (KGF) in the rat uterus - stimulation by estrogen. Biol Reprod 44: A536-1991.
90. Reynolds LP, Kirsch JD, Kraft KC, Knutson DL, McClaflin WJ, Redmer DA: Time-course of the uterine response to estradiol-17beta in ovariectomized ewes: uterine growth and microvascular development. Biol Reprod 59: 606-612, 1998.
91. Leibovich SJ, Polverini Pj, Shepard HM, Wiseman DM, Shively V, Nuseir N: Macrophage-induced angiogenesis is mediated by tumor necrosis factor-alpha. Nature 329: 630-632, 1987.
92. Le J, Vilcek J: Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 56: 234-248, 1987.
93. Tabibzadeh S, Satyaswaroop PG, von Wolff M, Strowitzki T: Regulation of TNF-alpha mRNA expression in endometrial cells by TNF-alpha and by estragen withdrawal. Mol Hum Reprod 5: 1141-1149, 1999.
94. von Wolff M, Classen-Linke I, Heid D, Krusche CA, Beier-Hellwig K, Karl C, Beier HM: Tumor necrosis factor-alpha (TNF-alpha) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. Mol Hum Reprod 5: 146-152, 1999.
95. Hunt JS, Chen HL, Hu XL, Tabibzadeh S: Tumor necrosis factor-alpha messenger ribonucleic acid and protein in human endometrium. Biol Reprod 47: 141-147, 1992.
96. Roby KF, Laham N, Hunt JS: Cellular localization and steroid hormone regulation of mRNA encoding tumor necrosis factor receptor I in mouse uterus. J Reprod Fertil 106: 285-290, 1996.
97. Laird SM, Tuckerman EM, Saravelos H, Li TC: The production of tumor necrosis factor alpha (TNF-alpha) by human endometrial cells in culture. Hum Reprod 11: 1318-1323, 1996.
98. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R: Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. J Biol Chem 273: 25381- 25387, 1998.
99. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N, Noguchi CT: Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci U S A 91: 3974-3978, 1994.
100. Carlini RG, Reyes AA, Rothstein M: Recombinant human erythropoietin stimulates angiogenesis *in vitro*. Kidney Int 47: 740-745, 1995.
101. Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan JA: Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. Proc Natl Acad Sci U S A 88: 21- 25, 1991.
102. Haining RE, Cameron IT, van Papendorp C, Davenport AP, Prentice A, Thomas EJ, Smith SK: Epidermal growth factor in human endometrium: proliferative effects in culture and immunocytochemical localization in normal and endometriotic tissues. Hum Reprod 6: 1200-1205, 1991.
103. Horowitz GM, Scott RTJ, Drews MR, Navot D, Hofmann GE: Immunohistochemical localization of transforming growth factor-alpha in human endometrium, decidua, and trophoblast. J Clin Endocrinol Metab 76: 786-792, 1993.
104. Murphy LJ, Gong Y, Murphy LC, Bhavnani B: Growth factors in normal and malignant uterine tissue. Ann N Y Acad Sci 622:383-391, 1991.

105. Kauma S, Matt D, Strom S, Eierman D, Turner T: Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. Am J Obstet Gynecol 163: 1430-1437, 1990.
106. Huynh HT, Pollak M: Insulin-like growth factor I gene expression in the uterus is stimulated by tamoxifen and inhibited by the pure antiestrogen ICI 182780. Cancer Res 53 : 5585-5588, 1993.
107. Giudice LC, Irwin JC: Roles of the insulinlike growth factor family in nonpregnant human endometrium and at the decidual: trophoblast interface. Semin.Reprod Endocrinol 17: 13-21, 1999.
108. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC: Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368: 419-424, 1994.
109. Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, Degen JL: The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Biol Chem 270: 16886-16894, 1995.
110. Carmeliet P, Kieckens L, Schoonjans L, Ream B, van Nuffelen A, Prendergast G, Cole M, Bronson R, Collen D, Mulligan RC: Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. J Clin Invest 92: 2746-2755, 1993.
111. Bacharach E, Itin A, Keshet E: *In vivo* patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc Natl Acad Sci U S A 89: 10686-10690, 1992.
112. Mazar AP, Henkin J, Goldfarb RH: The urokinase plasminogen activator system in cancer: implications for tumor angiogenesis and metastasis. Angiogenesis 3: 15-32, 1999.
113. Fernandez-Shaw S, Marshall JM, Hicks B, Barlow DH, Starkey PM: Plasminogen activators in ectopic and uterine endometrium. Fertil Steril 63: 45 -51, 1995.
114. Casslen B, Gustavsson B, Angelin B, Gafvels M: Degradation of urokinase plasminogen activator (UPA) in endometrial stromal cells requires both the UPA receptor and the low-density lipoprotein receptor-related protein/alpha2-macroglobulin receptor. Mol Hum Reprod 4: 585-593, 1998.
115. Casslen B, Astedt B: Occurrence of both urokinase and tissue plasminogen activator in the human endometrium. Contraception 28: 553-564, 1983.
116. Casslen B, Nordengren J, Gustavsson B, Nilbert M, Lund LR: Progesterone stimulates degradation of urokinase plasminogen activator (u-PA) in endometrial stromal cells by increasing its inhibitor and surface expression of the u-PA receptor. J Clin Endocrinol Metab. 80: 2776-2784, 1995.
117. Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ: Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell 95: 365-377, 1998.
118. Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F, Osteen KG: Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest 94: 946-953, 1994.
119. Irwin JC, Kirk D, Gwatkin RB, Navre M, Cannon P, Giudice LC: Human endometrial matrix metalloproteinase-2, a putative menstrual proteinase. Hormonal regulation in cultured stromal cells and messenger RNA expression during the menstrual cycle. J Clin Invest 97: 438-447, 1996.
120. Marbaix E, Kokorine I, Moulin P, Donnez J, Eeckhout Y, Courtoy PJ: Menstrual breakdown of human endometrium can be mimicked *in vitro* and is selectively and reversibly blocked by inhibitors of matrix metalloproteinases. Proc Natl Acad Sci U S A 93: 9120-9125, 1996.
121. Freitas S, Meduri G, Le Nestour E, Bausero P, Perrot-Applanat M: Expression of metalloproteinases and their inhibitors in blood vessels in human endometrium. Biol Reprod 61: 1070-1082, 1999.
122. Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP, Schweigerer L: The endogenous estragen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumor growth. Nature 368: 237-239, 1994.
123. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79: 315-328, 1994.
124. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88: 277-285, 1997.
125. D'Amato RJ, Loughnan MS, Flynn E, Folkman J: Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci U S A 91: 4082-4085, 1994.
126. Ingber D, Fujita T, Kishimoto S, Sudo K , Kanamaru T, Brem H, Folkman J: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature 348: 555-557, 1990.
127. Brown PD: Matrix metalloproteinase inhibitors. Breast Cancer Res Treat. 52: 125-136, 1998.
128. Li H, Griscelli F, Lindenmeyer F, Opolon P, Sun LQ, Connault E, Soria J, Soria C, Perricaudet M, Yeh P, Lu H: Systemic delivery of antiangiogenic adenovirus AdmATF induces liver resistance to metastasis and prolongs survival of mice. Hum Gene Ther 10: 3045-3053, 1999.

129. Rastinejad F, Polverini Pj, Bouck NP: Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56: 345-355, 1989.
130. DiPietro LA, Nebgen DR, Polverini Pj: Downregulation of endothelial cell thrombospondin 1 enhances *in vitro* angiogenesis. J Vasc Res 31: 178-185, 1994.
131. Evans RM: The steroid and thyroid hormone receptor superfamily. Science 240: 889-895, 1988.
132. Crum R, Szabo S, Folkman J: A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230: 1375-1378, 1985.
133. Cariou R, Harousseau JL, Tobelem G: Inhibition of human endothelial cell proliferation by heparin and steroids. Cell Biol Int Rep. 12: 1037-1047, 1988.
134. Derbyshire EJ, Comin GA, Yang YC, Overholser J, Watkins L, Thorpe PE: Anti-tumor and anti-angiogenic effects in mice of heparin conjugated to angiostatic steroids. Int J Cancer 63: 694-701, 1995.
135. Banerjeei SK, Zoubine MN, Sarkar DK, Weston AP, Shah JH, Campbell DR: 2-Methoxyestradiol blocks estrogen-induced rat pituitary tumor growth and tumor angiogenesis: possible role of vascular endothelial growth factor. Anticancer Res 20: 2641-2645, 2000.
136. Ashino-Fuse H, Takano Y, Oikawa T, Shimamura M, Iwaguchi T: Medroxyprogesterone acetate, an anti-cancer and anti-angiogenic steroid, inhibits the plasminogen activator in bovine endothelial cells. Int J Cancer 44: 859-864, 1989.
137. Jaggers DC, Collins WP, Milligan SR: Potent inhibitory effects of steroids in an *in vitro* model of angiogenesis. J Endocrinol 150: 457-464, 1996.
138. Hori Y, Hu DE, Yasui K, Smither RL, Gresham GA, Fan TP: Differential effects of angiostatic steroids and dexamethasone on angiogenesis and cytokine levels in rat sponge implants. Br J Pharmacol 118: 1584-1591, 1996.
139. Rees MC, Bicknell R: Angiogenesis in the endometrium. Angiogenesis 2: 29-35, 1998.
140. Gerritsen ME: Functional heterogeneity of vascular endothelial cells. Biochem Pharmacol 36: 2701-2711, 1987.
141. Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van der Vecht B, Helmerhorst FM, *et al*. Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. J Clin Endocrinol Metab 2001;86:3359-67.

7

EFFECTS OF OVARIAN STEROIDS ON HUMAN ENDOMETRIAL ENDOTHELIAL AND STROMAL CELLS. EVIDENCE FOR PARACRINE REGULATION OF ANGIOGENESIS

K. Kapiteijn
P. Koolwijk
E.L. Kaijzel
A.J. van Gool
F.M. Helmerhorst
V.W.M. van Hinsbergh

Submitted

Introduction

Angiogenesis is essential for tissue repair and the recovery of endometrial tissue during the menstrual cycle. It also plays a crucial role in the successful implantation of the embryo and its growth. Inadequate endometrial angiogenesis is likely involved in implantation failure and defective placentation, which may have a great impact on a patient's quality of life and pregnancy outcome[1-3]. As such, it is important to gain a better understanding of the process of endometrial angiogenesis.

The process of angiogenesis is under the control of angiogenic growth factors and hormones[4,5]. The ovarian steroids are main regulators of endometrial angiogenesis as already shown in early experiments by Markee and Abel[6,7]. Although many studies have indicated the involvement of these hormones in endometrial angiogenesis, the mechanisms by which 17β-estradiol (E_2) and progesterone act are still not well understood[8-11]. So far it is unclear whether the steroids directly influence the endometrial endothelium or whether they regulate endometrial angiogenesis indirectly via activation of other endometrial cells.

It is generally thought that the regulation of biological responses to estrogens and progesterone is mediated via the interaction of these hormones with their corresponding nuclear receptors; estrogen receptor-alpha (ERα), -beta (ERβ) and the progesterone receptor (PR). Bound to their receptors these hormones form a complex in the nucleus of the cell that binds to estrogen or progesterone response elements (EREs and PREs) in the promoter regions of many genes. This results in an altered expression of these genes. In addition ERs on the surface of endothelial cells participate in the rapid regulation of the vascular tone[12]. The existence of various splicing variants of the PR and ERs, which may have different transactivation properties *in vivo*, further adds to the complex regulation by the ovarian hormones[13-17]. Several studies reported on the expression of these receptors in endometrial cells *in vivo* and *in vitro*, but their conclusions have not been equivocal[16,18-21].

Mediators by which steroid hormones might influence the endometrial angiogenic process are locally produced angiogenic growth factors and cytokines. These factors affect endothelial proliferation, invasion and migration, which all play a role in angiogenesis. VEGF-A, in particular, is a potent mediator in the endometrium, as it is able to influence all these processes[1,19,20,22]. In the human endometrium VEGF-A is produced by epithelial cells, stromal cells and stromal leukocytes[23-25]. Steroid hormones may also affect angiogenesis by modulating factors that are involved in the local proteolytic remodeling of matrix proteins important for the invasion and migration of the endothelial cells, such as matrix metalloproteinases, urokinase (u-PA) and their inhibitors[4,22].

Endothelial cells from various tissues display organ-specific characteristics, which at least in part are retained in culture[26]. Therefore, endometrial angiogenesis is preferably studied with hEMVEC. As few other investigators, we have been able to isolate, culture and characterize hEMVEC[20,22,27,28]. In the present study we have examined the influence of E_2 and progesterone on hEMVEC and human stromal cells (hESC). This was done in order to get a better understanding of the steroidal regulation of endometrial angiogenesis and the mutual role of the hEMVEC and hESC in this process.

Materials and Methods

Materials

Medium 199 (M199) without phenol-red, and supplemented with 20 mM HEPES was obtained from BioWhittaker (Verviers, Belgium); newborn calf serum (NBCS) and human serum as previously described[22,29]. For experiments with steroids, charcoal-treated sera were used. Tissue-culture plastics, endothelial cell growth factor (ECGF), human bFGF, heparin, TNFα, thrombin, human fibrinogen, factor XIII and fibronectin were obtained as previously indicated[22,29]. Human recombinant VEGF-A was purchased from RELIATech (Braunschweig, Germany). E_2 (E-2758) and progesterone (P-8783) were purchased from Sigma (St Louis, USA) and ICI 182.780, a potent and specific anti-estrogen[30], (Faslodex (TM), fulvestrant) was from AstraZeneca (Alderley Park, UK). Stock solutions of steroids and ICI 182.780 (10mmol/L) were prepared in DMSO and stored at -20°C; further dilutions were made in M199 without phenol red with 0.1% pyrogen-free human serum albumin (HSA), and finally in incubation medium immediately before the start of an experiment. Oligonucleotides used for RT-PCR were obtained from Biosource Europe SA (Nivelles, Belgium).

Cell culture

HEMVEC were isolated from endometrial tissue as previously described and maintained in M199 without phenol-red supplemented with 20 mM HEPES (pH 7.3), 20% human serum, 10% heat-inactivated NBCS, 150 mg/mL ECGF, 5 ng/mL VEGF-A, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin (= hEMVEC culture medium)[22].

HESC were isolated from the primary heterogeneous cell population, which was obtained after the endometrial tissue was minced, incubated in collagenase and transferred into a culture dish. After 2-4 hours the non-adhered and CD31-negative cells (hESC and epithelial cells), were transferred and cultured in hEMVEC medium with

10% human serum and without VEGF-A in gelatin-coated dishes. Endometrial epithelial cells were quickly lost upon serial passage in culture. HESC were characterized as fibroblasts by immunofluorescence staining with anti-human fibroblast (ITK diagnostics, Uithoorn, The Netherlands). The cells were negative for the endothelial cell markers CD31 and von Willebrand Factor. Very few cells (<1%) at low passage number stained positive for the epithelial cell markers cytokeratine-8 and -18 or smooth-muscle actin (data not shown).

Cells were cultured on fibronectin-coated or gelatin-coated wells at 5% CO_2 / 95% air until confluence and subcultured with a split ratio of 1:3. The medium was renewed at 2-3 day intervals.

Immunohistochemistry

Human endometrial tissue specimens were embedded in paraffin and cut into 4 μm sections. After deparaffinization and blocking with 0.3% H_2O_2-methanol, the sections were washed in PBS. For antigen retrieval they were cooked in citrate buffer (0.01M, pH 6.0) in a microwave for 10 min. Subsequently they were washed and incubated for 15 min in a "block"-buffer (5% bovine serum albumin (BSA) in PBS) to reduce background staining. Then the specific steroid receptor antibody (ERα: DAKO 1D5, ERβ: Serotech MCA1974, PR: ABR PR-AT 4.14) was added (diluted in 1 % BSA/PBS), followed by an overnight incubation at 4ºC. The next day, after three washes in PBS, the sections were incubated with biotine-conjugated horse-anti-mouse Ig (1:300 in PBS-1% BSA) for 1 h at room temperature. After additional washing and amplification with Avidine Biotin Complex, the sections were stained with NOVA-RED for 10 min. They were counterstained with Mayers' haematoxylin.

RNA Isolation and RT-PCR

Total RNA from hEMVEC and hESC (30 cm^2/condition) was isolated as described by Chomczynski and Sacchi [31]. cDNA was synthesized from 1 μg total RNA with 0.5 μg oligo dT primer and 15 U AMV Reverse Transcriptase (Promega, Madison). PCR amplification of 1 μL cDNA was performed on a Robocycler (Stratagene) in 40 μL reaction mixtures containing 4μL 10x PCR buffer, 25 mM of each dNTP, 10 pmol of each primer, and 0.2 μL of *Taq* polymerase (Amersham Pharmacia Biotech Inc, Piscataway). The following cycling conditions were used: 94°C for 4 min; 35 cycles of 94°C for 1 min, 60°C (ERβ) and 67°C (ERα, PR) for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. After 35 cycles the PCR was stopped and amplification products were evaluated by 1% agarose gel electrophoresis.

Oligonucleotide primers

The following primer sequences were used in the RT-PCR to detect receptor mRNA: for the ERα mRNA: sense 5′-TGATGGGGAGGGCAGGGGTGAAGTG-3′ and antisense 5′-TAGGCGGTGGGCGTCCAGCATCTCC-3′[32]. For the ERβ mRNA: *set X*; sense 5′-TTGTGCGGAGACAGAGAAGTGC-3′ and antisense 5′-GGAATTGAGCAGGATCATGGCC-3′[33]. For the ERβ also another set of primers was designed which amplified part of the C-terminal region, *set Y*: sense 5′-CATGATCCTGCTCAATTCCA-3′ and antisense 5′-CTTGTTACTCGCATGCCTGA-3′. For the PR mRNA: sense 5′-GTGGGCGTTCCAAATGAAAGCCAAG-3′ and antisense 5′-AATTCAACACTCAGTGCCCGGGACT-3′[32]. For β-actin mRNA: sense 5′-AAGATGACCCAGATCATGTTTGAG-3′ and antisense 5′-AGGAGGAGCAATGATCTTGATCTT-3′.

Cell proliferation

Incorporation of ^{3}H-thymidine in DNA was determined as a measurement of endothelial and stromal cell proliferation as previously indicated[22].

In vitro angiogenesis assay

Human fibrin and collagen matrices were prepared as previously described[22,29]. Highly confluent hEMVEC were detached and seeded in a split ratio of 2:1 on the surface of the fibrin or collagen matrices and cultured for 24 h in M199 medium without indicator supplemented with 20% human serum, 10% NBCS, and penicillin/streptomycin. Subsequently hEMVEC were stimulated with the mediators indicated for 3-4 days to form capillary-like tubules. The culture medium with additions was renewed every day, because of the high turnover rate of E_2 and progesterone. The formation of tubular structures by hEMVEC in the three-dimensional matrix was quantified by non-phase contrast microscopy as previously given[22,29].

Assays

U-PA and PAI-1 were assayed by EIA as previously indicated[22]. VEGF antigen was determined by VEGF ELISA (R&D system, Minneapolis, USA).

Statistical analysis

The data are expressed as the mean ± SEM/range. Statistical evaluations of the data were performed using the Paired-Sample T-test after the control conditions were set at a 100%. $p < 0.05$ was considered statistically significant.

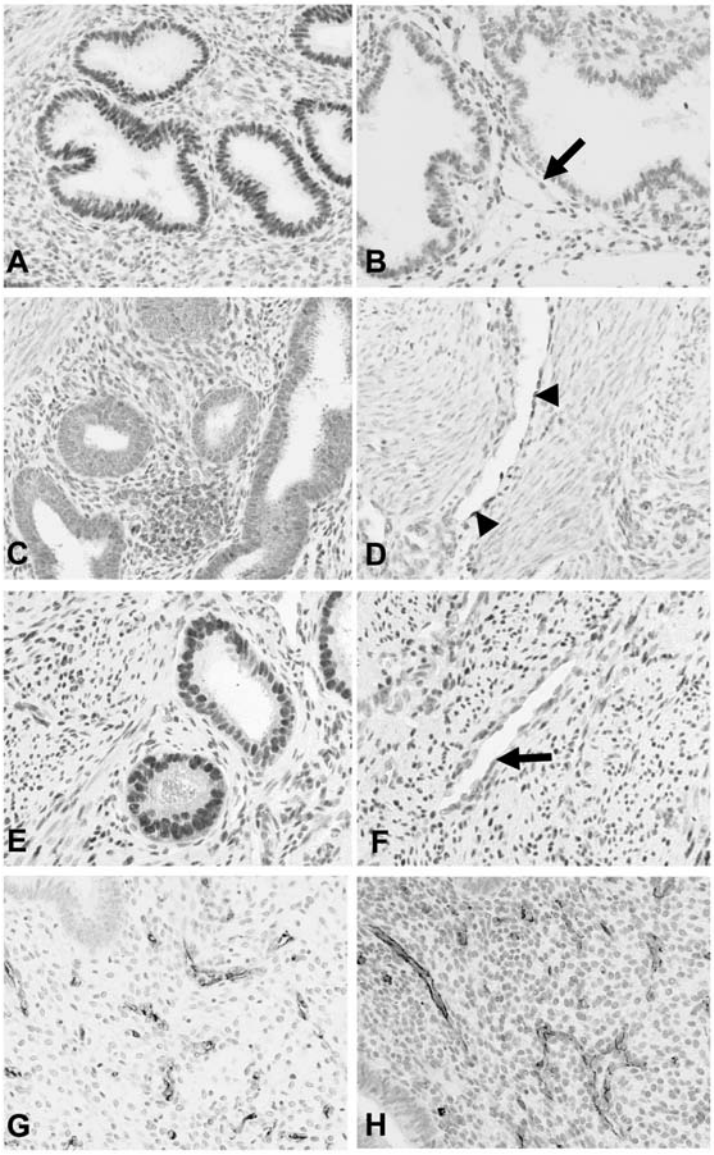


Figure 1. **Expression of ERα, ERβ and PR in human endometrial tissue.**
Immunohistochemistry was performed with labeled antibodies to ERα, ERβ and PR on paraffin sections of human endometrium, as described in the methods section. Panel A and B; brown staining shows ERα in the epithelium and in the stromal compartment, the endothelium is negative for the ERα. C and D; endometrial stroma, epithelium and endothelium show positive staining for ERβ. E and F; PR staining is seen in the epithelium and in the stroma, the endothelium stains negative for the PR. G and H; von Willebrand and CD31 staining were used to indicate the endothelial cells in the endometrium. Black arrow heads indicate an example of positive endothelial cells, and black arrows indicate negative endothelial cells. *[See appendix: color figures]*

Results

HEMVEC express ERβ, hESC express ERα, ERβ and PR

Immunohistochemical staining and RT-PCR analysis were performed to detect the expression of steroid receptors by hEMVEC and hESC. Endometrial tissue showed specific staining for the ERβ on endothelial cells and in the remaining stromal compartment. The stromal compartment also stained positive for the PR and ERα. The surface and glandular epithelium was highly positive for ERα and PR and also contained ERβ (Fig. 1).

Subsequently, we investigated the presence of ERs and PR in hEMVEC *in vitro*. Because the purification of hEMVEC includes several passages, the cells were evaluated from 5 passages onward. No notable ERα or PR mRNA was detected in hEMVEC from passages 5 to 14 of 4 different donors (Fig. 2A and E). In contrast, ERβ mRNA was clearly expressed in hEMVEC at passages 5 to 14 (cells from 2 different donors) (Fig. 2C); ERβ was detected by both *primer sets X* and *Y* (not shown). The ERβ remained expressed during serial passage of hEMVEC. In one of the 4 isolations a very weak signal for PR was shown in a higher passage (not shown).

In hESC the ERα was expressed in 2 (passages 4 and 6) out of 3 isolations from different donors. The hESC isolation that did not express the ERα was at passage 5 (Fig. 2A). HESC lost their expression of ERα above passage 5-6 (Fig. 2B). Unexpectedly, hESC, from passages 4 to 6 of 3 different donors, did not express ERβ when *primer set X* was used. However, when we used a different primer set, that amplified part of the C-terminal region of ERβ (*primer set Y*), ERβ mRNA was detected in hESC, suggesting an alternatively spliced ERβ (Fig. 2D).

Ovarian steroids and proliferation of hEMVEC and hESC

Increasing amounts of E_2 (10^{-10}-10^{-7}M) and progesterone (10^{-8}-10^{-6}M) were tested for their effect of hEMVEC proliferation under basal conditions (contains 0.75 ng/mL VEGF for hEMVEC maintenance) and in the presence of 6.25 ng/mL VEGF-A. E_2 had no effect on the basal and VEGF-A enhanced proliferation of hEMVEC (Fig. 3A). Progesterone did also not affect the basal and VEGF-A-mediated proliferation of hEMVEC (Fig. 3B).

The effects of E_2 and progesterone were subsequently evaluated in hESC in control medium (without additional growth factors) and in the presence of bFGF, which enhances hESC proliferation. A slight non-significant increase in basal proliferation of hESC was observed after 48 h incubation with high concentrations of E_2 only. This effect was absent when these cells were also stimulated by bFGF (Fig. 3C). Progesterone did not alter the proliferation of hESC either under control conditions, or in the presence of bFGF (Fig. 3D).

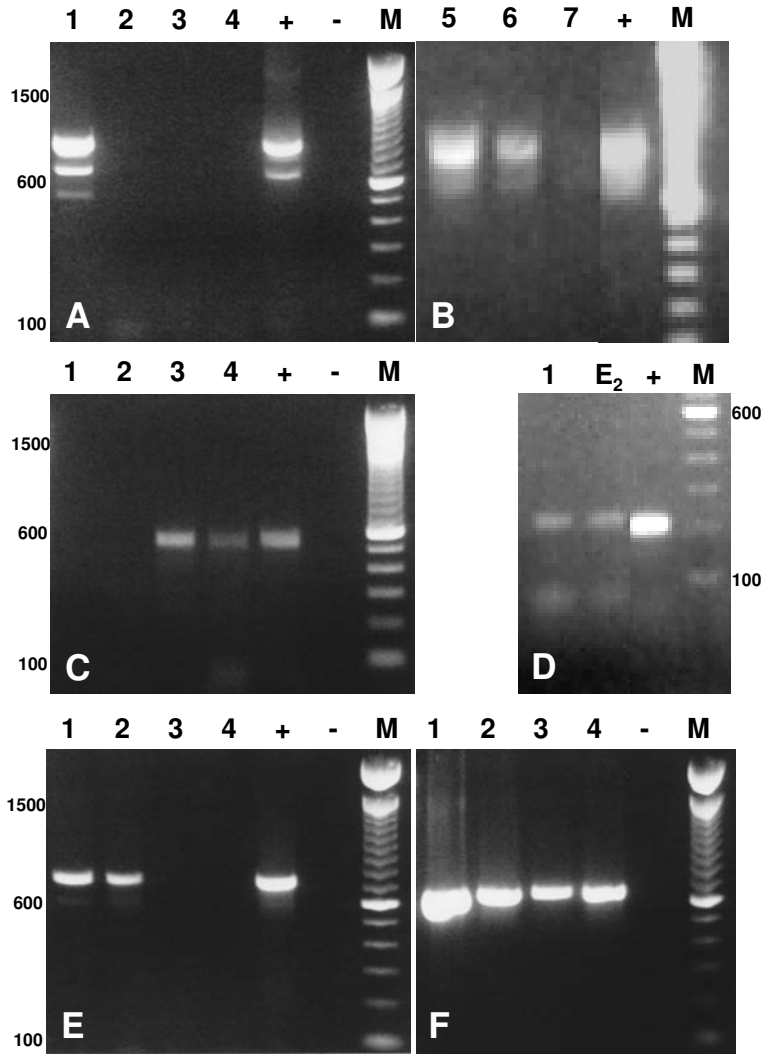


Figure 2. Expression of ERα, ERβ and PR in endometrial cells as determined by RT-PCR. HEMVEC and hESC were cultured till confluence on fibronectin- or gelatine-coated dishes in (hEMVEC) culture medium. RNA was isolated from these cells and cDNA was synthesized as described in Material and Methods. PCR amplification was performed with primers for ERα (panel A, B), ERβ (panel C, D) and PR (panel E) as described. The expected length of the amplified DNA fragment of the ERα is 832 bp, of the ERβ 541 bp (C) and 208 bp (D) and of the PR 737 bp. As a positive control for the expression of all three receptors, RNA isolated from T47D cells (human breast cancer cell line) was used. To check the quality of cDNA, primers specific to the human β-actin gene (panel F) were used; the expected length of the amplified DNA fragment was 647 bp. As negative control for the PCR reaction 1 μL of H_2O was used. Data were obtained with endometrial cells from different donors. Lane 1: hESC passage 4, lane 2: hESC passage 5, lane 3: hEMVEC passage 5, lane 4: hEMVEC passage 8, lane 5: hESC passage 1, lane 6: hESC passage 2, lane 7: hESC passage 3, +: positive control, -: negative control, M: molecular weight marker, E_2: hESC under E_2 stimulated conditions.

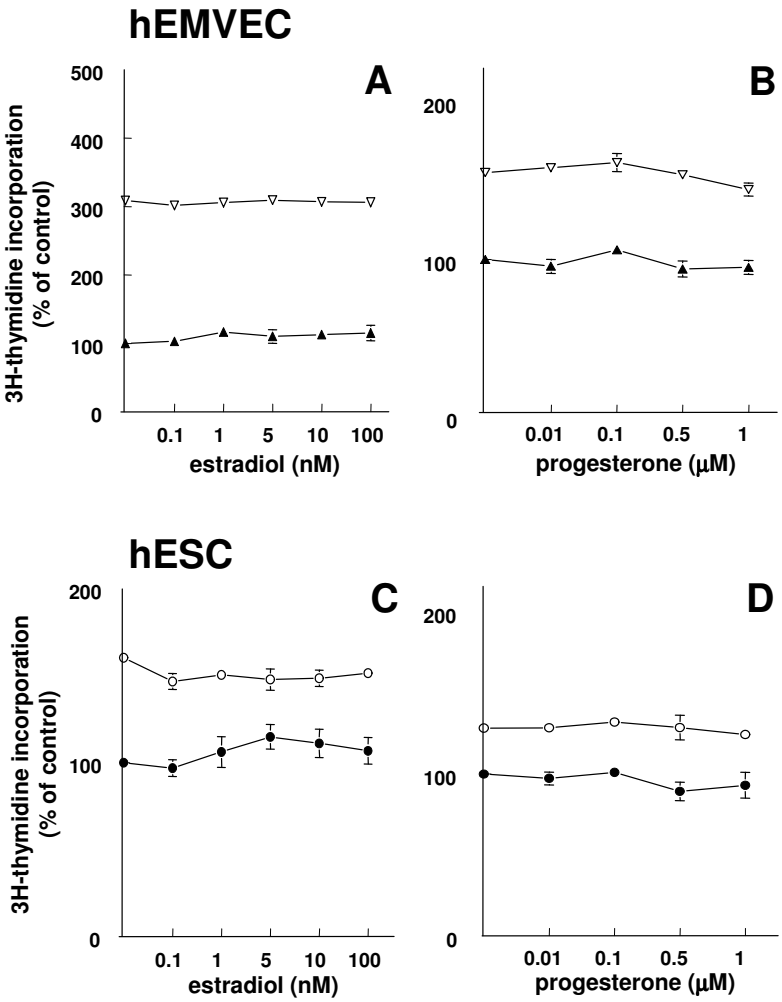


Figure 3. Effects of estradiol and progesterone on hEMVEC and hESC proliferation.
A,B: Non-confluent hEMVEC (passages 4 to 9 of 3 different donors) were cultured for 48 h in the presence of increasing amount of daily added E_2 (A) or progesterone (B) in M199 without phenol-red supplemented with 10% charcoal-treated NBCS and 0.75 ng/mL VEGF-A (-▲-) or 6.25 ng.mL VEGF-A (-▽-) . After 42 h, tracer amount of ^{3}H-thymidine was added to the medium and the incubation continued in the same medium for another 6 h and ^{3}H-thymidine incorporation was determined as described[23]. The data are expressed as a percentage of the control and represent mean ± SEM of 9 (E_2), 6 (P), 4 (E_2-or P+VEGF-A-) independent experiments performed in duplicate wells.
C,D: Non-confluent hESC, from passages 2 and 3 of 4 different donors, were cultured for 48 h in the absence or presence of an increasing amount of E_2 added daily (panel C) or progesterone (panel D) either in combination with (-○-) or without (-●-) bFGF (2.5 ng/mL) in M199 without phenol-red supplemented with 10% charcoal-treated NBCS. 3H-thymidine incorporation was assayed as given under A,B. The data are expressed as a percentage of the control and represent mean ± SEM/range of 4-5 (in the absence of bFGF) and 2-3 (in the presence of bFGF) independent experiments performed in duplicate wells.

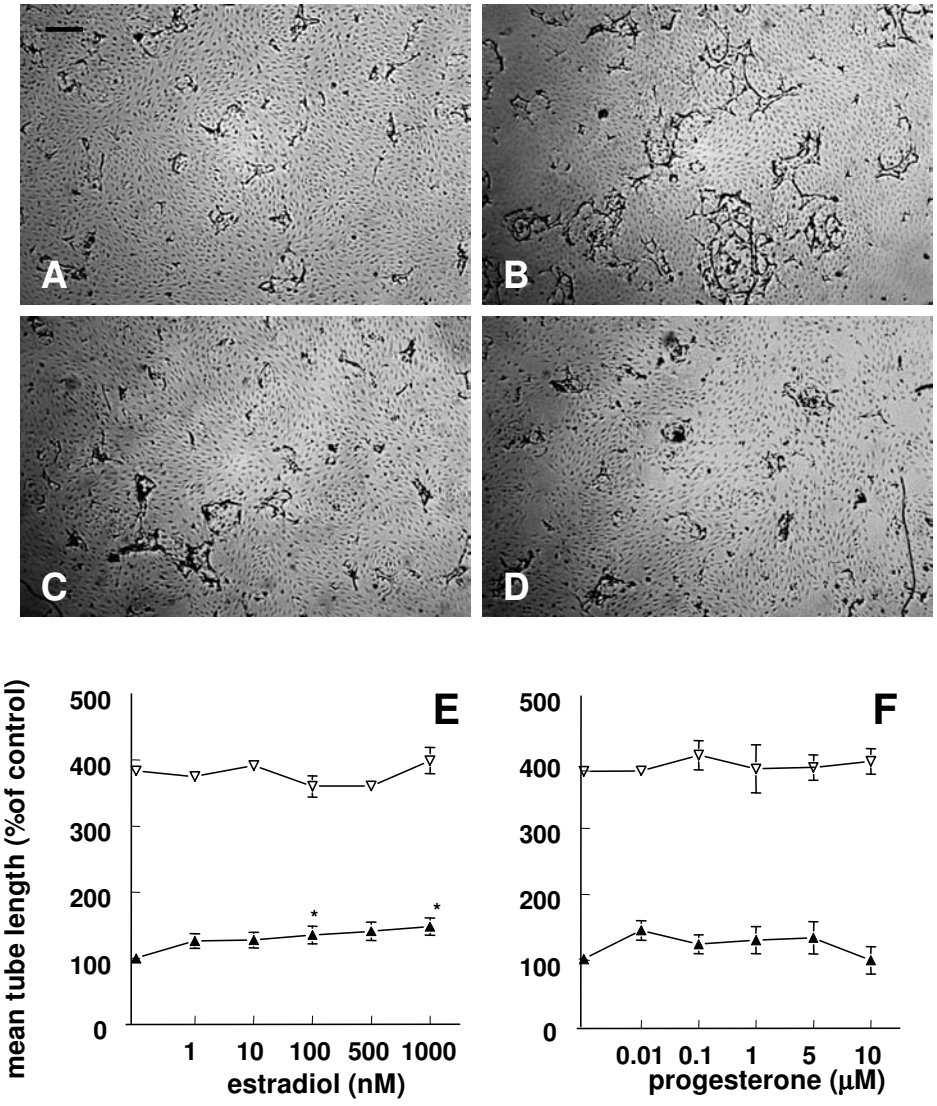


Figure 4. Effect of VEGF-A and ovarian steroids on capillary tube formation by hEMVEC.
A-D: Nonphase contrast views were taken which show growth of capillary-like tube structures in all panels. Panel A; control situation, panel B; stimulation with 10 ng/mL VEGF-A, panel C; stimulated with E_2 (10^{-7}M) and panel D; stimulation with progesterone (10^{-6}M). *Bar* = 300 μM.
E,F: hEMVEC, from passages 5-9 of 4 different donors, were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 20% human serum and 10% NBCS and stimulated with increasing amounts of E_2 (panel E) or progesterone (panel F) in the presence (-▽-) or absence (-▲-) of VEGF-A (10ng/mL). After 2-5 days of culturing, mean tube length was measured by image analysis as described and expressed as mean tube length as a percentage of the control ± SEM/range of 5 (absence of VEGF-A) or 2 (presence of VEGF-A) independent experiments performed in duplicate wells (* differs significantly, $p<0.05$, from 0 M condition).

Effect of VEGF-A, estradiol and progesterone on capillary-like tube formation

To evaluate whether ovarian hormones directly affected endothelial tube formation, we used an *in vitro* model, in which hEMVEC were cultured on top of a 3D-fibrin matrix[29]. Under basal conditions a limited number of hEMVEC invade the fibrin matrix and form tubular structures (fig. 4A). The presence of VEGF-A markedly enhanced the extent of capillary tube formation by hEMVEC (Fig. 4B, E). This increase amounted 3.8 ± 0.8-fold (11 independent experiments with hEMVEC from 4 different donors). The stimulation of capillary-like tube formation was completely prevented by the addition of VEGF-receptor-2/KDR blocking monoclonal antibodies (not shown).

Subsequently, the influence of increasing amounts of E_2 and/or progesterone on *in vitro* angiogenesis of hEMVEC was studied. A slight increase in tube formation was visible when hEMVEC were stimulated with E_2, which was borderline significant at 10^{-7}M (135±13%, $p=0.034$) and 10^{-6}M (148±13%, $p=0.046$, Fig. 4C,E). The minor stimulation by 10^{-7}M E_2 was completely inhibited by the addition of ICI 182.780 (not shown). Progesterone had no effect on tube formation (Fig. 4D, F). The amounts of u-PA and PAI-1 produced by the tube forming endothelial cultures were not significantly altered (not shown).

Various concentrations of E_2 or progesterone had no effect on VEGF-A-enhanced tube formation (Fig. 4E, F). Similarly, no effect on tube formation was seen, when E_2 (10^{-7}M) and progesterone (10^{-6}M) were simultaneously added, (not shown). When hEMVEC were cultured on collagen type-I matrices, E_2 (10^{-8}M) and/or progesterone (10^{-8}, 10^{-7}M), both with and without VEGF-A, had no significant effect on tube formation (not shown).

Estradiol and progesterone enhance VEGF-A production by hESC

A more distinct effect of E_2 and progesterone on endometrial angiogenesis might occur indirectly via activation of other endometrial cells that are in close contact with hEMVEC, in particular hESC. The presence of functional ER and PR in hESC was verified by measuring the IL-6 production in non-stimulated and IL-1β-stimulated hESC (0.014 ng/ml and 25 ng/mL IL-6, respectively). Both in non- and IL-1β-stimulated hESC, E_2 and progesterone (10^{-11}-10^{-8}M) reduced IL-6 production (Fig. 5).

Because hEMVEC responded well to VEGF-A, we evaluated whether E_2 and/or progesterone induced VEGF-A production by hESC. Increasing amounts of E_2 stimulated the VEGF-A production 10- to 29-fold (Fig. 6). ICI 182,780 blocked this effect by 50%. Increasing amounts of progesterone stimulated the VEGF-A production 9- to 15-fold. Progesterone did not further enhance the E_2-increased VEGF-A production (Fig. 6).

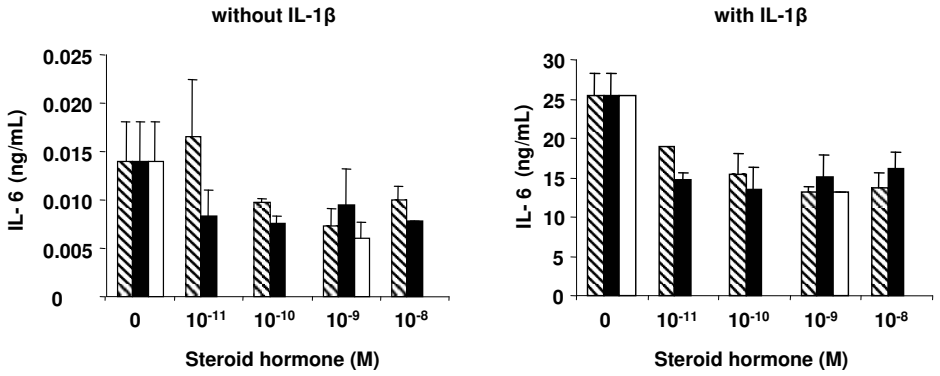


Figure 5. Effect of ovarian steroids on IL-6 production by hESC.
HESC were incubated for 20 h in the presence of the indicated concentrations of steroid hormones in 0.25 ml/cm^2 M199 medium (phenol-free) supplemented with 10% bovine charcoal-treated serum and 10% human charcoal-treated serum. After 1 h 125 pg/ml hr-IL-1β was added to part of the wells (right panel). After the 20 h incubation period IL-6 was determined in the conditioned medium by ELISA. Hatched bars, E_2; closed bars, progesterone; open bars (none and 10^{-9} M), E_2 plus progesterone. Note the difference in scale of the two panels. The data (mean ± range) are given for a representative experiment. Similar data were obtained with hESC cultures derived from another donor.

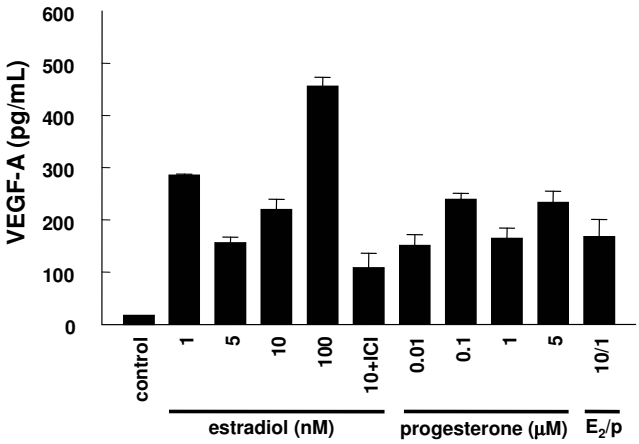


Figure 6. Estradiol and progesterone stimulate VEGF-A expression by hESC.
Confluent hESC on gelatin-coated wells were preincubated with M199 without phenol-red supplemented with 10% charcoal-treated NBCS for 24 h and subsequently stimulated with increasing amounts of E_2 or progesterone, or 10 nM E_2 plus 10 μM ICI 182.780 (ICI was added 1 h before E_2 was spiked) or the combination of 10 nM E_2 and 1 μM progesterone in M199 without phenol-red supplemented with 10% charcoal-treated NBCS. After 24 h E_2 and progesterone were spiked in the medium. After 48 h, supernatants were collected. VEGF-A was determined in 48 h conditioned medium by ELISA. The data are expressed as mean ± range of duplicate wells and are representative of two experiments performed with hESC from different donors.

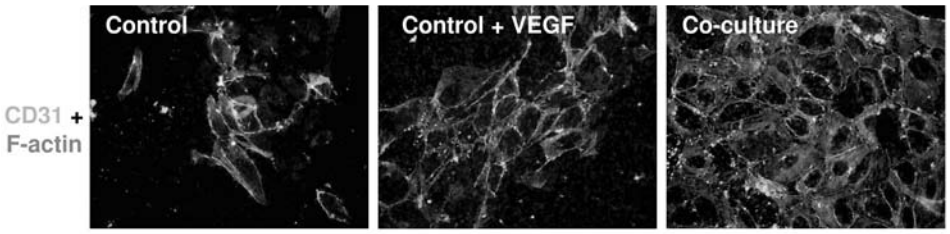


Figure 7. HESC and VEGF contribute to maintenance of hEMVEC monolayers.
Cultures of hEMVEC and hESC were detached and seeded on the surface of a filter (hEMVEC) or dish (hESC) of a transwell™ system (Costar). The next day the hEMVEC-covered filters were transferred into the wells in which hESC had been grown or wells without cells (control). To half of the control conditions VEGF-A (10 ng/mL) was added. HEMVEC were immunostained for CD 31 (green) and F-actin was visualized by rhodamine-falloidin (red). HEMVEC monolayers remained intact in co-culture, but showed holes in control cells. The addition of VEGF-A improved the quality of the monolayers. *[See appendix: color figures]*

Interaction between hESC and hEMVEC

In the absence of VEGF, hEMVEC showed discontinuities in their monolayer due to cell detachment. Addition of VEGF-A prevented hEMVEC cell death, and induced the maintenance of intact monolayers by the hEMVEC. When hEMVEC and hESC were co-cultured separated from each other by a porous filter, the monolayers of these hEMVEC maintained the characteristic regular cobblestone pattern even in the absence of VEGF (Fig. 7).This suggests that hESC provide factors, including VEGF-A, that stimulate the maintenance of hEMVEC.

Discussion

In this study we have shown that hESC expressed ERα, ERβ and PR and responded to the ovarian steroids by an increase in VEGF-A expression. HEMVEC express ERβ and show only a marginal angiogenic response to E_2. HEMVEC cultured in close contact with hESC survived better, probably due to paracrine VEGF production by hESC.

The occurrence of ER and PR in endometrial cells

In endometrial tissue, ERα, ERβ and PR were detected in endometrial epithelial cells, stromal cells and ERβ and PR in (perivascular) smooth muscle cells[18,34-39]. In the epithelial cells, and less clearly in the stromal cells, the receptors reflected a cycle-depended expression pattern[18,24,34,37-39]. Endothelial cells showed specific staining for the ERβ but no staining for ERα or PR, which is in agreement with previous reports[18,37,38]. Only

Lecce *et al*. detected, although only occasionally, both ERβ and ERα in endometrial endothelial cells and an up-regulation of ERβ during the late secretory phase[38].

In cultured hESC we detected ERα, ERβ and PR mRNA expression. The ERβ expression was less prominent than that of ERα, in agreement with previous observations[38,40,41]. Interestingly, the ERβ could only be detected in hESC by the primer set that amplified part of the C-terminal region of ERβ. HESC lost their ERα upon serial passage under these culture conditions. It is likely that the single hESC isolation, that did not express the ERα, had already lost its ability to express the ERα. The expression of PR appeared to be less dependent on the passage number of the hESC.

Cultured hEMVEC expressed ERβ, in accordance with *in vivo* studies[18,21,38]. Iruela-Arispe *et al*. reported the presence of ER on hEMVEC[19,20]. As these authors made no distinction between ERα and β, the ER detected may well have been ERβ. Because different splicing variants of ERα have been described caution should be taken to conclude definitively on the absence of ERα in endothelial cells[15,16]. However, none of the presently known splicing variants could be detected in hEMVEC in our experimental conditions.

The precise physiological function and importance of ERβ in the endometrium, as well as in other organs, is still unclear[42]. It has been shown that in the human uterus ERβ is less abundant compared to ERα. ERβ-knockout mice were fertile but showed small litter size, likely due to impaired ovarian function, and multiple resorbed fetuses[42,43]. Furthermore, it has been suggested that a role of ERβ may be antagonizing and/or modulating ERα-mediated actions. Examples of this are the exaggerated induction of VEGF by E_2 via ERα in ERβ-knockout mice and the absence of the physiological down-regulation of PR in the luminal epithelium by E_2 via ERα in ERβ-knockout mice[42,44]. When both ERα and ERβ are co-expressed, they can form homo-or heterodimers and it seems that ERβ preferentially forms heterodimers when ERα is present[45,46].

Krikun *et al*.[21] found, in addition to ERβ, also PR mRNA in hEMVEC. However, they could not confirm the presence of PR immunohistochemically. We detected PR mRNA in only one hEMVEC isolation, although to a very minor extent. In the present and other immunohistochemical studies PR could not be detected in endometrial endothelial cells[18,37]. The faint PCR signal for PR mRNA may reflect a minor contamination of the culture with hESC that has escaped our inspection, or indicate that the cells, when at a higher passage, undergo some kind of decidualization, a condition in which endothelial cells have been described as expressing PR and reacting to ovarian steroids[47,48]. Iruela-Arispe *et al*. detected PR on hEMVEC, in addition to ER, although the levels of PR were significantly lower than those displayed by stromal cells[19,20,49]. These authors reported that only a subpopulation of endothelial cells in normal endometrium stained positive for PR[19].

The effects of E_2 and progesterone on hEMVEC

E_2 and progesterone did not affect the proliferation of hEMVEC to a biologically significant level in our study. Iruela-Arispe *et al*. reported a stimulatory effect of E_2 and an inhibitory effect of progesterone on comparable cells, but only in the presence of high concentrations of VEGF-A and bFGF[20]. Peek *et al*., who examined decidual endothelial cells, found an increased proliferation upon exposure to E_2; a lower dose of E_2 and a high dose of progesterone inhibited proliferation[48]. A positive proliferative response to E_2 was described for hUVEC, but other investigators were unable to confirm this[50-52]. On the contrary, hESC responded well to estrogens. In agreement with Irwin *et al*., we found that hESC showed a slight, though not significant, proliferative response to E_2[53]. When our study was completed, Kayisli *et al.* reported that both E_2 and progesterone stimulated hEMVEC proliferation, albeit to a limited extent, and tube formation in a collagen matrix[54]. However, their finding that the hEMVEC responded to 10^{-12} M progesterone is difficult to explain in the light of their own finding that these cells did not express PR. Taken together, our data and those by other investigators indicate that the effect of E_2 and progesterone on hEMVEC is absent or very small as compared to the effect of these ovarian hormones on hESC.

The meaning of the observed minor effect of E_2 on *in vitro* angiogenesis by hEMVEC is doubtful. This effect was only very small compared to the effect seen after stimulation with VEGF-A[22]. Estrogens can rapidly up-regulate VEGF expression in endometrial stromal and epithelial cells by the direct transcriptional action of the ER. VEGF has been shown to be responsive to E_2 and progesterone[23,41,55,56]. Functional DNA sequences, called estrogen response elements (ERE), have been identified in the VEGF gene that functions as a classical enhancer for both ERα and ERβ. Although the ERE can bind both ERs, it might exhibit a more selective response to ERα[57,58]. Also progestins have a direct effect on VEGF gene transcription as analysis of the sequence of the VEGF promoter revealed three functional progesterone response elements (PREs) and full VEGF promoter activation required all three. Although PR-mediated transcriptional regulation of the VEGF-promoter appeared to be complex and could not be localized to confined PRE sequences, other response-element motifs are thus likely to play a contributory role[59].

When the hEMVEC were co-cultured with hESC, improved survival of hEMVEC was seen. This is probably due to the local generation of VEGF, which resembles the *in vivo* situation, in which a positive correlation between stromal VEGF immunostaining and endothelial cell density was found[24]. Other (angiogenic) factors, such as PDGF and bFGF, may be involved as well, as hEMVEC survived better in co-culture than after sole addition of VEGF-A. The epithelial cells of the endometrium also express VEGF-A. Albrecht *et al*. found that myometrial endothelial cells in co-culture with endometrial epithelial cells formed more tube-like structures than with stromal cells[56]. However, it remains un-

certain to what extent the VEGF-A produced by these cells is available to the endothelial cells, as a mainly apical secretion has been described[60].

To summarize, this study indicates that hEMVEC proliferation and *in vitro* angiogenesis is not much influenced by the ovarian steroids despite ERβ expression in these cells. Ovarian steroids stimulate hESC to produce VEGF-A, a factor to which hEMVEC highly respond. These findings suggest that E_2 and progesterone are indirect regulators of endometrial angiogenesis.

Acknowledgements

We thank Dr. J.J. Emeis, and Dr. G.P. van Nieuw Amerongen for their help. Furthermore, we would like to thank Dr. R. M. F. van der Weiden (St. Franciscus Gasthuis, Rotterdam, The Netherlands) and Dr. R.A. Verwey and colleagues (Bronovo Hospital, The Hague, The Netherlands), who provided us with endometrial tissue.

References

1. Smith SK 1998 Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 4:509-519
2. Krikun G, Schatz F, Lockwood CJ 2004 Endometrial angiogenesis: from physiology to pathology. Ann N Y Acad Sci 1034:27-35
3. Augustin HG 2005 Angiogenesis in the female reproductive system. EXS 35-52
4. Pepper MS 1997 Manipulating angiogenesis. From basic science to the bedside. Arterioscler Thromb Vasc Biol 17:605-619
5. Carmeliet P 2003 Angiogenesis in health and disease. Nat Med 9:653-660
6. Abel MH 1985 Prostanoids and menstruation. Mechanisms of menstrual bleeding 25:139-156
7. Markee JE 1940 Menstruation in intraocular endometrial transplants in the rhesus monkey. Contrib.Embryol 177:221-308
8. Gargett CE, Rogers PA 2001 Human endometrial angiogenesis. Reproduction 121:181-186
9. Smith SK 2001 Regulation of angiogenesis in the endometrium. Trends Endocrinol Metab 12:147-151
10. Rees MC, Bicknell R 1998 Angiogenesis in the endometrium. Angiogenesis 2:29-35.
11. Albrecht ED, Pepe GJ 2003 Steroid hormone regulation of angiogenesis in the primate endometrium. Front Biosci 8:d416-d429
12. Mendelsohn ME, Karas RH 1994 Estrogen and the blood vessel wall. Curr Opin Cardiol 9:619-626
13. Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA 1998 Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. Endocrinology 139:1082-1092
14. Yamanaka T, Hirata S, Shoda T, Hoshi K 2002 Progesterone receptor mRNA variant containing novel exon insertions between exon 4 and exon 5 in human uterine endometrium. Endocr J 49:473-482
15. Figtree GA, McDonald D, Watkins H, Channon KM 2003 Truncated estrogen receptor alpha 46-kDa isoform in human endothelial cells: relationship to acute activation of nitric oxide synthase. Circulation 107:120-126
16. Rey JM, Pujol P, Dechaud H, Edouard E, Hedon B, Maudelonde T 1998 Expression of oestrogen receptor-alpha splicing variants and oestrogen receptor-beta in endometrium of infertile patients. Mol Hum Reprod 4:641-647
17. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, Sonntag-Buck V, Gannon F 2000 Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J 19:4688-4700
18. Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR, Saunders,PT 2001 Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. J Clin Endocrinol Metab 86:1370-1378
19. Rodriguez-manzaneque JC, Graubert M, Iruela-Arispe ML 2000 Endothelial cell dysfunction following prolonged activation of progesterone receptor. Hum Reprod 15 Suppl 3:39-47
20. Iruela-Arispe ML, Rodriguez-manzaneque JC, Abujawdeh G 1999 Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue response to angiogenic growth factors. Microcirculation 6:127-140
21. Krikun G, Schatz F, Taylor R Critchley HO, Rogers PA, Huang SJ, Lockwood CJ 2004 Endometrial endothelial cells steroid receptor expression and steroid effects on gene expression. J Clin Endocrinol Metab 90:1812-1818
22. Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van Der Weiden RM, Helmerhorst FM, van Hinsbergh VW 2001 Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. J Clin Endocrinol Metab 86:3359-3367
23. Taylor RN, Lebovic DI, Hornung D, Mueller MD 2001 Endocrine and paracrine regulation of endometrial angiogenesis. Ann N Y Acad Sci 943:109-121
24. Charnock-Jones DS, Macpherson AM, Archer DF, Leslie S, Makkink WK, Sharkey AM, Smith SK 2000 The effect of progestins on vascular endothelial growth factor, oestrogen receptor and progesterone receptor immunoreactivity and endothelial cell density in human endometrium. Hum Reprod 15 Suppl 3:85-95
25. Mueller MD, Lebovic DI, Garrett E, Taylor RN 2000 Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. Fertil Steril 74:107-112
26. Gerritsen ME 1987 Functional heterogeneity of vascular endothelial cells. Biochem Pharmacol 36:2701-2711
27. Schatz F, Soderland C, Hendricks-Munoz KD, Gerrets RP, Lockwood CJ 2000 Human endometrial endothelial cells: isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. Biol Reprod 62:691-697

28. Nikitenko LL, Mackenzie IZ, Rees MC, Bicknell R 2000 Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. Mol Hum Reprod 6:811-819
29. Plaisier M, Kapiteijn K, Koolwijk P, Fijten C, Hanemaaijer R, Grimbergen JM, Mulder-Stapel A, Quax PH, Helmerhorst FM, van Hinsbergh VW 2004 Involvement of membrane-Type matrix metalloproteinases (MT-MMPs) in capillary tube formation by human endometrial microvascular endothelial cells: role of MT3-MMP. J Clin Endocrinol Metab 89:5828-5836
30. Wakeling AE, Dukes M, Bowler J 1991 A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867-3873
31. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159
32. Perrot-Applanat M, Cohen-Solal K, Milgrom E, Finet M 1995 Progesterone receptor expression in human saphenous veins. Circulation 92:2975-2983
33. Mosselman S, Polman J, Dijkema R 1996 ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49-53
34. Garcia E, Bouchard P, De Brux J, Berdah J, Frydman R, Schaison G, Milgrom E, Perrot-Applanat M 1988 Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. J Clin Endocrinol Metab 67:80-87
35. Critchley HO, Henderson TA, Kelly RW, Scobie GS, Evans LR, Groome NP, Saunders PT 2002 Wild-type estrogen receptor (ERbeta1) and the splice variant (ERbetacx/beta2) are both expressed within the human endometrium throughout the normal menstrual cycle. J Clin Endocrinol Metab 87:5265-5273
36. Taylor AH, Al Azzawi F 2000 Immunolocalisation of oestrogen receptor beta in human tissues. J Mol Endocrinol 24:145-155
37. Press MF, Udove JA, Greene GL 1988 Progesterone receptor distribution in the human endometrium. Analysis using monoclonal antibodies to the human progesterone receptor. Am J Pathol 131:112-124
38. Lecce G, Meduri G, Ancelin M, Bergeron C, Perrot-Applanat M 2001 Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. J Clin Endocrinol Metab 86:1379-1386
39. Fujishita A, Nakane PK, Koji T, Masuzaki H, Chavez RO, Yamabe T, Ishimaru T 1997 Expression of estrogen and progesterone receptors in endometrium and peritoneal endometriosis: an immunohistochemical and in situ hybridization study. Fertil Steril 67:856-864
40. Brandenberger AW, Lebovic DI, Tee MK, Ryan IP, Tseng JF, Jaffe RB, Taylor RN 1999 Oestrogen receptor (ER)-alpha and ER-beta isoforms in normal endometrial and endometriosis-derived stromal cells. Mol Hum Reprod 5:651-655
41. Classen-Linke I, Alfer J, Krusche CA, Chwalisz K, Rath W, Beier HM 2000 Progestins, progesterone receptor modulators, and progesterone antagonists change VEGF release of endometrial cells in culture. Steroids 65:763-771
42. Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA 2000 Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A 97:5936-5941
43. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 95:15677-15682
44. Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson JA 2000 Estrogen receptors alpha and beta in the rodent mammary gland. Proc Natl Acad Sci U S A 97:337-342
45. Fujimoto J, Hirose R, Sakaguchi H, Tamaya T 1999 Expression of oestrogen receptor-alpha and -beta in ovarian endometriomata. Mol Hum Reprod 5:742-747
46. Cowley SM, Hoare S, Mosselman S, Parker MG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem 272:19858-19862
47. Wang JD, Fu Y, Shi WL, Zhu PD, Cheng J, Qiao GM, Wang YQ, Greene GL 1992 Immunohistochemical localization of progesterone receptor in human decidua of early pregnancy. Hum Reprod 7:123-127
48. Peek MJ, Markham R, Fraser IS 1995 The effects of natural and synthetic sex steroids on human decidual endothelial cell proliferation. Hum Reprod 10:2238-2243
49. Vazquez F, Rodriguez-manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML 1999 Progesterone regulates proliferation of endothelial cells. J Biol Chem 274:2185-2192
50. Kim-Schulze S, McGowan KA, Hubchak SC, Cid MC, Martin MB, Kleinman HK, Greene GL, Schnaper HW 1996 Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. Circulation 94:1402-1407
51. Soares R, Guo S, Russo J, Schmitt F 2003 Role of the estrogen antagonist ICI 182,780 in vessel assembly and apoptosis of endothelial cells. Ultrastruct Pathol 27:33-39
52. Keck C, Herchenbach D, Pfisterer J, Breckwoldt M 1998 Effects of 17beta-estradiol and progesterone on

interleukin-6 production and proliferation of human umbilical vein endothelial cells. Exp Clin Endocrinol Diabetes 106:334-339
53. Irwin JC, Kirk D, King RJ, Quigley MM, Gwatkin RB 1989 Hormonal regulation of human endometrial stromal cells in culture: an *in vitro* model for decidualization. Fertil Steril 52:761-768
54. Kayisli UA, Luk J, Guzeloglu-Kayisli O, Seval Y, Demir R, Arici A 2004 Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids. J Clin Endocrinol Metab 89:5794-5802
55. Perrot-Applanat M, Ancelin M, Buteau-Lozano H, Meduri G, Bausero P 2000 Ovarian steroids in endometrial angiogenesis. Steroids 65:599-603
56. Albrecht ED, Babischkin JS, Lidor Y, Anderson LD, Udoff LC, Pepe GJ 2003 Effect of estrogen on angiogenesis in co-cultures of human endometrial cells and microvascular endothelial cells. Hum Reprod 18:2039-2047
57. Hyder SM, Nawaz Z, Chiappetta C, Stancel GM 2000 Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res 60:3183-3190
58. Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN 2000 Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. Proc Natl Acad Sci U S A 97:10972-10977
59. Mueller MD, Vigne JL, Pritts EA, Chao V, Dreher E, Taylor RN 2003 Progestins activate vascular endothelial growth factor gene transcription in endometrial adenocarcinoma cells. Fertil Steril 79:386-392
60. Hornung D, Lebovic DI, Shifren JL, Vigne JL, Taylor RN 1998 Vectorial secretion of vascular endothelial growth factor by polarized human endometrial epithelial cells. Fertil Steril 69:909-915

8

HUMAN EMBRYO-CONDITIONED MEDIUM STIMULATES *IN VITRO* ENDOMETRIAL ANGIOGENESIS

K. Kapiteijn
P. Koolwijk
R.M.F. van der Weiden
G.P. van Nieuw Amerongen
M. Plaisier
V.W.M. van Hinsbergh
F.M. Helmerhorst

Fertility and Sterility 2006;85 Suppl 1,1232-1239

Introduction

Successful implantation and subsequent placentation depend on the interaction between a receptive decidualized endometrium and an intrusive blastocyst. Angiogenesis plays a major role in the formation of a receptive endometrium and an adequate functioning of the placenta.

When the blastocyst enters the uterine cavity, its survival depends on endometrial secretion. After attachment and invasion, it is fed and oxygenated by the decidualized endometrium.

Maternal blood supply, via an extensive endometrial vascular network, to the embryo is indispensable for further growth. In the peri-implantation period, local enhancement of angiogenesis is necessary to support further differentiation of the endometrium, ultimately leading to the formation of the maternal part of the placenta. The stimulus for this process might very well come from the implanting blastocyst itself, which in this way optimizes its implantation site[1]. Inadequate angiogenesis in the peri-implantation phase may lead to a less receptive endometrium. This can result in implantation failure or aberrant placental formation, which in turn may affect the pregnancy outcome, as demonstrated in morphological studies demonstrating poor placental vascular development in intrauterine growth restriction[2].

Before the embryo and the endometrium can make physical contact, an interaction by signaling molecules must have been established[3]. The exact nature of this interaction is not fully resolved yet. It is unknown whether the blastocyst is able to induce the angiogenic process in the endometrium directly or indirectly via the epithelial or stromal cells[4-7]. Following penetration of the epithelial lining, the embryo has to establish a closer contact with endothelial cells. A direct regulation of angiogenesis by the blastocyst/early trophoblast might be possible during that phase of implantation.

The production of cytokines and hormones varies at specific stages of embryonic differentiation. Human blastocysts produce activin, colony stimulating factor (CSF)-1, epidermal growth factor (EGF), interferon (IFN) γ, insulin-like growth factor (IGF) I and II, interleukin (IL) 1α and -β, IL-6, IL-10, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) α and β, tumor necrosis factor (TNF) α, vascular endothelial growth factor (VEGF)-A, and hCG, whereas human first trimester trophoblasts produce EGF, IGF-II, placental growth factor (PLGF), TGFα and β, TNFα, and hCG. These molecules may enable the implanting embryo to induce angiogenesis locally at the implantation site. Among these factors, VEGF-A is known to be a highly specific mitogen for endothelial cells[8]. It induces angiogenesis and increases the permeability of blood vessels[9].

Here we have investigated the influence of the embryo on endometrial angiogenesis

by evaluating the effect of conditioned medium from human embryos (IVF culture medium) on isolated human endometrial microvascular endothelial cells (hEMVEC) in an *in vitro* angiogenesis model, which we have previously characterized[10]. Furthermore, individual recombinant cytokines, known to be expressed by the human embryo and first trimester trophoblast, together with hCG were tested on hEMVEC to determine which factors are involved in inducing angiogenesis at the time of implantation.

Materials and methods

Materials

Penicillin/streptomycin, L-glutamine and medium 199 (M199) with and without phenol red and supplemented with 20 mmol/L HEPES was obtained from BioWhittaker (Verviers, Belgium); Newborn calf serum (NBCS) was obtained from Life Technologies (Grand Island, NY, USA). Human serum (HS) was obtained from a local blood bank and was prepared from fresh blood from 10-20 healthy donors, pooled and stored at 4°C; it was heat-inactivated before use. Human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Tissue culture plastics and microtiter plates came from Costar/Corning (Cambridge, MA) and Falcon (Becton Dickinson, Bedford, MA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al*[11]. Heparin and thrombin were obtained from Leo Pharmaceutics Products (Weesp, the Netherlands). Human fibrinogen was purchased from Chromogenics (Mölndal, Sweden). Dr. H. Metzner and Dr. G. Seeman (Aventis Behring, Marburg, Germany) generously provided factor XIII. Fibronectin was a gift from Dr. J. van Mourik (CLB, Amsterdam, The Netherlands). Human recombinant VEGF-A and PLGF were purchased from RELIATech (Braunschweig, Germany); soluble VEGF receptor 1 (sVEGFR-1) was a generous gift from Dr. H.A. Weich (GBF, Braunschweig, Germany). Tumor necrosis factor α was a gift from Dr. J. Travernier (Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ). Recombinant human activin was obtained from Dr. Pawson via the National Hormone and Pituitary Program, The National Institute of Diabetes and Digestive and Kidney disease, The National Institute of Child Health and Human Development, and the US Department of Agriculture (Bethessa, MD). Recombinant human EGF, human CSF, IFNγ, IGF-I and –II, IL-1α, -1β, -6 and -10 and TGFα and -β were commercially obtained from PrepoTech (Rocky Hill, NJ). Human chorionic gonadotrophin (Pregnyl) was obtained from Organon (Oss, The Netherlands). Recombinant human LIF was purchased from Chemicon International (Temecula, CA) and PDGF B/B from Roche (Mannheim, Germany).

Human embryo conditioned medium

The study was conducted according to the guidelines of the Institutional Review Board, and informed consent was obtained from each patient. The IVF culture media used in our experiments were obtained from the IVF Department of the Reinier de Graaf Group (Diaconessenhuis, Voorburg, The Netherlands) and the Infertility Centre from the Gent University Hospital (Gent, Belgium). The media were collected during a period of 1 and 2 years. Early stage human embryos obtained after oocyte pick-up and IVF were cultured in media until embryo transfer. The culture media used were: GPO medium (for the exact compounds see Rijnders *et al* 1998[12]), Complete P-1 and Complete Blastocyst Irvine media (Irvine Scientific, Santa Ana, Ca) and Earle's medium supplemented with 0.08% (w/v) HSA, penicillin G (8 mg/L), sodium pyruvate (0.10 g/L), and sodium bicarbonate (2.1 g/L).

Early stage human embryos produce and accumulate mediators in the medium in which they are cultured. Pool A, B and C consisted of medium in which 12, 40 and 79 embryos (2-8 cell stage) were cultured originating from 7, 10 and 17 patients respectively. Pool D consisted of medium from 90 blastocysts originating from an unknown number of patients.

Earle's medium or GPO medium (pool A, B and C) were refreshed after one or three days respectively. The Irvine medium (pool D) was changed on day 3 from Complete-1 Irvine medium to Complete Blastocyst Irvine medium. No data are available on the success rate of implantation of these embryos.

Other materials used have been specified in the methods described or in the related references mentioned.

Cell culture

Human endometrial microvascular endothelial cells (hEMVEC) were isolated from endometrial tissue (collected according to the guidelines of the Institutional Review Board and informed consent was obtained from each patient) as previously described[10] and maintained in indicator-free M199 supplemented with 20 mM HEPES (pH 7.3), 20% human serum, 10% heat-inactivated NBCS, 150 μg/mL ECGF, 5 ng/mL VEGF-A, 5 U/mL heparin, 100 IU/mL penicillin, and 100 mg/mL streptomycin (= hEMVEC culture medium). HEMVEC were cultured on fibronectin-coated wells at 5% CO_2 / 95% air until confluence was reached and were subsequently detached with 0.05% trypsin / 0.025% EDTA and transferred into coated dishes at a split ratio of 1:3. Fresh medium was given three times a week with twice a two day interval and once a three day interval (weekend).

Incorporation of ^{3}H-thymidine

Incorporation of ^{3}H-thymidine into DNA was determined as the measurement of endothelial cell proliferation. Confluent cultures of endothelial cells (passages 5 to 9 of three different donors) were detached by trypsin/EDTA solution and allowed to adhere and spread at a density of 10^4 cells per cm^2 on fibronectin-coated dishes in indicator-free M199-HEPES supplemented with 10% heat-inactivated and charcoal-treated NBCS, penicillin/streptomycin and 0.75 ng/mL VEGF-A for 18 h. The 0.75 ng/mL VEGF-A was added as a maintenance factor to prevent hEMVEC death under these control culture conditions. Then the cells were stimulated with conditioned medium, increasing concentrations of cytokines or hCG in the presence or absence of extra 6.25 ng/mL VEGF-A, as indicated in the text. After a total incubation period of 42 h, ^{3}H-thymidine was added and the cells were incubated for another 6 h period. Subsequently, the ^{3}H-labeled DNA was precipitated and counted in a liquid scintillation counter and the stimulation index was calculated as previously described[10].

In vitro angiogenesis model

Human fibrin matrices were prepared as described by Koolwijk *et al*[10]. Confluent hEMVEC (passages 6 to11 of two different donors) were detached and seeded in a split ratio of 2:1 on the surface of the fibrin matrices and cultured for 24 h in indicator-free M199 medium supplemented with 20% human serum, 10% NBCS, and penicillin/streptomycin. Then the endothelial cells were cultured with the mediators indicated for 2 - 5 days. Invading cells and the formation of capillary-like structures of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy; the total length of the tube-like structures was measured as described by Kroon *et al*[13].

Enzyme-Linked Immunosorbent Assays (ELISA)

The VEGF-A antigen determinations were performed by the commercially available DuoSet ELISA Development Kit for human VEGF-A (R&D system, Minneapolis, MN), which recognizes VEGF-A_{165} and VEGF-A_{121}. Human recombinant VEGF-A_{165} (R&D systems) was used as a standard.

Statistics

The data are expressed as the mean ± SD/SEM or range. Statistical evaluations of the data were performed using the paired *t* test and Wilcoxon rank test after the control conditions were set at a 100%. $p < 0.05$ was considered statistically significant.

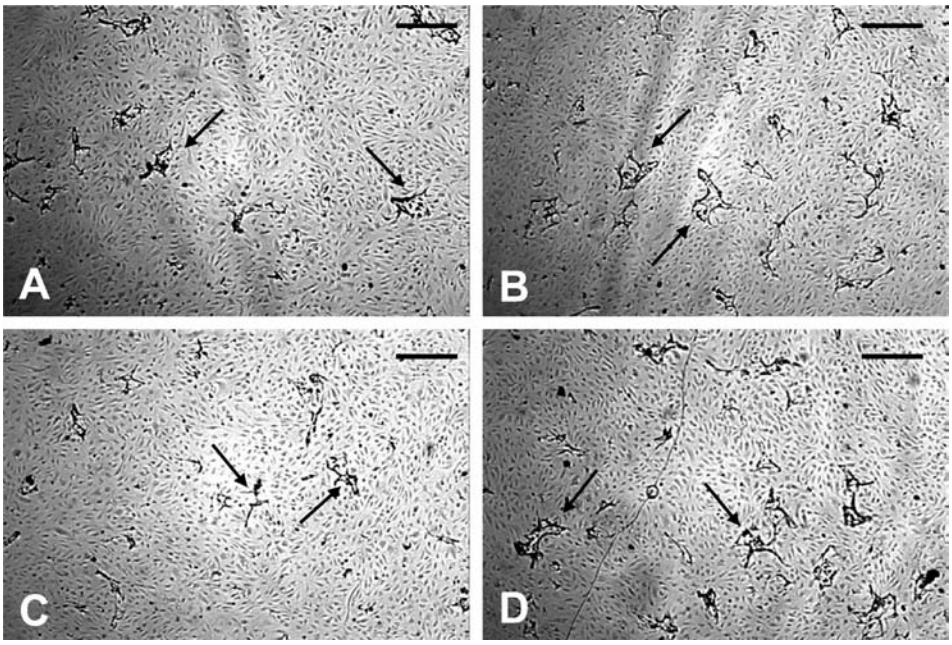


Figure 1. Conditioned medium of early stage embryos stimulate *in vitro* hEMVEC tube formation.
Phase-contrast pictures were taken of hEMVEC cultured on top of a three-dimensional fibrin matrix under control conditions (A), after stimulation with VEGF-A (10 ng/mL, B), 5% (v/v) control IVF medium (C), or 5% (v/v) pooled conditioned medium (D). The number of tubular structures (examples indicated by arrows) increased after stimulation with VEGF-A or 5% pooled conditioned medium. *Bar* = 500 μM.

Results

Early stage embryo conditioned medium enhances hEMVEC proliferation and tube formation

We have used conditioned IVF culture medium of pool A to evaluate the effect of cytokines and other mediators produced by early stage human embryos on hEMVEC proliferation and the media of pools B, C and the blastocysts derived pool D on *in vitro* angiogenesis.

An increase in hEMVEC proliferation was observed when hEMVEC were stimulated with pool A. When 5% of the conditioned media was added, a maximum stimulation was observed, although this increase was not significant as compared with the control condition (with 0.75 ng/mL VEGF-A as maintenance factor, data not shown). When 5% of non-conditioned culture media was added, no effect on hEMVEC proliferation was seen.

Pools B, C and D independently induced an increase in tube formation by hEMVEC when these cells were stimulated with 2.5-10% of the conditioned media (Fig. 1). The

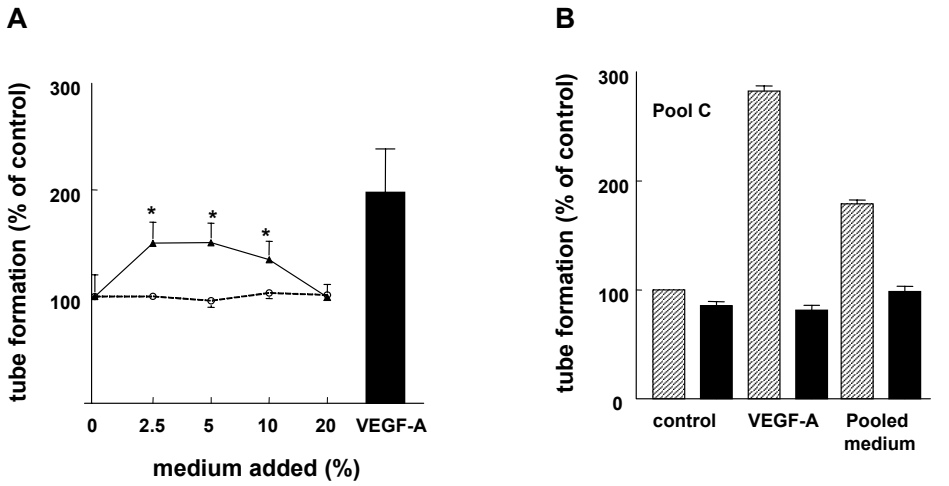


Figure 2. Conditioned medium of embryos stimulate *in vitro* hEMVEC tube formation, an effect which is VEGF-A-mediated.

A. HEMVEC, from passage 5-10 of one donor, were cultured on top of a three-dimensional fibrin matrix and stimulated with 2.5%, 5%, 10%, and 20% (v/v) of pooled medium (pool B,C, or D; ▲ with solid line). As control, the cells were stimulated with 2.5%, 5%, and 10%, and 20% (v/v) of the IVF culture medium in which no embryos were grown (○ with dotted line) or 10 ng/ml VEGF (solid bars). After 3-5 days of culturing, mean tube length was measured. The data are expressed as a percentage of the control and represent mean ± SEM of four independent experiments performed in duplicate wells. * = $p < 0.05$ compared to control condition. The mean tube length of the controls was 63.4 mm/cm^2.

B. Addition of 0.5 μg/mL sVEGFR-1 to control (M199 supplemented with 10% NBCS and 20% HS) and VEGF-A stimulated conditions, inhibited the amount of capillary-like structures formed. The enhanced formation of capillary-like structures by hEMVEC after stimulation with 10% (v/v) embryo culture medium was also reduced by sVEGFR-1. Mean tube length was measured and expressed as a percentage of the control ± range. Hatched bars: without sVEGFR-1, solid bars: with sVEGFR-1. The mean tube length of the controls was 77.4 mm/cm^2. The experiments were performed in duplicate wells.

results of the three pools were taken together for statistical analysis. Quantification of the tube formation revealed that the effect was significant when 2.5-10% of the conditioned medium was used and showed an increase of 150%, 151% and 135% when 2.5%, 5%, and 10%, respectively, of the conditioned medium was used (control set at 100%)(Fig. 2A). At higher concentrations (20%) the ingrowth of vascular structures declined. The nonconditioned IVF culture medium (control) had no effect on tube formation (Fig. 2A).

To test whether the enhancement of tube formation was due to expression of VEGF-A by the early stage embryos, sVEGFR-1 was added to the pools. Soluble VEGFR-1 captures VEGF-A and prevents its binding to cellular receptors. The presence of sVEGFR-1 completely prevented VEGF-A-induced tube formation under our standard conditions. Addition of

sVEGFR-1 inhibited the tube formation that was enhanced by pool C (Fig. 2B). Similar results were obtained with pool B and D (data not shown). This suggests that early stage embryos are able to express VEGF-A to such a level that it was involved in the stimulation of tube formation by hEMVEC.

To confirm the presence of VEGF-A in the conditioned medium of day 2-3 and blastocyst-stage embryos, VEGF-A concentrations in the media of pool B, C and D were assayed by ELISA. In the pooled media B, C and D, 10,700 pg/mL, 5200 pg/mL and 14 pg/mL VEGF-A antigen was detected respectively. The control medium of the pooled media B and C, in which no embryos were grown, contained 70 pg/mL of VEGF-A, whereas no VEGF-A antigen was detectable in the control medium of pool D.

Effect of cytokines and hCG produced by the early embryo and first trimester trophoblast on hEMVEC proliferation

Because conditioned medium of early embryos was able to induce hEMVEC proliferation and tube formation, the question arose which factors produced by the human embryo could be held responsible for these effects. As such, recombinant cytokines and hCG, known to be expressed by the human embryo and first trimester trophoblast, were tested on hEMVEC proliferation and tube formation, both in the absence or presence of 6.25 ng/mL VEGF-A.

VEGF-A was a potent stimulator of hEMVEC proliferation, as measured by ^{3}H-thymidine incorporation[10] and confirmed by an increase in cell number (determined at 48h; data not shown).

Under control conditions (with 0.75 ng/mL VEGF-A as maintenance factor), IL-1α and activin (only the highest concentration) significantly inhibited hEMVEC proliferation, whereas all the other tested cytokines (EGF, LIF, CSF-1, IFNγ, IGF-I, IGF-II, IL-1β, IL-6, IL-10, PDGF, TGFα, TGFβ, PLGF and hCG) did not have a significant effect. Only incubation with increasing concentrations of IL-1α had a significant inhibitory effect on hEMVEC proliferation induced by 6.25 ng/mL VEGF-A (data not shown).

Cell death in hEMVEC cultures was caused by TNFα, at concentrations of 1 and 2.5 ng/mL, but addition of 6.25 ng/mL VEGF-A prevented the TNFα-induced cell death (data not shown).

Effect of cytokines and hCG produced by the early embryo and first trimester trophoblast on *in vitro* angiogenesis by hEMVEC

Subsequently, the effect of factors expressed by the human embryo and first trimester trophoblast on *in vitro* angiogenesis by hEMVEC was studied in the absence or presence of VEGF-A.

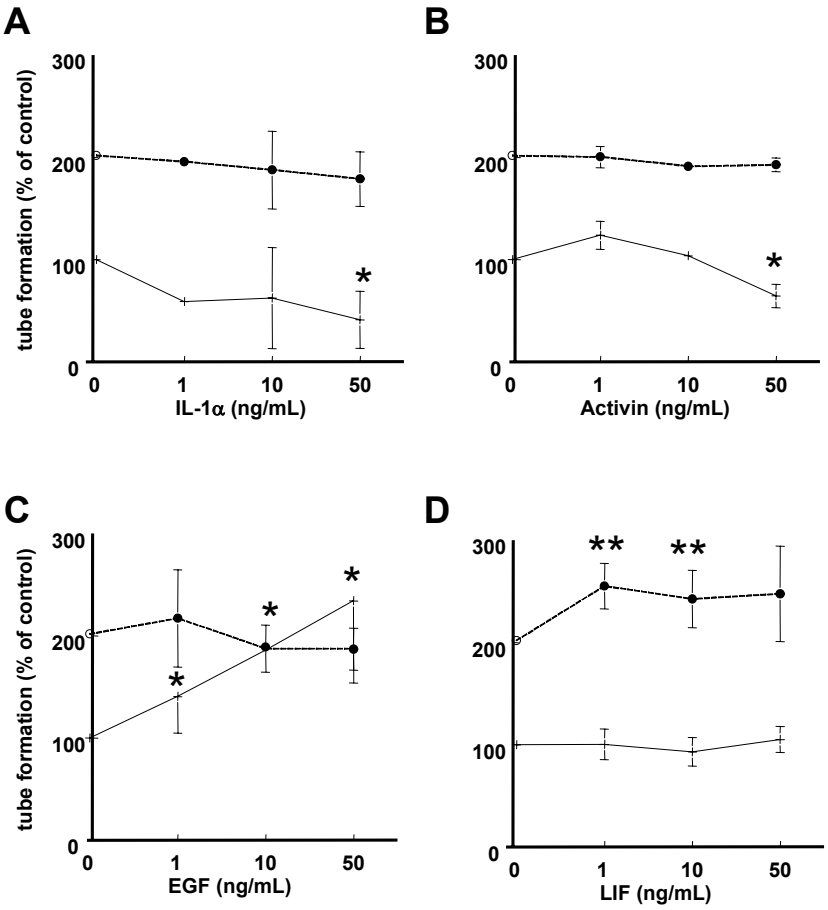


Figure 3. The influence of recombinant cytokines and hCG on the formation of capillary-like structures by hEMVEC.

HEMVEC were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 20% human serum and 10% NBCS and stimulated with increasing amounts of different cytokines and hCG in the absence (solid line) or presence (dotted line) of VEGF-A (10 ng/mL). After 2-5 days of culturing, mean tube length was measured by image analysis as described and expressed as a percentage of the control ± range of two independent experiments performed in duplicate wells. * = $p < 0.05$ compared to control condition, ** = $p < 0.05$ compared to VEGF-stimulated condition.

In the absence of VEGF-A, EGF significantly stimulated tube formation concentration dependently. However, in combination with VEGF-A no additive effect of EGF was observed (Fig. 3C). Similarly to their inhibitory effect on hEMVEC proliferation, high concentrations of activin (Fig. 3B), IL-1α (Fig. 3A) and IL-1β (not shown) significantly inhibited the amount of tubes formed. Interestingly, LIF (1 and 10 ng/mL) significantly stimulated the VEGF-A-enhanced tube formation but did not alter basal tube formation

(Fig. 3D). Interleukin-10, TGFβ, PLGF, hCG, CSF-1, IFNγ, IGF-I/-II, IL-6, PDGF, and TGFα in the indicated concentrations had no effect on tube formation (data not shown).

Discussion

The data presented here demonstrate that conditioned media of human embryos contained VEGF-A and stimulated *in vitro* endometrial angiogenesis, an effect counteracted by sVEGFR-1. The VEGF-A was the most potent mediator in stimulating hEMVEC proliferation and tube formation among the known mediators expressed by the human embryo and first trimester trophoblast, of which LIF could increase the VEGF-mediated tube formation.

Adequate interaction between embryo and endometrium is essential for successful implantation and placentation. The embryo locally prepares the endometrium for its nidation by producing various mediators[3,14-17]. Previously, Sakkas *et al*.[18] described that the human blastocyst directly induces changes in endometrial epithelial cells. Therefore it was suggested that the human embryo might also directly affect the endometrial endothelium, thus regulating endometrial angiogenesis, an important factor in the preparation process[19]. Studies in rats support the hypothesis that angiogenesis at the implantation site is a localized process controlled by the embryo, whereas angiogenesis, which occurs in the entire endometrium is maternally controlled[20,21]. Our data indicate that the human embryo is able to produce detectable concentrations of active VEGF-A and thus to stimulate local angiogenesis in the endometrium during the peri-implantation phase.

Krüssel *et al*[22,23] previously demonstrated that human embryos from the 10-cell up to the blastocyst stage were able to express mRNA, encoding for four different isoforms of VEGF-A (121, 145, 165, and 189). Relatively highly expressed were isoforms 121 and 165, which are both secreted VEGF-A isoforms. However, they were not able to detect VEGF-A protein in the embryo culture medium, presumably because it was below the detection limit of the ELISA, which only detected VEGF-A 165. The ELISA we used detected both the 121 and 165 isoform. Furthermore, Krüssel *et al*. used a larger volume of conditioned medium per embryo (50 μL vs. 10-30 μL in our experiments).

The human embryos in our study were the only source of VEGF-A production, because little or no VEGF-A protein could be detected in the control IVF medium in which no embryos were cultured. After 24 (pool A to C) or 72 (pool D) h of culture the medium was changed. This eliminates other potential sources of cytokine production (by granulosa cells, cumulus cells and sperm cells). Krüssel *et al.*[22,24] almost ruled out the possibility of paternal contamination by using embryos, which resulted from intracytoplasmic sperm

injection. They found that unfertilized oocytes did not express VEGF-A mRNA, which proved that the VEGF-A mRNA was truly embryonic[23].

The amount of VEGF-A detected by ELISA varied in the pools. This could not be explained only by the difference in numbers of embryos from which the pools were derived. Differences in the culture media used in pools A to C on the one hand and pool D on the other, differences in embryonic stage and viability, or reuptake of VEGF-A by the blastocyst itself[25] may contribute to and explain the phenomenon.

It should be noted that the proliferation and outgrowth of tubular structures was reduced at high concentrations of embryo-conditioned medium. It is plausible that in addition to the stimulatory VEGF-A, also angiogenesis-inhibiting compounds also are present, which only become effective at relatively high concentrations and point to a delicate balance between angiogenesis-stimulating and -inhibiting factors which strictly control angiogenesis.

Heterozygous and homozygous deletion of the VEGF-A gene in mouse embryos resulted in embryonic mortality at midgestation and in impaired placental development due to abnormal formation of intra- and extraembryonic vessels[26-28]. This further underlines the importance of VEGF-A. However, the embryo might also produce other mediators which are able to induce or enhance angiogenesis at the site of implantation. Such cytokines derived from the human embryo may act directly on angiogenesis or may affect angiogenesis indirectly by inducing VEGF-A (e.g. IL1β, hCG[29,30]), or its receptors in endometrial cells.

One other candidate mediator that, in the presence of VEGF, might be involved in angiogenesis at the site of implantation is LIF. In our studies LIF had an indirect effect on angiogenesis. It increased the VEGF-mediated tube formation, whereas LIF by itself did not have an effect.

In vivo, it has been shown that LIF was able to induce angiogenesis in the rabbit cornea[31] and that female mice lacking a functional LIF gene are fertile but their blastocysts fail to implant and their uteri were found to be poorly vascularized[32,33]. However, *in vitro*, inconsistent effects of LIF on endothelial cells are described. Leukemia inhibitor factor, either or not in the presence of an angiogenic factor, was found to inhibit[34-36] or stimulate[31,37] endothelial cell proliferation and tube formation. Also on the influence of LIF on the proteolytic potential of endothelial cells, an important phase in angiogenesis, opposite results were found[29,31,34]. These discrepancies can be attributed to the biological versatility of LIF which depends on cell species and origin.

Although the embryo only produces small amounts of angiogenic factors, locally high concentrations are reached owing to close contact between the embryo and maternal blood vessels. Together with the increased sensitivity of the endometrial endothelium to angiogenic factors at the time of implantation, the embryo might very well cause an increased angiogenic response at the implantation site.

In conclusion, VEGF-A has been recognized as an important mediator in the process of endometrial angiogenesis during the menstrual cycle[10,38,39]. The results of this study support a crucial role for embryonic VEGF-A in the process of angiogenesis during the peri-implantation phase. By the expression of VEGF-A, the embryo enables itself to induce angiogenesis directly at its implantation site, and as such creates an environment necessary for its survival and growth.

In contrast to tumor angiogenesis, endometrial angiogenesis at the time of implantation and placentation seems to be strictly orchestrated. Further studies should focus on the exact nature of the interactions between the human embryo and the endometrium regarding angiogenesis in the peri-implantation phase and during the formation of placenta, and on the role of defective angiogenesis in implantation failure, which affects the outcome of gestation.

Acknowledgements

We wish to thank Ellen Rijnders, Ilse de Croo and their collegues from the IVF Department of the Reinier de Graaf Group (Diaconessenhuis, Voorburg, The Netherlands) and the Infertility Centre from the Gent University Hospital (Gent, Belgium) for collecting the IVF culture media. Furthermore we would like to thank Dr. R.A. Verwey and colleagues of the Bronovo Hospital (Den Haag, The Netherlands), who provided us with endometrial tissue and Erna Peters for her technical assistance.

References

1. van der Weiden RM, Wisse LJ, Helmerhorst FM, Keirse MJ, Poelmann RE. Immunohistochemical and ultrastructural localization of prostaglandin H synthase in the preimplantation mouse embryo. J Reprod Fertil 1996;107:161-66.
2. Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. Placenta 2000;21 Suppl A:S16-24.
3. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. N Engl J Med 2001;345:1400-08.
4. Armant DR, Wang J, Liu Z. Intracellular signaling in the developing blastocyst as a consequence of the maternal-embryonic dialogue. Semin Reprod Med 2000;18:273-87.
5. Salamonsen LA, Dimitriadis E, Robb L. Cytokines in implantation. Semin Reprod Med 2000;18:299-310.
6. Kimber SJ. Molecular interactions at the maternal-embryonic interface during the early phase of implantation. Semin Reprod Med 2000;18:237-53.
7. Krussel JS, Huang HY, Hirchenhain J, Bielfeld P, Cupisti S, Jeremias L, *et al*. Is there a place for biochemical embryonic preimplantational screening? J Reprod Fertil Suppl 2000;55:147-59.
8. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 2001;280:C1358-66.
9. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol. 1995;146:1029-39.
10. Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van der Vecht B, Helmerhorst FM, *et al*. Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. J Clin Endocrinol Metab 2001;86:3359-67.
11. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc Natl Acad Sci USA 1979;76:5674-78.
12. Rijnders PM, Jansen CA. The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod 1998;13:2869-73.
13. Kroon ME, Koolwijk P, van Goor H, Weidle UH, Collen A, van der Pluijm G, *et al*. Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. Am J Pathol 1999;154:1731-42.
14. van der Weiden RM, Helmerhorst FM, Keirse MJ. Influence of prostaglandins and platelet activating factor on implantation. Hum Reprod 1991;6:436-42.
15. Lindhard A, Bentin-Ley U, Ravn V, Islin H, Hviid T, Rex S,*et al*. Biochemical evaluation of endometrial function at the time of implantation. Fertil Steril 2002;78:221-33.
16. Tabibzadeh S, Babaknia A. The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. Hum Reprod 1995;10:1579-1602.
17. Lopata A. Blastocyst-endometrial interaction: an appraisal of some old and new ideas. Mol Hum Reprod 1996;2:519-25.
18. Sakkas D, Lu C, Zulfikaroglu E, Neuber E, Taylor HS. A soluble molecule secreted by human blastocysts modulates regulation of HOXA10 expression in an epithelial endometrial cell line. Fertil Steril 2003;80:1169-74.
19. Geva E, Ginzinger DG, Zaloudek CJ, Moore DH, Byrne A, Jaffe RB. Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2. J Clin Endocrinol Metab 2002;87:4213-24.
20. Abberton KM, Rogers PA. Production of an endothelial cell migratory signal in rat endometrium during early pregnancy. Cell Tissue Res 1995;279:215-20.
21. Goodger AM, Rogers PA. Uterine endothelial cell proliferation before and after embryo implantation in rats. J Reprod Fertil 1993;99:451-57.
22. Krüssel JS, Behr B, Milki AA, Hirchenhain J, Wen Y, Bielfeld P, *et al*. Vascular endothelial growth factor (VEGF) mRNA splice variants are differentially expressed in human blastocysts. Mol Hum Reprod 2001;7:57-63.
23. Krüssel JS, Behr B, Hirchenhain J, Wen Y, Milki AA, Cupisti S, *et al*. Expression of vascular endothelial growth factor mRNA in human preimplantation embryos derived from tripronuclear zygotes. Fertil Steril 2000;74:1220-26.
24. Krüssel JS, Casan EM, Raga F, Hirchenhain J, Wen Y, Huanh HY, *et al*. Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sflt in cycling human endometrium. Mol Hum Reprod 1999;5:452-58.
25. Hornung D, Lebovic DI, Shifren JL, Vigne JL, Taylor RN. Vectorial secretion of vascular endothelial growth factor by polarized human endometrial epithelial cells. Fertil Steril 1998;69:909-15.

26. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, *et al*. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435-39.
27. Carmeliet P, Moons L, Dewerchin M, Mackman N, Luther T, Breier G, *et al*. Insights in vessel development and vascular disorders using targeted inactivation and transfer of vascular endothelial growth factor, the tissue factor receptor, and the plasminogen system. Ann N Y Acad Sci 1997;811:191-206.
28. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, *et al*. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439-42.
29. Sunder S, Lenton EA. Endocrinology of the peri-implantation period. Baillieres Best Pract Res Clin Obstet Gynaecol 2000;14:789-800.
30. Licht P, Russu V, Wildt L. On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. Semin Reprod Med 2001;19:37-47.
31. Vasse M, Pourtau J, Trochon V, Muraine M, Vannier J, Lu H, *et al*. Oncostatin M induces angiogenesis *in vitro* and in vivo. Arterioscler Thromb Vasc Biol 1999;19:1835-42.
32. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, *et al*. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992;359:76-9.
33. Chen HF, Shew JY, Ho HN, Hsu WL, Yang YS. Expression of leukemia inhibitory factor and its receptor in preimplantation embryos. Fertil Steril 1999;72:713-19.
34. Pepper MS, Ferrara N, Orci L, Montesano R. Leukemia inhibitory factor (LIF) inhibits angiogenesis *in vitro*. J Cell Sci 1995;108:73-83.
35. Hatzi E, Murphy C, Zoephel A, Ahorn H, Tontsch U, Bamberger A, *et al*. N-myc oncogene overexpression down-regulates leukemia inhibitory factor in neuroblastoma. Eur J Biochem 2002;269:3732-41.
36. Ferrara N, Winer J, Henzel WJ. Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: identification as leukemia inhibitory factor. Proc Natl Acad Sci USA 1992;89:698-702.
37. Gendron RL, Adams LC, Paradis H. Tubedown-1, a novel acetyltransferase associated with blood vessel development. Dev Dyn 2000;218:300-15.
38. Smith SK. Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 1998;4:509-19.
39. Rogers PA, Gargett CE. Endometrial angiogenesis. Angiogenesis 1998;2:287-94.

9

GENERAL DISCUSSION

1. Results of the studies

In Chapter 2 we demonstrated that women with a low birth weight had a higher risk of myocardial infarction than women with a normal birth weight. In Chapter 3 we found that singleton IVF pregnancies had significantly worse perinatal outcomes than spontaneously conceived pregnancies in subfertile women.

In Chapters 4 - 8 the isolation of human endometrial microvascular endothelial cells (hEMVEC) is described together with their high angiogenic capacity, which was likely due to their high response to VEGF-A and their high expression of u-PA. Besides the u-PA/plasmin system, MMPs, in particular MT3-MMP, contributed to hEMVEC tube formation. Furthermore, we demonstrated that human endometrial stromal cells (hESC) expressed ERα, ERβ and PR and responded to the ovarian steroids by an increase in VEGF-A expression. In contrast, hEMVEC expressed only ERβ and showed at best a marginal angiogenic response to E_2. HEMVEC cultured in close contact with hESC survived better, probably due to paracrine VEGF production by hESC.

The data presented in Chapter 8 demonstrate that conditioned media of human embryos contained VEGF-A and stimulated *in vitro* endometrial angiogenesis, an effect which was counteracted by sVEGFR-1. Among the factors expressed by human embryonic tissue, VEGF-A was obviously the most potent in stimulating hEMVEC proliferation and tube formation.

With these results we elucidate more of the processes involved in angiogenesis, which was our main goal.

2. From clinical questions to implantation and angiogenesis

Our clinical studies confirm the "Barker hypothesis" that low birth weight is a serious risk factor for cardio-vascular disease in later life and, furthermore, indicate that controlled ovarian hyperstimulation (COHS) in assisted procreation is related with low birth weight. In order to prevent fetal growth retardation, which might be induced by assisted procreation, we must understand more about the mechanisms involved.

For a healthy fetal development, an optimal environment needs to be created early in life, during the process of implantation. When the implantation site is located in the uterus, the preparations for an optimal environment already start before fertilization, during the proliferative and secretory phase of the menstrual cycle. Optimalization of the implantation site is subsequently established by the embryo itself, which interacts with its implantation site. Also in an unprepared extra-uterine milieu the

embryo is able to accommodate its implantation site as proven by advanced ectopic pregnancies.

Angiogenesis is considered to be one of the most critical adaptive changes during implantation and placentation. It is essential to establish the vascular structures involved in maternal-fetal exchange. Adaptation of the maternal vasculature to the rising needs of the embryo and fetus occurs, in addition to angiogenesis, through vasodilation, increased permeability, and maturation[1-3]. Although direct embryonic contact with the maternal circulation is not well established until the beginning of the second trimester of pregnancy[4], an extensive vascular network needs to be established to support the endometrial cells (e.g. the epithelial cells) that supply the embryo with nutrition until then. Furthermore, the extending vascular network is necessary for the process of decidualization and placentation. Contact between the embryo and the maternal circulation that occurs too early might lead to pregnancy loss, as excessive entry of maternal blood at a very early stage inside the developing embryonic placenta results in oxidative stress and subsequent degeneration of villous tissue[59,60].

Several studies, both in rodents and in the human, elucidate the importance of a well established endometrial vasculature in implantation;

1) In rodents:
   - administration of an angiogenesis inhibitor (AGM-1470) to pregnant mice resulted in complete failure of embryonic growth due to interference with, among others, decidualization and placental development. When non-pregnant mice were treated with AGM-1470, inhibition of endometrial maturation was observed[5].
   - Sibug *et al.*[6] showed that COHS, more specifically urinary gonadotrophins, negatively affects angiogenic factors (VEGF and VEGF-R expression) in the mouse uterus during implantation. This led to a delay in embryo implantation and smaller size of the implantation site, which might be caused by the decrease in angiogenic factors as other studies have shown that the number of implantation sites was significantly reduced after VEGF antibody treatment[7,8].
2) In the human:
   - uterine perfusion appears to be involved in human endometrial receptivity as a high intra-uterine blood flow resistance is associated with unexplained recurrent miscarriages[9].
   - morphological studies[10,11] show poor placental vascular development in intra uterine growth retardation (IUGR).
   - inadequate transformation of the maternal vasculature is associated with early pregnancy loss, and a higher perinatal morbidity and mortality caused by preterm delivery, pre-eclampsia, and/or intra-uterine growth restriction[12-22].

3. Endometrial angiogenesis

Most of the findings in Chapter 4 - 8 are the result of *in vitro* experiments. They provide us with more knowledge about the regulation, production and physiological responses of the endometrial vasculature. But can we transpose these results to the *in vivo* situation?

3.1 Endometrial endothelial cells

Endothelial cells are heterogeneous and differ in structure, function, antigen composition, metabolic properties and response to growth factors. In this way, the cells can adapt to different (micro-) environmental needs. The endometrial vasculature enhances during preimplantation (proliferative and secretory phase), decidualization, implantation, and placentation[23,24]. For this purpose, the endometrial endothelial cells must be able to react adequately to angiogenic factors in order to prepare the endometrium for implantation and placentation. Endometrial endothelial cells in culture obviously demonstrated rapid responses, particularly when compared with endothelial cells originating from other tissues.

VEGF plays an important role in a variety of angiogenic processes[25]. Hence, it was to be expected that the endometrial endothelial cells are highly sensitive to VEGF as well. What did surprise us was, beside the fact that the cells already formed tubes under control conditions, the huge enhancement of tube formation after the addition of VEGF. This could be explained by the high expression of VEGFR-2 on these cells. *In vivo*, the presence of VEGF and its receptors in the human endometrium is manifest[26-30]. The sub-epithelial-capillary plexus in the endometrium, with which the embryo comes into first contact during implantation, consists of endothelial cells that are not associated with pericytes or a vascular smooth muscle cells. It was shown that such endothelial cells are more susceptible to variations in local levels of VEGF and undergo apoptosis (programmed cell death) upon withdrawal of VEGF more readily than endothelial cells that were stabilized by contact with pericytes or smooth muscle cells[31].

An explanation for the rapid ingrowth in the 3-D fibrin matrix is the relative high expression of u-PA by hEMVEC. A relatively high u-PA expression was found *in vivo* as well.

HEMVEC displayed their enhanced angiogenic capacity *in vitro* in 3-D fibrin and collagen matrices. The endometrial ECM consists of several proteins, among which collagens, and its composition and fibrin contents vary during the menstrual cycle. The *in vitro* angiogenesis model that we used in our experiments has its limitations, as it only

consists of fibrin (which is actually a model for wound healing), collagen or a combination of both. It would be desirable to mimic the cyclic changes of the matrix as occur in vivo, but this will be extremely difficult. Nevertheless, we believe that our *in vitro* model reflects the *in vivo* situation adequately.

3.2 The role of MMP's in endometrial angiogenesis

To be able to respond quickly to an angiogenic stimulant, the endometrial endothelial cells need adequate proteolytic enzymes for pericellular proteolysis, as this is essential in endothelial cell migration, invasion and tube formation. The endometrial endothelial cells make use of both the u-PA/plasmin system and the MMP's for this purpose. The expression of MMPs by human endometrial endothelial cells *in vitro* was in accordance with the observations found *in vivo*[32-36]. Apparently, the endothelial cells maintain this ability when brought into culture and therefore our results may apply to the *in vivo* situation.

The experiments with TIMP-1 and 3, which are also expressed by the endothelial cells *in vivo*[32,36,37], proved that human endometrial endothelial cells express a quite unique pattern of (MT-)MMP's compared with endothelial cell originating from other tissues[38-41]. Our results suggest an important role for MT3-MMP in endometrial endothelial cell tube formation which may apply for the *in vivo* situation as well, as an increase in MT3-MMP is found in endometrium in the proliferative phase of the menstrual cycle, during which angiogenesis definitely takes place[34].

In combination with the high expression of u-PA, these results might further explain the specific angiogenic behavior of the endometrial endothelial cells compared to other endothelial cells. It is very likely that the unique environmental cyclic changes in the endometrium (the ECM) challenge the cells to adjust and express specific proteolytic enzymes necessary for angiogenesis, tissue desquamation and repair.

3.3 Regulation by the ovarian steroids

Development of the endometrium to a receptive state is primarily dependent on the coordinated effects of the ovarian steroids. Therefore it is to be expected that angiogenesis, essential in this process, should also be controlled by an overall regulation of these steroids.

Experiments with steroids require adequate precautions, as steroids, due to their structure, pass the cell membrane very easily and have a high turnover rate. We tried to

approach the *in vivo* situation as much as possible by creating a steroid free environment (charcoal-treated serum) and adding the steroids in the experiments daily to maintain a steady steroid concentration available for the cells.

In our *in vitro* and *in vivo* experiments we found that the endometrial cells do not loose their steroid receptors when kept in culture, except for hESC, which lost ERα at higher passages. From our results, an indirect regulation of endometrial angiogenesis by the ovarian steroids appears most likely. Krikun *et al*.[42] found no effects of E_2 or progesterone on endometrial endothelial expression of angiogenic factors, which supports the idea of an indirect action of the steroids on endometrial angiogenesis.

The endometrial stromal cells, which are not defined in most studies, but which we have identified by immunocytological staining as fibroblasts, appear to play an important role as intermediate cells between the ovarian steroids and the endothelial cells. Other studies support this role for stromal cells. Matsui *et al*.[23] demonstrated that VEGF production by endometrial stromal cells increases in association with decidualization of the cells, a process first induced by steroids. Nayak *et al*.[43], studying the Rhesus Macaque, found that the midproliferative peak in stromal VEGF expression, which did not occur in the absence of estradiol, coincided with the peak in endothelial cell proliferation and that VEGF expression in the stroma, not in the epithelium, was significantly correlated with vascular proliferation. They also doubted whether epithelium derived VEGF plays a role in endometrial angiogenesis. In the endometrial stroma, other cell types are present in addition to the stromal cells we have characterized. These include granulocytes, neutrophils and natural killer cells. These leucocytes may also act as intermediate cells between the ovarian steroids and the endothelial cells, as these cells produce VEGF, come in contact with the endothelial cells, and are attracted to the endometrium via chemokines. The expression of these chemokines is induced by ovarian steroids[28,44-46].

Nayak *et al.*[43] suggested that progesterone, besides stimulating VEGF expression, plays a role in vascular remodeling during implantation and early pregnancy. In our *in vitro* experiments we found no indication for this phenomenon, as we did not see morphological differences in tube formation after stimulation with progesterone compared with estradiol or control conditions

3.4 Interaction between embryo and endometrium; the role of VEGF

Adequate interaction and synchronization between the developing embryo and the endometrium is essential for successful implantation and placentation. In animal studies it was shown that the endometrial vasculature undergoes expansion during preimplantation stages and, even more prominently, after implantation[24,47]. This suggests that the

embryo itself is responsible for further enhancement of angiogenesis at its implantation site. Already during the pre-implantation phase, before there is any physical contact between the embryo and endometrium, pregnancy-related endometrial vascular changes are seen throughout the whole endometrium. When the embryo approaches the endometrium, more localized changes occur[48,49].

Inadequate vascular transformation can have embryonic and/or maternal causes. Cross *et al*.[50] observed an inadequate vascular transformation in pregnancies in which the trophoblast failed to invade. Impaired early stage vascular remodeling in case of an ectopic pregnancy suggests that there could be primary maternal causes[51].

In agreement with our findings, several studies indicate that the embryo prepares its implantation site by stimulating local angiogenesis via the production of VEGF-A. Das *et al.*[47] showed in their rabbit studies a pronounced in situ hybridization signal of VEGF transcripts present in the trophoblast that attached and invaded the endometrium. Furthermore, they detected high levels of VEGF receptor-2 (VEGFR-2) mRNAs on blood vessels during implantation. More indications that VEGF plays a crucial role during this phase are given by the study of Vuorela *et al*.[52], who examined cases of recurrent miscarriage and found a diminished expression of VEGF in trophoblastic tissue and a weaker expression of its receptors in maternal decidual endothelium. Furthermore, Krussel *et al.*[53] showed that the VEGF gene is one of the earliest genes activated during human preimplantation embryo development.

Soluble VEGFR-1 (flt-1), important in modulating the actions of VEGF in angiogenesis, is secreted by the placenta and expected to function as a VEGF antagonist. He *et al*.[54] demonstrated in mice that an increase in the ratio of VEGF to sVEGFR-1 results in an increase in the number of resorption sites. High concentrations of VEGF can harm to the process of angiogenesis during implantation. This emphasizes that a balance in angiogenesis promoters and inhibitors is crucial in angiogenesis.

Taken together, it is very likely that VEGF plays a key role in the interaction between the embryo and the endometrium at the time of implantation. The way it is expressed strongly suggests that it is involved in angiogenesis on both maternal and fetal sides of the placenta[12]. And as the embryo appears to be able to stimulate local angiogenesis in the endometrium, it might very well stimulate angiogenesis in ectopic sites, thereby preparing an alternative implantation site.

4. Future research and clinical implications

Knowing all this, one questions how we can relate and intervene our results to the physiological and moreover, the pathological *in vivo* situation.

To widen our view concerning the physiological situation, one could further optimize the *in vitro* model and study the factors involved in extended detail. To this end, in future research one might use a three dimensional angiogenesis model consisting of a mixture of endometrial ECM components, and further elucidate the role of MT(3)-MMP and the ERβ-mediated signaling pathway in the process of endometrial angiogenesis. Furthermore, one might seek for other angiogenic factors which might be up regulated in endometrial cells by ovarian or pregnancy induced hormones.

In pathological processes in which in endometrial angiogenesis is involved, one would like to be able to therapeutically intervene, this way improving perinatal outcome by optimizing the implantation site and fetal intrauterine environment. Important additional information could be obtained from tissue samples of failed implantations (spontaneous abortions) and of abortus provocatus. In these cases, the determination of angiogenic factors in tissue samples of decidua basalis compared with decidua parietalis and secretory endometrium might enhance our understanding in the role of (disrupted) angiogenesis at this time and why it should contribute to the failure of implantation.

Moreover, studying the influence of the human blastocyst and its signaling factors on other types of endothelium and stromal cells, like those of the mesothelium could deepen our insight into ectopic implantation. More research is necessary to determine which additional early embryonic factors are able to control the process of angiogenesis. A comparison between the factors produced by embryo's which, in retrospect, did and did not implant could be of interest and might be beneficial for IVF/ICSI strategies in the future.

One way of optimizing the embryo implantation site in case of IVF/ICSI seems to be embryo transfer in a natural cycle. So far, the direct effect of COHS on endometrial angiogenesis was studied in animals but not in humans. It would be informative to test different COHS in our *in vitro* angiogenesis model and determine whether this might negatively influence the endometrial environment.

5. Conclusions

Endometrial vascular maladaptation prior and during implantation may lead to serious complications (pregnancy loss, IUGR, pre-term delivery, pre-eclampsia) which may have consequences during pregnancy, perinatally, but also later in the neonates' life. The consequences in later life often appear to be related to an endothelial dysfunction, which is in agreement with our findings. This endothelial dysfunction appears to exist already at a young age[55-57]. Endometrial vascular maladaptation has an embryonic cause, a maternal cause or a combination of both. A less well developed embryo might be impaired

in signaling the endometrial endothelial cells leading to inadequate adaptation of the vessels. On the other side, (pre-existing) maternal endothelial dysfunction may also lead to defective maternal vascular remodeling. Such endothelial dysfunction might be either autogenously (e.g. in diabetes, auto-immune diseases, pre-eclampsia?) or caused by exogenous factors (e.g. smoking, COHS?)[51,58]. We have shown that COHS adversely affects pregnancy outcome, whether COHS negatively affects the endometrial vasculature needs further study.

The experiments have shown that the isolated human endometrial endothelial cells display a high angiogenic capacity, which is further enhanced upon exposition to VEGF-A. The u-PA/plasmin system and MT3-MMP play an important in this process and the ovarian steroids overall regulate this process indirectly, via the endometrial stromal cells. During implantation, the embryo takes over as the main (local) regulator by inducing angiogenesis locally at its implantation site through the expression of VEGF. With the results described in this thesis, we provide more insight in the (patho-)physiology of endometrial angiogenesis, and in the role of the embryo in these phenomena.

References

1. Zygmunt M, Herr F, Keller-Schoenwetter S et al. Characterization of human chorionic gonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab. 2002;87:5290-5296.
2. Herr F, Liang OD, Herrero J et al. Possible angiogenic roles of insulin-like growth factor II and its receptors in uterine vascular adaptation to pregnancy. J Clin Endocrinol Metab. 2003;88:4811-4817.
3. Reynolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive system. FASEB J. 1992;6:886-892.
4. Jauniaux E, Watson AL, Hempstock J et al. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol. 2000;157:2111-2122.
5. Klauber N, Rohan RM, Flynn E, D'Amato RJ. Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. Nat Med. 1997;3:443-446.
6. Sibug RM, de Koning J, Tijssen AM et al. Urinary gonadotrophins but not recombinant gonadotrophins reduce expression of VEGF120 and its receptors flt-1 and flk-1 in the mouse uterus during the peri-implantation period. Hum Reprod. 2005;20:649-656.
7. Rabbani MM, Rogers PA. Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats. Reproduction 2001; 122:85-90.
8. Rockwell LC, Pillai S, Olson CE, Koos RD. Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. Biol Reprod. 2002;67:1804-1810.
9. Habara T, Nakatsuka M, Konishi H et al. Elevated blood flow resistance in uterine arteries of women with unexplained recurrent pregnancy loss. Hum Reprod. 2002;17:190-194.
10. Krebs C, Macara LM, Leiser R et al. Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. Am J Obstet Gynecol. 1996;175:1534-1542.
11. Macara L, Kingdom JC, Kaufmann P et al. Structural analysis of placental terminal villi from growth-restricted pregnancies with abnormal umbilical artery Doppler waveforms. Placenta. 1996;17:37-48.
12. Sharkey AM, D.S., Boocock CA, Brown KD, Smith SK. Expression of mRNA for vascular endothelial growth factor in human placenta. J Reprod Fertil. 1993;99:609-615.
13. Findlay JK. Angiogenesis in reproductive tissues. J Endocrinol. 1986;111:357-366.
14. Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br J Obstet Gynaecol. 1986;93:1049-1059.
15. Kitzmiller JL, Watt N, Driscoll SG. Decidual arteriopathy in hypertension and diabetes in pregnancy: immunofluorescent studies. Am J Obstet Gynecol. 1981;141:773-779.
16. Labarrere CA. Acute atherosis. A histopathological hallmark of immune aggression? Placenta. 1988;9:95-108.
17. Althabe O, Labarrere C, Telenta M. Maternal vascular lesions in placentae of small-for-gestational-age infants. Placenta. 1985;6:265-276.
18. Ward K, Hata A, Jeunemaitre X et al. A molecular variant of angiotensinogen associated with preeclampsia. Nat Genet. 1993;4:59-61.
19. Frusca T, Morassi L, Pecorelli S, Grigolato P, Gastaldi A. Histological features of uteroplacental vessels in normal and hypertensive patients in relation to birthweight. Br J Obstet Gynaecol. 1989;96:835-839.
20. Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD. Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol. 2003;110 Suppl:S10-S18.
21. Taylor RN, Grimwood J, Taylor RS *et al*. Longitudinal serum concentrations of placental growth factor: evidence for abnormal placental angiogenesis in pathologic pregnancies, Am J Obstet Gynecol. 2003;188:177-82.
22. Meegdes BH, Ingenhoes R, Peeters LL, Exalto N. Early pregnancy wastage: relationship between chorionic vascularization and embryonic development. Fertil Steril. 1988;49:216-220.
23. Matsui N, Kawano Y, Nakamura S, Miyakawa I. Changes in vascular endothelial growth factor production associated with decidualization by human endometrial stromal cells *in vitro*. Acta Obstet Gynecol Scand. 2004;83:138-143.
24. Hafez ES, Tsutsumi Y. Changes in endometrial vascularity during implantation and pregnancy in the rabbit. Am J Anat. 1966;118:249-282.
25. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-676.
26. Li XF, Gregory J, Ahmed A. Immunolocalisation of vascular endothelial growth factor in human endometrium. Growth Factors. 1994;11:277-282.
27. Bausero P, Cavaille F, Meduri G, Freitas S, Perrot-Applanat M. Paracrine action of vascular endothelial growth

factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis 1998;2:167-182.
28. Gargett CE, Lederman F, Heryanto B, Gambino LS, Rogers PA. Focal vascular endothelial growth factor correlates with angiogenesis in human endometrium. Role of intravascular neutrophils. Hum Reprod 2001;16:1065-1075.
29. Meduri G, Bausero P, Perrot-Applanat M. Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. Biol Reprod. 2000;62:439-447.
30. Krussel SS, Casan EM, Raga F et al. Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sflt in cycling human endometrium. Mol Hum Reprod. 1999;5:452-458.
31. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development. 1998;125:1591-1598.
32. Freitas S, Meduri G, Le Nestour E, Bausero P, Perrot-Applanat M. Expression of metalloproteinases and their inhibitors in blood vessels in human endometrium. Biol Reprod. 1999;61:1070-1082.
33. Skinner JL, Riley SC, Gebbie AE, Glasier AF, Critchley HO. Regulation of matrix metalloproteinase-9 in endometrium during the menstrual cycle and following administration of intrauterine levonorgestrel. Hum Reprod. 1999;14:793-799.
34. Goffin F, Munaut C, Frankenne F et al. Expression Pattern of Metalloproteinases and Tissue Inhibitors of Matrix-Metalloproteinases in Cycling Human Endometrium. Biol Reprod. 2003;69:976-984.
35. Zhang J, Hampton AL, Nie G, Salamonsen LA. Progesterone inhibits activation of latent matrix metalloproteinase (MMP)-2 by membrane-type 1 MMP: enzymes coordinately expressed in human endometrium. Biol Reprod 2000;62:85-94.
36. Maatta M, Soini Y, Liakka A, Autio-Harmainen H. Localization of MT1-MMP, TIMP-1, TIMP-2, and TIMP-3 messenger RNA in normal, hyperplastic, and neoplastic endometrium. Enhanced expression by endometrial adenocarcinomas is associated with low differentiation. Am J Clin Pathol 2000;114:402-411.
37. Rodgers WH, Matrisian LM, Giudice LC et al. Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest. 1994;94:946-953.
38. Galvez BG, Matias-Roman S, Albar JP, Sanchez-Madrid F, Arroyo AG. Membrane type 1-matrix metalloproteinase is activated during migration of human endothelial cells and modulates endothelial motility and matrix remodeling. J Biol Chem 2001;276:37491-37500.
39. Lafleur MA, Handsley MM, Knauper V, Murphy G, Edwards DR. Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). J Cell Sci 2002;115:3427-3438.
40. Collen A, Hanemaaijer R, Lupu F et al. Membrane-type matrix metalloproteinase-mediated angiogenesis in a fibrin-collagen matrix. Blood. 2003;101:1810-1817.
41. Hotary KB, Yana I, Sabeh F et al. Matrix metalloproteinases (MMPs. regulate fibrin-invasive activity via MT1-MMP-dependent and -independent processes. J Exp Med 2002;195:295-308.
42. Krikun G, Schatz F, Taylor R et al. Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. J Clin Endocrinol Metab. 2005;90:1812-1818.
43. Nayak NR, Brenner RM. Vascular proliferation and vascular endothelial growth factor expression in the rhesus macaque endometrium. J Clin Endocrinol Metab. 2002;87:1845-1855.
44. Taylor RN, Lebovic DI, Hornung D, Mueller MD. Endocrine and paracrine regulation of endometrial angiogenesis. Ann N Y Acad Sci 2001;943:109-121.
45. van der MA, Lukassen HG, van Lierop MJ et al. Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. Mol Hum Reprod. 2004;10:189-195.
46. Sentman CL, Meadows SK, Wira CR, Eriksson M. Recruitment of uterine NK cells: induction of CXC chemokine ligands 10 and 11 in human endometrium by estradiol and progesterone. J Immunol. 2004;173:6760-6766.
47. Das SK, Chakraborty I, Wang J, Dey SK, Hoffman LH. Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. Biol Reprod. 1997;56:1390-1399.
48. Rosario GX, Modi DN, Sachdeva G, Manjramkar DD, Puri CP. Morphological events in the primate endometrium in the presence of a preimplantation embryo, detected by the serum preimplantation factor bioassay. Hum Reprod. 2005;20:61-71.
49. Wakuda K, Takakura K, Nakanishi K et al. Embryo-dependent induction of embryo receptivity in the mouse endometrium. J Reprod Fertil. 1999;115:315-324.
50. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science. 1994;266:1508-1518.
51. Craven CM, Morgan T, Ward K. Decidual spiral artery remodelling begins before cellular interaction with cytotrophoblasts. Placenta. 1998;19:241-252.

52. Vuorela P, Carpen O, Tulppala M, Halmesmaki E. VEGF, its receptors and the tie receptors in recurrent miscarriage. Mol Hum Reprod. 2000;6:276-282.
53. Krussel JS, Bielfeld P, Polan ML, Simon C. Regulation of embryonic implantation. Eur J Obstet Gynecol Reprod Biol. 2003;110 Suppl:S2-S9.
54. He Y, Smith SK, Day KA et al. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. Mol Endocrinol. 1999;13:537-545.
55. Poston L. Intrauterine vascular development: programming effects of nutrition on vascular function in the new born and adult. Nutr Health. 2001;15:207-212.
56. Norman M, Martin H. Preterm birth attenuates association between low birth weight and endothelial dysfunction. Circulation. 2003;108:996-1001.
57. McAllister AS, Atkinson AB, Johnston GD, McCance DR. Relationship of endothelial function to birth weight in humans. Diabetes Care. 1999;22:2061-2066.
58. Kadyrov M, Kosanke G, Kingdom J, Kaufmann P. Increased fetoplacental angiogenesis during first trimester in anaemic women. Lancet. 1998;352:1747-1749.
59. Jauniaux E, Burton GJ. Pathophysiology of histological changes in early pregnancy loss. Placenta. 2005;26:114-123.
60. Jauniaux E, Johns J, Burton GJ. The role of ultrasound imaging in diagnosing and investigating early pregnancy failure. Ultrasound Obstet Gynecol. 2005;25:613-624.

10

SUMMARY & SAMENVATTING

Summary

In Chapter 1, the background is given of the studies in which it is hypothesized that negative effects prior or during implantation may lead to aberrant placenta development and/or fetal development, which may give rise to diseases later in life. Maternal angiogenesis, the formation of new blood vessels from pre-existing ones, plays a key role in the process of implantation and placentation, whereas endothelial (dys)function might represent a link to fetal programming.

The first part of this thesis consists of two epidemiological studies which refer to the abovementioned. The main part of this thesis is about the process of endometrial angiogenesis in relation to implantation en placentation.

In the development towards a successful outcome of human procreation, implantation seems to be a highly inefficient process. Assisted procreation may compensate for the inefficiency to some extent. This compensation can be reached by selection of gametes and embryos and the induction of, regrettably, multiple pregnancy. For selection of the best oocytes, controlled ovarian hyperstimulation (COHS) is required. However, it adversely affects perinatal outcome in some cases and might do this by exerting a negative influence on the implantation process. The Barker hypothesis proposes that several diseases later in life are related to low birth weight, which is the result of intra-uterine undernourishment. The underlying biological mechanisms behind the association of low birth weight and adult disease have not been explained yet: endothelial dysfunction might play a role in this association.

An important function of the endothelium is angiogenesis. The different phases of angiogenesis are described in Chapter 1. The angiogenic capacity of endothelial cells depends largely on the ability to degrade and remodel its extracellular matrix. Two known main proteolytic enzymes systems are involved in this process: the urokinase-type plasminogen activator (u-PA)/plasmin cascade and the matrix-metalloproteinase (MMP) system. Vascular endothelial growth factor-A (VEGF-A), which was detected in endometrium in *in vivo* studies, is the growth factor that is generally assumed to play a stimulating role in angiogenesis. The ovarian steroids have the overall orchestration of endometrial angiogenesis. However, whether they exert their influence directly on the endothelium is unclear.

Most studies in which the Barker hypothesis is challenged, are cohort studies carried out in different countries. In Chapter 2, we performed a case-control study amongst Dutch women, in whom an association between low birth weight and myocardial infarction later in life was investigated.

A nationwide population-based case-control study was used. The study included

152 patients with a first myocardial infarction before the age of 50 years and 568 control women who had not had a myocardial infarction. Patients and their controls were matched for age, calendar year of the index event, and area of residence.

The results showed that birth weight in the patient group was significantly lower than in control women (3214 g vs. 3370 g, mean difference 156.3 g (95%CI -9.5 to -303.1 g). The odds ratio (OR) for myocardial infarction in patients with a birth weight lower than 3000 gram (20th percentile in controls) as compared to higher than 3000 g, was 1.7 (95%CI 1.1-2.7). The OR for myocardial infarction in patients with a low birth weight (<2000 g) as compared to a birth weight $\geq$ 2000 g, was 2.4 (95%CI 1.0-5.8). Both these outcomes did not change after adjustment for putative confounders (age, education level, body mass index, waist-hip ratio, hypertension, diabetes, hypercholesterolemia, smoking, and family history of cardiovascular disease).

The conclusion of this study was that low birth weight is associated with an increased risk of myocardial infarction before the age of 50 years in Dutch women. As such, these results support the Barker hypothesis.

In Chapter 3, our primary objective was to investigate whether subfertility explains poor perinatal outcome after assisted conception. A secondary objective was to test the hypothesis that ovarian hyperstimulation, rather than the IVF procedure itself, may influence the perinatal outcome.

For this purpose, data of a Dutch population-based historical cohort of 26,428 women treated for subfertility was used. We compared perinatal outcome of singletons conceived after COHS and IVF (IVF+COHS; n=2,239) with perinatal outcome of singletons in subfertile women who conceived spontaneously (subfertile controls; n=6,343) and in women who only received COHS (COHS-only, n=84). Furthermore, we compared perinatal outcome of singletons conceived after transfer of thawed embryos with (Stim+Cryo; n=66) and without COHS (Stim–Cryo; n=73).

The OR for very low birth weight (<1500 g) was 2.8 (95% confidence interval (CI) 1.9-3.9) in the IVF+COHS group as compared to the subfertile control group. The ORs for low birth weight (<2500 g), very preterm birth (<32 weeks) and for preterm birth (<37 weeks) were 1.6 (95%CI 1.3-1.8), 2.0 (95%CI 1.4-2.9) and 1.5 (95%CI 1.3-1.8), respectively. Adjustment for confounders did not change these risk estimates. The difference in risk between the COHS-only group and the subfertile group was significant only for very low birth weight (OR 3.5; 95%CI 1.1-11.4), but the association became weaker after adjustment for maternal age and primiparity (OR 3.1 (95%CI 1.0-10.2). No significant difference in perinatal outcome was found between the group of children conceived after embryo transfer of thawed embryos either in a cycle with ovarian stimulation / ovulation induction (Stim+Cryo) or in a spontaneous cycle (Stim–Cryo).

In conclusion, the association between assisted conception and poor perinatal outcome could not be explained by subfertility in this study.

The following two chapters focus on the establishment of an optimal intra-uterine environment of which the basis is formed by a well functioning, receptive endometrium with angiogenesis as the most important factor in this process.

In Chapter 4, the isolation and (angiogenic) characterization of human endometrial microvascular endothelial cells (hEMVEC) derived from premenopausal endometrium is described.

Unlike human foreskin microvascular endothelial cells (hFMVEC), which proliferated better upon stimulation by basic fibroblast growth factor (bFGF), hEMVEC were much more sensitive to VEGF-A stimulation. This is probably due to enhanced VEGF receptor 2 expression. In addition, hEMVEC displayed an enhanced expression of u-PA as compared to hFMVEC. No differences were found in tissue-type PA, PA inhibitor-1, and u-PA receptor expression. The high *in vitro* expression of u-PA by hEMVEC was also found in vivo, in tissue sections.

HEMVEC formed capillary-like structures when cultured in 20% human serum on top of three-dimensional (3D) fibrin matrices. VEGF-A and bFGF increased this tube formation. This was in contrast to hFMVEC, which formed tubes only after simultaneous stimulation by a growth factor and tumor necrosis factor-α. The high basal level of u-PA contributed to and may explain the high angiogenic properties of hEMVEC (*in vitro*).

In Chapter 5, we further investigated the proteolytic properties of hEMVEC as this might explain their typical angiogenic behavior. We studied the expression of proteases by hEMVEC and their involvement in the formation of capillary tubes, and compared these requirements with those of hFMVEC.

Inhibition of urokinase and MMPs both reduced tube formation in a fibrin or fibrin/collagen matrix. Cultured hEMVEC expressed various MMP mRNAs and proteins; in particular, MMP-1, MMP-2, MT1-, MT3- and MT4-MMPs. MT3- and MT4-MMP mRNA levels were significantly higher in hEMVEC than in hFMVEC. Other MT-MMP mRNAs and MMP-9 were hardly detectable. Immunohistochemical analysis confirmed the presence of MT3-MMP in endothelial cells of endometrial tissue. Overexpression of TIMP-1 or TIMP-3 by adenoviral transduction of hEMVEC reduced tube formation to the same extent, whereas only TIMP-3 was able to inhibit tube formation by hFMVEC. Tube formation by hEMVEC was partly inhibited by the presence of anti-MT-3-MMP antibodies.

Thus, in contrast to tube formation by hFMVEC, which largely depends on MT1-MMP, capillary-like tube formation by hEMVEC is, at least partly, regulated by MT3-MMP.
Sex-steroid hormones affect angiogenesis in the endometrium during the menstrual

cycle. Little information has been available regarding the exact mechanisms by which these steroids exert their function on the process of angiogenesis.

In Chapter 6, a survey is given on factors important for endometrial angiogenesis that have been found (or suggested) to respond to ovarian steroids. These factors include VEGF, fibroblast growth factors (FGFs), tumor necrosis factor α (TNF-α), erythropoietin (Epo) and trombospondin-1 (TSP-1). In addition, the influence of steroids on the expression of matrix-degrading proteases, in particular the u-PA/plasmin system and MMPs, are reviewed.

Since it was not clear whether the ovarian steroids regulate endometrial angiogenesis directly or indirectly via adjacent cells, the influence of 17β-estradiol (E_2) and progesterone on hEMVEC and human endometrial stromal cells (hESC) was examined.

The results, as described in Chapter 7, show that hEMVEC express estrogen receptor (ER)β, whereas hESC express ERα, ERβ (detected at its C-terminal part) and progesterone receptor (PR). This was revealed by immunohistochemical staining of steroid receptors and RT-PCR. Functional ER and PR activities were confirmed by the abilities of E_2 and progesterone (10^{-11}-10^{-8}M) to reduce IL-6 production. The addition of E_2 and progesterone had no significant effect on basal and VEGFA-enhanced proliferation of hEMVEC. The formation of capillary tubular structures was evaluated with hEMVEC grown on a 3D-fibrin matrix. Basal tube formation was enhanced 3.8-fold by VEGF-A. E_2 and progesterone had no significant effect, neither under the basal conditions nor in the presence of VEGF-A. However, both progesterone and E_2 stimulated VEGF-A production by hESC 9- to 29-fold. Furthermore, co-culture of hESC with hEMVEC improved the maintenance of hEMVEC.

In conclusion, ovarian steroids did not have a major effect on proliferation and tube formation by hEMVEC, although they express ERβ. However, E_2 and progesterone stimulate VEGF-A production by hESC markedly. It is proposed that ovarian steroids have an indirect effect on regulation of endometrial angiogenesis.

In Chapter 8, we studied the direct influence of the human embryo on *in vitro* endometrial angiogenesis. Conditioned media (CM) of human embryos were used to stimulate hEMVEC grown in an *in vitro* angiogenesis model.

It was shown that CM of human embryos, containing significant amounts of VEGF-A as determined by enzyme-linked immunosorbent assay (ELISA), caused an increase in hEMVEC tube formation. This effect was prevented by soluble VEGFR-1, which quenches VEGF-A activity. Recombinant EGF, alone and leukemia inhibitory factor in combination with VEGF-A, stimulated hEMVEC tube formation. None of the other tested recombinant mediators, which have been described to be produced by the early embryo/trophoblast

(interleukin (IL) 10, transforming growth factor (TGF) β, placental growth factor (PLGF), hCG, colony-stimulating factor 1, interferon-γ, insulin-like growth factor I and II, IL-6, platelet-derived growth factor, and TGFα), had an effect on tube formation by hEMVEC.

For the first time, it was shown that the human embryo is able to stimulate *in vitro* endometrial angiogenesis at the time of implantation. This process was shown to be mediated by VEGF-A.

In Chapter 9, the results of the studies are summarized and discussed in a broader perspective. In short, vascular maladaptation prior and during implantation may lead to serious complications during pregnancy, perinatally, but also later in life (Barker hypothesis). The consequences later in life often appear to be related to endothelial dysfunction, which is in agreement with our results. Furthermore, we have shown that COHS adversely affects pregnancy outcome. Whether COHS negatively affects the endometrial vasculature needs further study.

The experiments have shown that the isolated human endometrial endothelial cells display a high angiogenic capacity, which is further enhanced by exposition to VEGF-A. The u-PA/plasmin system and MT3-MMP play an important role in this process and the ovarian steroids overall regulate this process indirectly via the endometrial stromal cells. During implantation, the embryo takes over as the main (local) regulator by inducing angiogenesis at its implantation site through the expression of VEGF. With the results described in this thesis, we provide more insight in the (patho)physiology of endometrial angiogenesis and in the role of the embryo in this process.

Samenvatting

Tijdens de innesteling (implantatie) vindt er communicatie over en weer plaatst tussen het embryo en de implantatieplaats (endometrium; baarmoederslijmvlies). Als deze communicatie, welke plaats vindt door middel van biochemische signalen, niet goed verloopt, kan dit leiden tot een verstoorde implantatie en moederkoekvorming (placentatie). Dit kan een miskraam tot gevolg hebben, maar ook een verstoorde foetale ontwikkeling, leidend tot intra-uteriene groeivertraging.

Barker stelt in zijn hypothese 'fetal origins of adult disease' dat een laag geboortegewicht, veroorzaakt door intra-uteriene ondervoeding, gerelateerd is aan het meer voorkomen van bepaalde (voornamelijk hart -en vaat-) ziekten op latere leeftijd.

Een goed verlopend implantatieproces is dus van groot belang. Angiogenese, de vorming van nieuwe bloedvaatjes uit reeds bestaande bloedvaatjes, aan de maternale zijde van de placenta, speelt een essentiële rol tijdens de implantatie en placentatie. Endotheelcellen bekleden de binnenzijde van bloedvaten. Een verstoorde endotheelfunctie, met dientengevolge een verstoorde angiogenese, zou mogelijk een rol kunnen spelen bij "fetal programming" (Barker hypothese).

In dit proefschrift worden in het eerste deel twee epidemiologische studies beschreven die betrekking hebben op de Barker hypothese. Het andere deel van het proefschrift bestrijkt het onderzoek naar het proces van angiogenese.

In Hoofdstuk 1 wordt de achtergrond gegeven van de Barker hypothese en van het proces van implantatie en angiogenese. In de voortplanting van de mens blijkt het proces van implantatie zeer inefficiënt te zijn. Geassisteerde conceptie kan dit enigszins beïnvloeden door selectie van gameten en embryo's, maar mede ook, door iatrogene meerlingzwangerschappen. Gecontroleerde ovariële hyperstimulatie (COHS) is nodig voor selectie van de beste oöcyten. Daarnaast kan COHS, mogelijk via zijn invloed op de implantatie, de perinatale uitkomst negatief beïnvloeden. Er zijn nog geen duidelijke biologische mechanismen bekend die de associatie tussen implantatie, geboortegewicht en ziekten op latere leeftijd kunnen verklaren. Een verstoorde endotheelfunctie kan hierbij een rol spelen.

Een belangrijke functie van endotheelcellen is angiogenese. De verschillende fases van angiogenese worden beschreven in Hoofdstuk 1. De angiogene capaciteit van de endotheelcellen hangt grotendeels samen met hun vermogen tot het degraderen en herschikken van hun extracellulaire matrix. Twee belangrijke proteolytische systemen spelen hierbij een belangrijke rol: het urokinase-type plasminogeen activator (u-PA)/plasmine systeem en het matrix-metalloproteinase (MMP) systeem. Een groeifactor die in *in vivo* studies is aangetoond in het endometrium, is "vascular endothelial growth factor-A"

(VEGF-A). Van VEGF-A is bekend dat deze een stimulerende rol in de angiogenese heeft. De ovariële steroïden besturen het proces van angiogenese in het endometrium, maar of zij een directe invloed uitoefenen op het endotheel is onbekend.

De meeste studies waarin de Barker hypothese is getest, betreffen cohort studies die zijn uitgevoerd in verschillende landen. In Hoofdstuk 2 beschrijven wij een case-control studie met vrouwen, waarin de associatie tussen een laag geboortegewicht en een myocardinfarct op latere leeftijd wordt beschreven.

De case-control studie is gedaan in een Nederlandse populatie. Honderdtweeënvijftig patiënten met een eerste myocardinfarct voor hun 50^{e} levensjaar en 568 controle vrouwen die nog nooit een myocardinfarct hadden gehad, werden bestudeerd. Patiënten en hun controles werden gematched voor leeftijd, index datum en postcode.

De resultaten laten zien dat het geboortegewicht van de vrouwen in de patiëntengroep significant lager was dan in de controle groep (3214 g vs. 3370 g, gemiddeld verschil 156,3 g (95% betrouwbaarheidsinterval (BI) -9,5 tot -303,1 g). De odds ratio (OR) voor een myocardinfarct in de geboortegewicht groep $<$ 3000 g (20e percentiel in de controle groep), vergeleken met de geboortegewicht groep $\geq$ 3000 g, was 1,7 (95%BI 1,1-2,7). De OR voor het krijgen van een myocardinfarct voor vrouwen met een geboortegewicht $<$ 2000 g ten opzichte van een geboortegewicht $\geq$ 2000 g, was 2,4 (95%BI 1,0-5,8). Deze resultaten veranderden niet na correctie voor eventueel verstorende factoren (leeftijd, opleiding, "body mass index", middel-heup ratio, hypertensie, diabetes, hypercholesterolemie, roken en een positieve familie anamnese voor cardiovasculaire aandoeningen).

De conclusie van de studie was dat een laag geboortegewicht geassocieerd is met een toegenomen risico op een myocardinfarct voor het 50e levensjaar in Nederlandse vrouwen. Deze bevindingen zijn in overeenstemming met de hypothese van Barker.

Bij geassisteerde conceptie, waarbij men gebruik maakt van COHS eventueel in combinatie met IVF of ICSI, is gebleken dat de perinatale uitkomsten slechter zijn. Dit uit zich in lagere geboortegewichten en/of vroeggeboorten (partus prematurus). In Hoofdstuk 3 hebben we ten eerste onderzocht of subfertiliteit, gedefinieerd als het uitblijven van een zwangerschap na één jaar regelmatig onbeschermde coïtus, de slechtere perinatale uitkomst kan verklaren na geassisteerde conceptie. Ten tweede hebben we gekeken of ovariële hyperstimulatie, en niet zozeer de IVF procedure zelf, de perinatale uitkomst beïnvloedt.

Hiervoor hebben we data gebruikt uit een Nederlandse retrospectieve cohort studie bestaande uit 26.428 vrouwen die onder controle waren in verband met subfertiliteit. We hebben de perinatale uitkomsten (geboortegewicht en partus prematurus) van eenlingen die geboren waren na het gebruik van COHS en IVF ("IVF+COHS"; n=2.239) vergeleken

met perinatale uitkomsten in een groep van subfertiele vrouwen die uiteindelijk spontaan zwanger waren geworden (“subfertile controls”; n=6.343) en een groep vrouwen die zwanger waren geworden na het gebruik van alleen COHS (“COHS-only”, n=84). Verder hebben we de perinatale uitkomsten bestudeerd tussen eenlingzwangerschappen, die ontstaan zijn na het terugplaatsen van een ontdooid embryo in een met COHS gestimuleerde (“Stim+Cryo”; n=66), en ongestimuleerde cyclus (“Stim–Cryo”; n=73).

De OR van zeer laag geboortegewicht (< 1500 g) was 2,8 (95%BI 1,9-3,9) ten opzichte van de “IVF+COHS” groep, vergeleken met de “subfertile control” groep. De OR’s voor laag geboortegewicht (<2500 g), zeer preterme geboorte (<32 weken) en preterme geboorte (<37 weken) waren achtereenvolgens 1,6 (95%BI 1,3-1,8), 2,0 (95%BI 1,4-2,9) en 1,5 (95%BI 1,3-1,8). Correctie voor mogelijke verstorende factoren deed deze resultaten niet veranderen. Er was een significant verschil in risico op een zeer laag geboortegewicht tussen de “COHS-only” groep en de “subfertile” groep (OR 3,5; 95%BI 1,1-11,4). Na correctie voor maternale leeftijd en primipariteit werd het risico minder (OR 3,1 (95%BI 1,0-10,2). In de groepen met ovariële stimulatie/ovulatie inductie gestimuleerde cyclus (“Stim+Cryo”) of ongestimuleerde spontane cyclus (“Stim–Cryo”), werden geen significante verschillen gevonden in geboortegewicht en voorkomen van een partus prematurus.

Concluderend, de slechtere perinatale uitkomsten na geassisteerde conceptie kunnen niet verklaard worden door de factor subfertiliteit.

De volgende twee hoofdstukken richten zich op het creëren van een optimale intrauteriene omgeving voor het embryo. Angiogenese is essentieel voor een goed functionerend en receptief endometrium.

In Hoofdstuk 4 wordt de isolatie en (angiogene) karakterisering van humane endometriale microvasculaire endotheelcellen (hEMVEC), afkomstig uit premenopauzaal endometrium, beschreven.

In tegenstelling tot humane voorhuid microvasculaire endotheelcellen (hFMVEC), die het beste prolifereren na stimulatie met “basic fibroblast growth factor” (bFGF), reageerden hEMVEC veel beter op stimulatie met VEGF-A. Dit is waarschijnlijk te verklaren door de verhoogde expressie van VEGF receptor 2 op hEMVEC. Een ander verschil met hFMVEC was dat hEMVEC het “urokinase type plasminogeen activator” (u-PA) in veel hogere mate uitscheidden. Er werden geen verschillen gevonden in expressie van “tissue-type PA”, “PA inhibitor-1” en u-PA receptor. De hoge u-PA expressie door hEMVEC *in vitro* kon ook worden aangetoond *in vivo* in endometrium coupes.

Als we hEMVEC lieten groeien op een drie-dimensionale (3D) fibrine matrix in de aanwezigheid van 20% humaan serum, vormden zij spontaan capillair-achtige structuren

(verder "vaatjes" te noemen). VEGF-A en bFGF zorgden voor verdere stimulatie van deze vaatvorming. Dit in tegenstelling tot bij hFMVEC, waarbij pas vaatjes werden gevormd nadat de cellen gestimuleerd waren met een groeifactor en "tumor necrosis factor-α". De hoge basale u-PA expressie zal zeker bijgedragen hebben aan de sterke angiogene eigenschappen van hEMVEC (*in vitro*).

In Hoofdstuk 5 zijn we verder ingegaan op de proteolytische eigenschappen van hEMVEC, aangezien dit hun typische angiogene gedrag kan verklaren. We hebben gekeken naar de expressie van verschillende proteasen door hEMVEC en hun rol in de vaatvorming. De resultaten van hEMVEC hebben we vergeleken met die van hFMVEC.

Blokkade van u-PA en/of MMP's remde de vaatvorming in een fibrine en fibrine/collageen matrix. Gekweekte hEMVEC brachten verschillende MMP mRNA's en eiwitten tot expressie. Duidelijk tot expressie kwamen: MMP-1, MMP-2, MT1-, MT3- en MT4-MMP's. De hoeveelheid MT3- en MT4-MMP mRNA was significant hoger in hEMVEC dan in hFMVEC. MT2-, MT5-, MT6-MMP en MMP-9 mRNAs, waren nauwelijks detecteerbaar. Immunohistochemische analyse bevestigde de aanwezigheid van MT3-MMP in endotheelcellen in het endometrium. Overexpressie van TIMP-1 en TIMP-3 door adenovirale transductie gaf een identieke remming van de vaatvorming door hEMVEC, terwijl alleen TIMP-3 in staat was om de vaatvorming door hFMVEC te remmen. Vaatvorming door hEMVEC kon deels worden geremd door toevoegen van anti-MT-3-MMP antilichamen.

Dus, in tegenstelling tot de vaatvorming door hFMVEC, die grotendeels afhankelijk is van MT1-MMP, wordt de vaatvorming door hEMVEC, in ieder geval gedeeltelijk, gereguleerd door MT3-MMP.

Ovariële steroïden beïnvloeden de angiogenese in het endometrium tijdens de menstruele cyclus. Er is weinig bekend over de manier waarop de steroïden hun invloed uitoefenen op het proces van angiogenese.

In Hoofdstuk 6 wordt een overzicht gegeven van de factoren die de angiogenese in het endometrium stimuleren of remmen en die onder invloed staan van steroïden. Deze factoren zijn: VEGF, fibroblast growth factors (FGFs), tumor necrosis factor α (TNF-α), erythropoietine (Epo) en trombospondine-1 (TSP-1). Daarnaast wordt de invloed van de steroïden op de expressie van matrix-afbrekende proteasen, zoals het u-PA/plasmine systeem en de MMP's, beschreven.

Het was tot voor kort nog onduidelijk of de ovariële steroïden direct danwel indirect (via andere endometriale cellen) de angiogenese in het endometrium reguleren. Om dit te onderzoeken werd in hoofdstuk 7 de invloed van 17β-oestradiol (E_2) en progesteron op hEMVEC en humane endometriale stromale cellen (hESC) onderzocht.

De resultaten laten zien dat hEMVEC oestrogeen receptor (ER) β tot expressie brengen, terwijl hESC ERα, ERβ en progesteron receptor (PR) tot expressie brengen. Dit kon worden aangetoond door middel van immunohistochemische kleuring van de steroidreceptoren en RT-PCR. Dat de ER en PR op de cellen ook functioneel actief waren, werd bevestigd doordat zowel E_2 als progesteron (10^{-11}-10^{-8}M), de interleukine-6 (IL-6) productie deden verminderen. Het toevoegen van E_2 en progesteron aan hEMVEC had geen significant effect op de basale en VEGF-A-geïnduceerde proliferatie. Tevens werd de invloed van steroïden op de vaatvorming door hEMVEC op een 3D-fibrine matrix onderzocht. De basale vaatvorming nam met een factor 3.8 toe indien VEGF-A was toegevoegd. E_2 en progesteron hadden geen significant effect; niet onder basale condities, maar ook niet in combinatie met VEGF-A. Echter, zowel progesteron als E_2 stimuleerden de VEGF-A productie (9- tot 29-voudig) door hESC. Tevens viel op dat als hEMVEC in coculture met werd gebracht met hESC, de conditie van hEMVEC werden verbeterd.

Concluderend, de ovariële steroïden hadden geen grote invloed op de proliferatie en vaatvorming door hEMVEC, ondanks dat ze ERβ tot expressie brengen. E_2 en progesteron stimuleerden de VEGF-A productie door hESC wel evident. Aldus lijkt een indirecte regulatie van de endometriale angiogenese door ovariële steroïden aannemelijk.

In Hoofdstuk 8 hebben we de directe invloed van het humane embryo op de *in vitro* endometriale angiogenese onderzocht. In een *in vitro* angiogenese model werd geconditioneerd medium (CM) van humane embryo's gebruikt om hEMVEC te stimuleren.

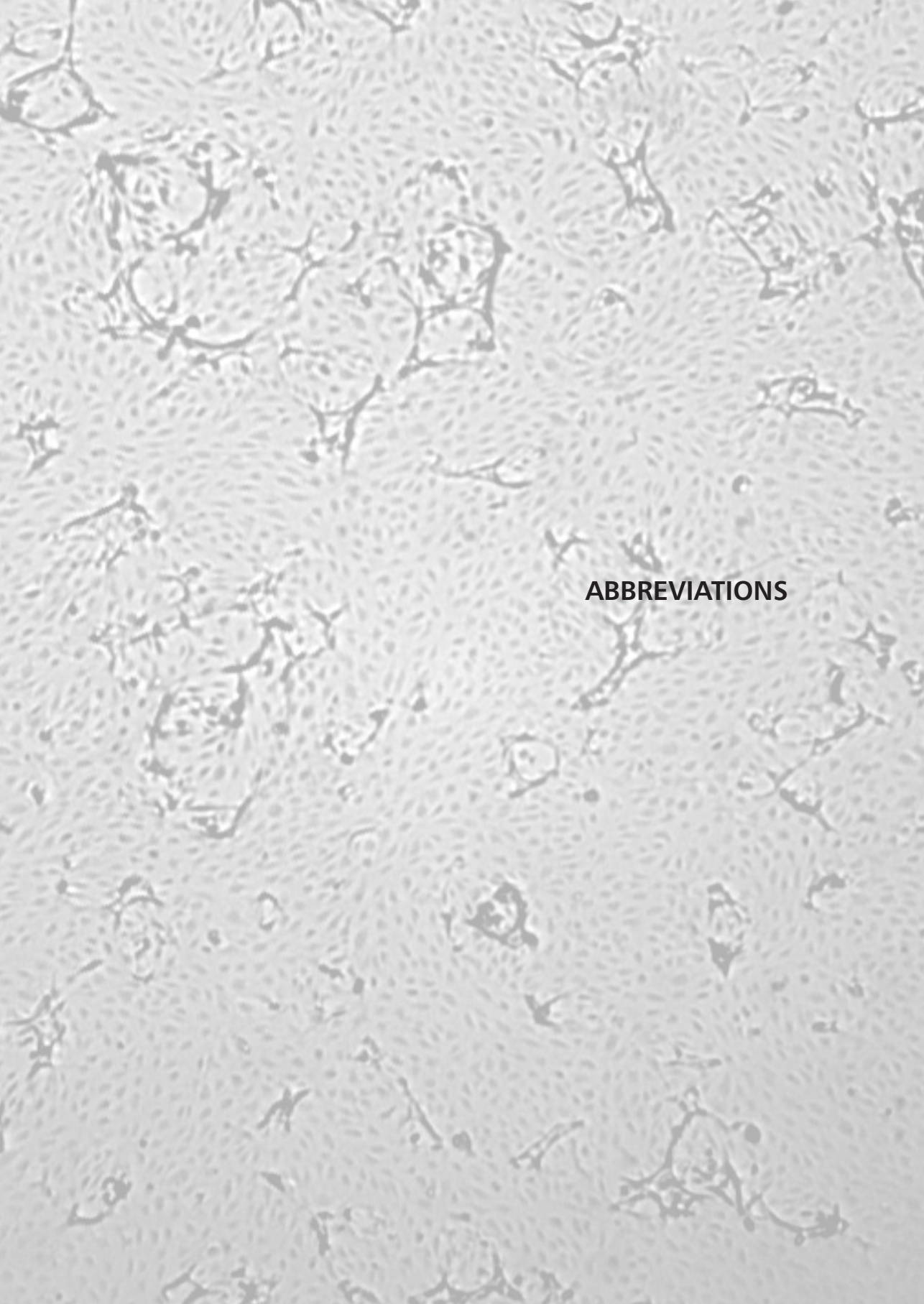
Het CM van humane embryo's bleek significante hoeveelheden VEGF-A te bevatten, hetgeen kon worden aangetoond met behulp van "enzyme-linked immunosorbent assay" (ELISA). Dit veroorzaakte een toename in vaatvorming door hEMVEC. Dit effect werd tegengegaan door "soluble VEGFR-1", dat VEGF wegvangt. Recombinant "epidermal growth factor" (EGF) alleen en "leukemia inhibitory factor" (LIF) in combinatie met VEGF-A, stimuleerden vaatvorming door hEMVEC. Andere geteste recombinant factoren waren "interleukin (IL) 10", "transforming growth factor" (TGF) β, "placental growth factor" (PLGF), hCG, "colony-stimulating factor 1","interferon-γ","insulin-like growth factor I en II", IL-6, "platelet-derived growth factor" en TGFα. Van deze factoren is beschreven dat zij geproduceerd worden door het vroege embryo en/of eerste trimester trofoblast. Echter, deze factoren hadden in onze studie geen effect op de vaatvorming door hEMVEC.

Voor het eerst werd in deze studie aangetoond dat het humane embryo, door de expressie van VEGF-A, in staat is om *in vitro* endometriale angiogenese te stimuleren.
In Hoofdstuk 9 zijn de resultaten van de verschillende studies samengevat en bediscussieerd. Een verstoorde vasculaire ontwikkeling voorafgaand aan en tijdens de implantatie, kan leiden tot ernstige complicaties met gevolgen voor de vrucht tijdens de

graviditeit, voor de neonaat perinataal, maar mogelijk ook later in het kind zijn/haar leven. De aandoeningen die later in het leven optreden blijken vaak gerelateerd te zijn aan het disfunctioneren van het endotheel. Dit komt overeen met onze bevindingen. We hebben laten zien dat COHS de perinatale uitkomst negatief beïnvloed. Of dit komt doordat COHS een negatieve invloed uitoefent op de endometriale vaten moet verder onderzocht worden.

De experimenten laten zien dat geïsoleerde humane endometriale endotheelcellen grote angiogene capaciteiten hebben, vooral wanneer ze blootgesteld worden aan VEGF-A. Het u-PA/plasmine en het MT3-MMP systeem spelen een belangrijke rol in de endometriale angiogenese waarvan de ovariële steroïden de aanvoerders zijn. E_2 en progesteron reguleren het proces indirect via de endometriale stromacellen. Tijdens de implantatie neemt het embryo de aanvoerdersrol over door de angiogenese ter plaatse van zijn implantatie te stimuleren door de expressie van VEGF.

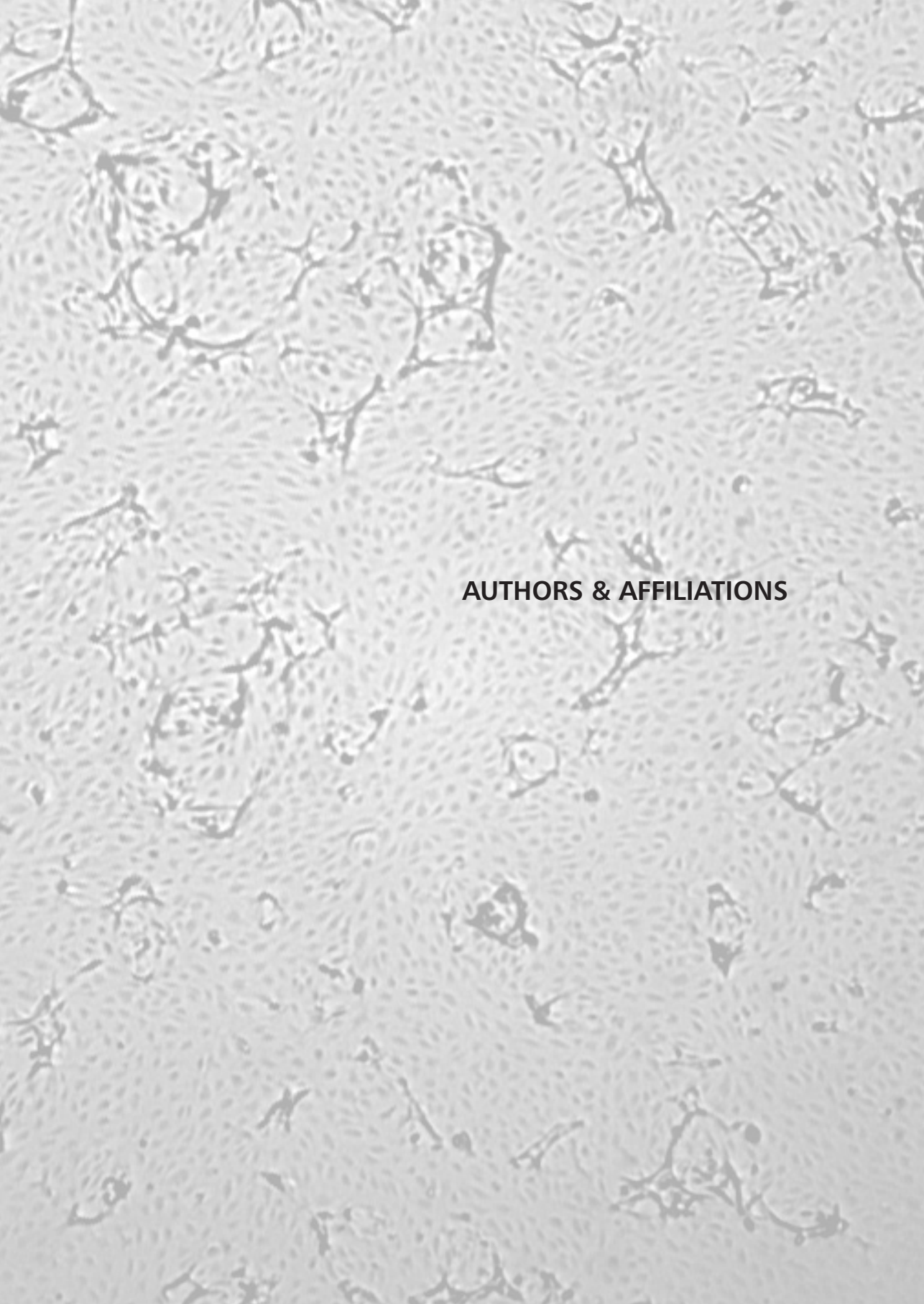
De resultaten beschreven in dit proefschrift geven ons meer inzicht in de (patho)fysiologie van de angiogenese in het endometrium en de rol van het embryo hierin.



ABBREVIATIONS

bFGF	basic fibroblast growth factor
BM	basal membrane
BMI	body mass index
CM	conditioned medium
COHS	controlled ovarian hyperstimulation
CSF-1	colony stimulating factor
DNA	deoxyribonucleic acid
E_2	17β-estradiol
ECM	extracellular matrix
ECGF	endothelial cell growth factor
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Epo	erythropoietin
ER	estrogen receptor
ERE	estrogen response elements
ET	embryo transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hCG	human chorionic gonadotropin
hEMVEC	human endometrial microvascular endothelial cells
hESC	human endometrial stromal cells
hFMVEC	human foreskin microvascular endothelial cells
HRE	hormone response elements
HS	human serum
HSA	human serum albumin
hUVEC	human umbilical cord endothelial cells
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IVF	*in vitro* fertilization
ICSI	intracytoplasmic sperm injection
IUGR	intra uterine growth retardation
LIF	leukemia inhibitory factor
MPA	methoxyprogesterone acetate
MMP	matrix metalloproteinase
MT-MMP	membrane-type MMP
NBCS	new born calf serum
OR	odds ratio
PAI	plasminogen activator inhibitor

PE	pre-eclampsia
Plg	plasminogen
Plg-R	plasminogen receptor
PLGF	placental growth factor
PDGF	platelet-derived growth factor
PR	progesterone receptor
PRE	progesterone response elements
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
sVEGFR	soluble vascular endothelial growth factor receptor
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase-type plasminogen receptor
sc-u-PA	single-chain urokinase-type plasminogen
tc-u-PA	two-chain urokinase-type plasminogen
t-PA	tissue-type plasminogen activator
TSP	thrombospondin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

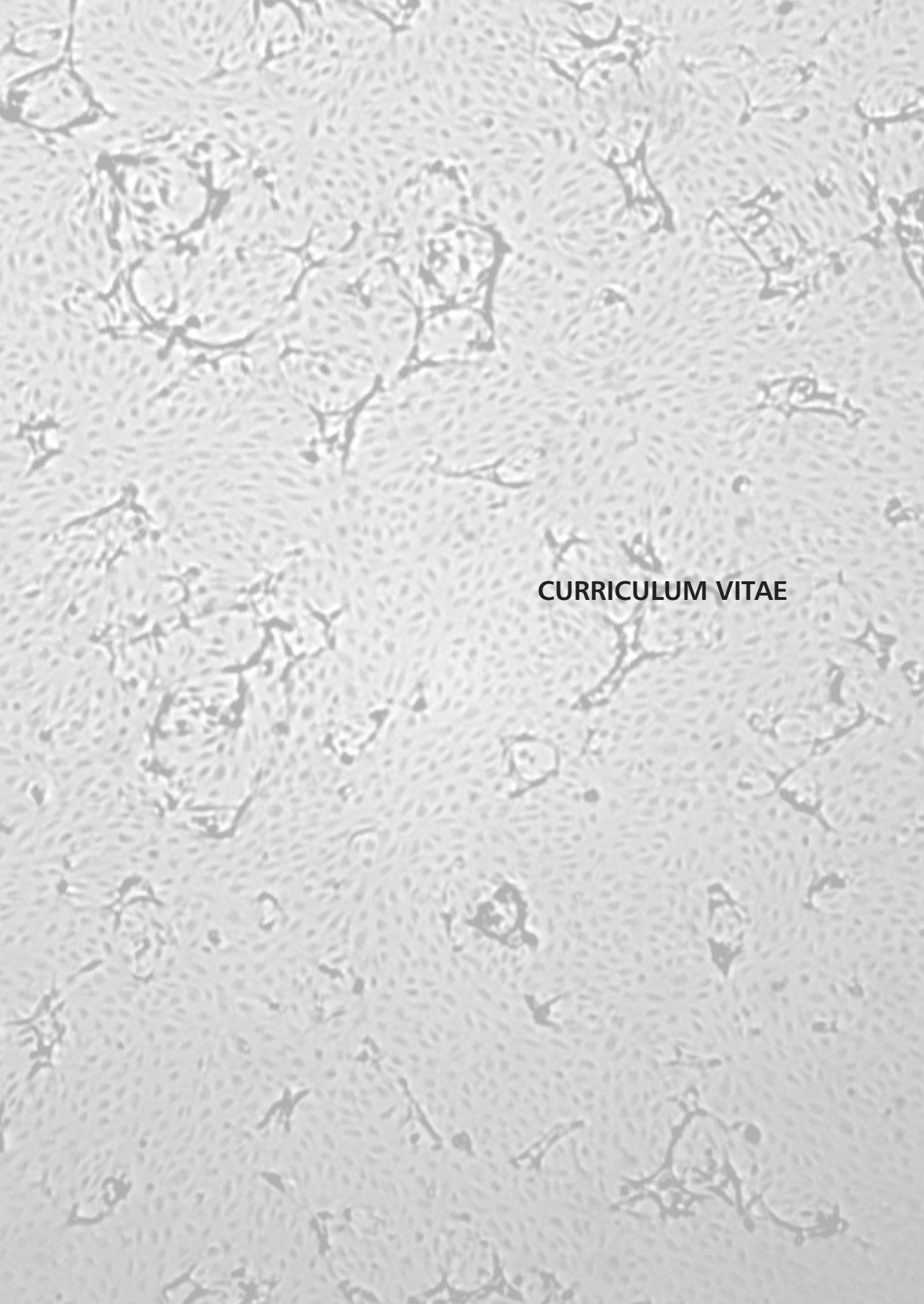


AUTHORS & AFFILIATIONS

E. de Boer	Department of Epidemiology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
C.S. de Bruijn	Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands.
C.W. Burger	Department of Obstetrics & Gynaecology, Erasmus Medical centre, Rotterdam, The Netherlands.
A.J.M. de Craen	Department of Gerontology & Geriatrics, Leiden University Medical Center, Leiden, The Netherlands.
C. Fijten	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
A.J. van Gool	Target Discovery Unit, NV Organon, Oss, The Netherlands.
J.M. Grimbergen	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
R.M. Hage	Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands.
R. Hanemaaijer	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
F.M. Helmerhorst	Department of Gynaecology, division Reproductive Medicine, Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands.
V.W.M. van Hinsbergh	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands. Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands.
E.L. Kaijzel	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
K. Kapiteijn	Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands. Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
T. Kooistra	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.

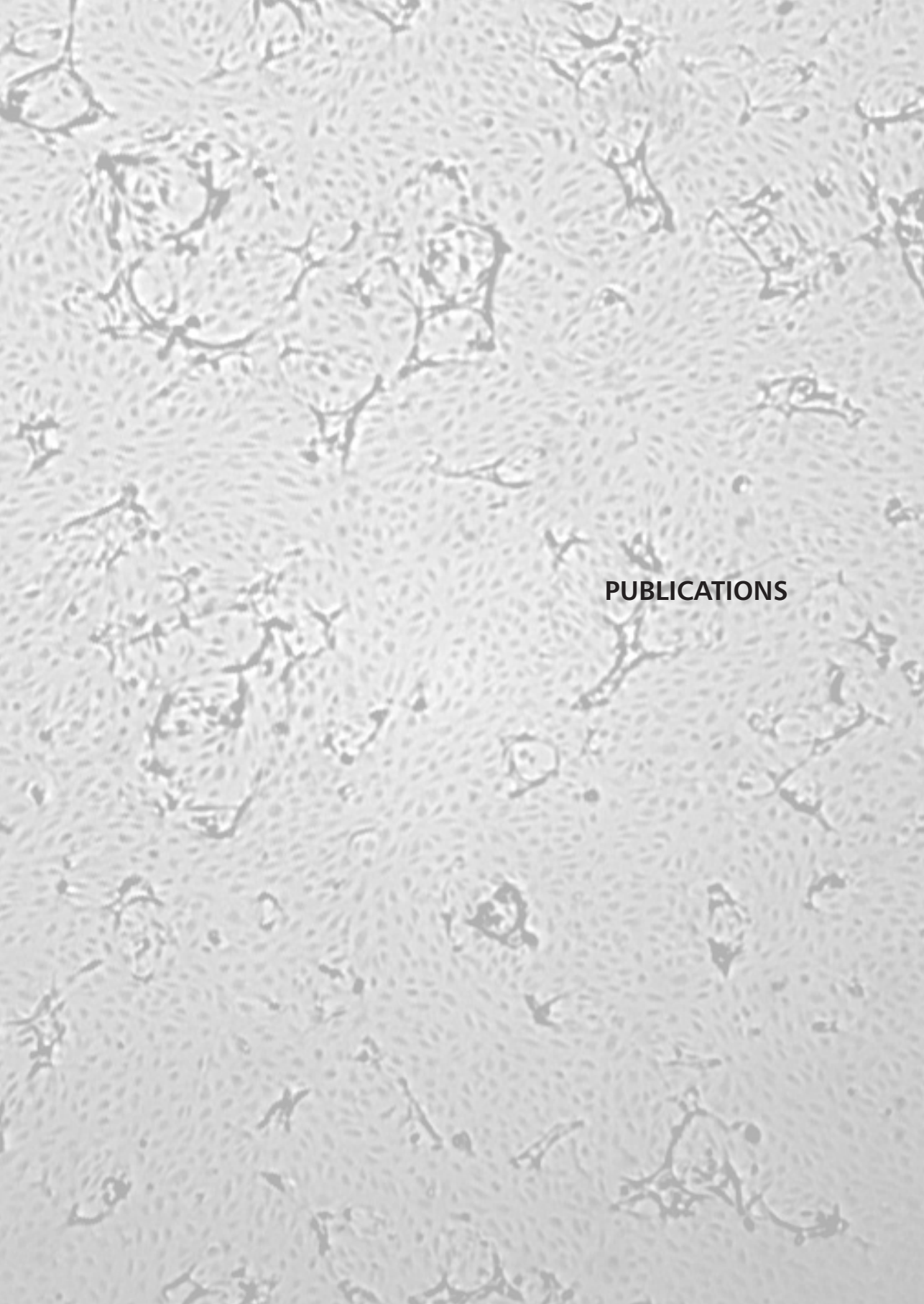
P. Koolwijk	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands. Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands.
F.E. van Leeuwen	Department of Epidemiology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
B. Molenaar	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands. Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands.
A. Mulder-Stapel	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
G.P. van Nieuw Amerongen	Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands.
M. Plaisier	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands. Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands.
P.H.A. Quax	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.
F.R. Rosendaal	Department of Hematology, Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands.
E. van Spronsen	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands. Department of Gynaecology, division Reproductive Medicine, Leiden University Medical Center, Leiden, The Netherlands.
B.C. Tanis	Department of Hematology, Leiden University Medical Center, Leiden, the department of Internal Medicine, Groene Hart Hospital Gouda, The Netherlands.
B. van der Vecht	Division of Biomedical Research, TNO Quality of Life, Leiden, The Netherlands.

R.M.F. van der Weiden — Department of Obstetrics & Gynaecology, Sint Franciscus Gasthuis, Rotterdam, The Netherlands.



CURRICULUM VITAE

Kitty Kapiteijn werd geboren op 29 november 1971 te Sassenheim. Zij behaalde het VWO diploma aan het Rijnlands Lyceum Sassenheim in 1990, waarna zij in datzelfde jaar begon aan de studie Geneeskunde aan de Rijksuniversiteit Leiden. Vanaf 1993 deed zij onderzoek naar de laparoscopische chirurgie in de gynaecologie onder begeleiding van Dr. F.W. Jansen, LUMC. Haar afstudeerproject betrof een studie naar de verschillen tussen het adeno- en plaveiselcel cervixcarcinoom en een klinische stage op de afdeling gynaecologische oncologie van het Groote Schuur Ziekenhuis te Kaapstad, Zuid-Afrika (Prof. Dr. A.A.W. Peters en Dr. R. Soeters). Na het behalen van het doctoraal examen in 1995, begon zij met haar co-schappen. Als keuze co-schap zette zij het onderzoek voort naar de laparoscopische chirurgie in de gynaecologie. In 1997 behaalde zij haar artsexamen, waarna zij werkzaam was als AGNIO op de afdeling gynaecologie en obstetrie van het Leyenburg Ziekenhuis te Den Haag. Het jaar daarop was zij werkzaam als AGNIO op de afdeling gynaecologie en obstetrie van het Bronovo Ziekenhuis te Den Haag. In 1999 begon zij als AGIKO aan het in dit proefschrift beschreven onderzoek. Dit onderzoek was een samenwerkingsverband tussen TNO Quality of Life Leiden (Prof. Dr. V.W.M. van Hinsbergh, Dr. P. Koolwijk) en de afdeling gynaecologie van het LUMC (Prof. Dr. F.M. Helmerhorst). Vanaf 2002 is zij in opleiding tot gynaecoloog in het Groene Hart Ziekenhuis te Gouda (opleider: Dr. J.C.M. van Huisseling) en in het LUMC (opleiders: Prof. Dr. H.H.H. Kanhai en Prof. Dr. G.G. Kenter) te Leiden.



PUBLICATIONS

Jansen F.W., *Kapiteijn K.*, Hermans J., Trimbos-Kemper G.C.M. A survey on (operative) laparoscopy in the Netherlands in 1992. European Journal of Obstetrics & Gynecology and Reproductive Biology 1996;64,105-109.

Jansen F.W., *Kapiteijn K.*, Trimbos-Kemper G.C.M. Laparoscopie in Nederland: Resultaten van de follow-up enquête. Nederlands Tijdschrift voor Obstetrie & Gynaecologie 1996;109,280-281.

Jansen F.W., *Kapiteijn K.*, Trimbos-Kemper G.C.M., Hermans J., Trimbos J.B. Complications of laparoscopy; a prospective multicentre observational study. British Journal of Obstetrics and Gynaecology 1997;104,595-600.

Koolwijk P., *Kapiteijn K.*, Molenaar B., van Spronsen E., van der Vecht B., Helmerhorst F.M., van Hinsbergh V.W.M. Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. Journal of Clinical Endocrinology & Metabolism 2001;86,3359-3367.

Kapiteijn K., Koolwijk P., van der Weiden R.M.F., Helmerhorst F.M., Kooistra T., van Hinsbergh V.W.M. Steroids and cytokines in endometrial angiogenesis. Anticancer Research 2001;21,4231-4242.

Kapiteijn K., Plaisier M., Koolwijk P., Fijten C., Hanemaaijer R., Grimbergen J.M., Mulder-Stapel A., Quax P.H., Helmerhorst F.M., van Hinsbergh V.W.M. Involvement of membrane-type matrix metalloproteinases (MT-MMPs) in capillary tube formation by human endometrial microvascular endothelial cells: role of MT3-MMP. Journal of Clinical Endocrinology and Metabolism 2004;89,5828-5836.

Tanis B.C., *Kapiteijn K.*, Hage R.M., Rosendaal F.R., Helmerhorst F.M. Dutch women with a low birth weight have an increased risk of myocardial infraction later in life: a case control study. Reproductive Health 2005;2,1.

Helmerhorst F.M., van Dunne F.M., *Kapiteijn K.*, Perquin D.A.M., Plaisier M., Sibug R. Implantatie: cytokinen en groeifactoren. Nederlands Tijdschrift voor Obstetrie & Gynaecologie 2005;118,230-231.

Kapiteijn K., Koolwijk P., van der Weiden R.M.F., van Nieuw Amerongen G., Plaisier M., van Hinsbergh V.W.M., Helmerhorst F.M. Human embryo-conditioned medium stimulates *in vitro* endometrial angiogenesis. Fertility and Sterility 2006;85 Suppl 1,1232-1239.

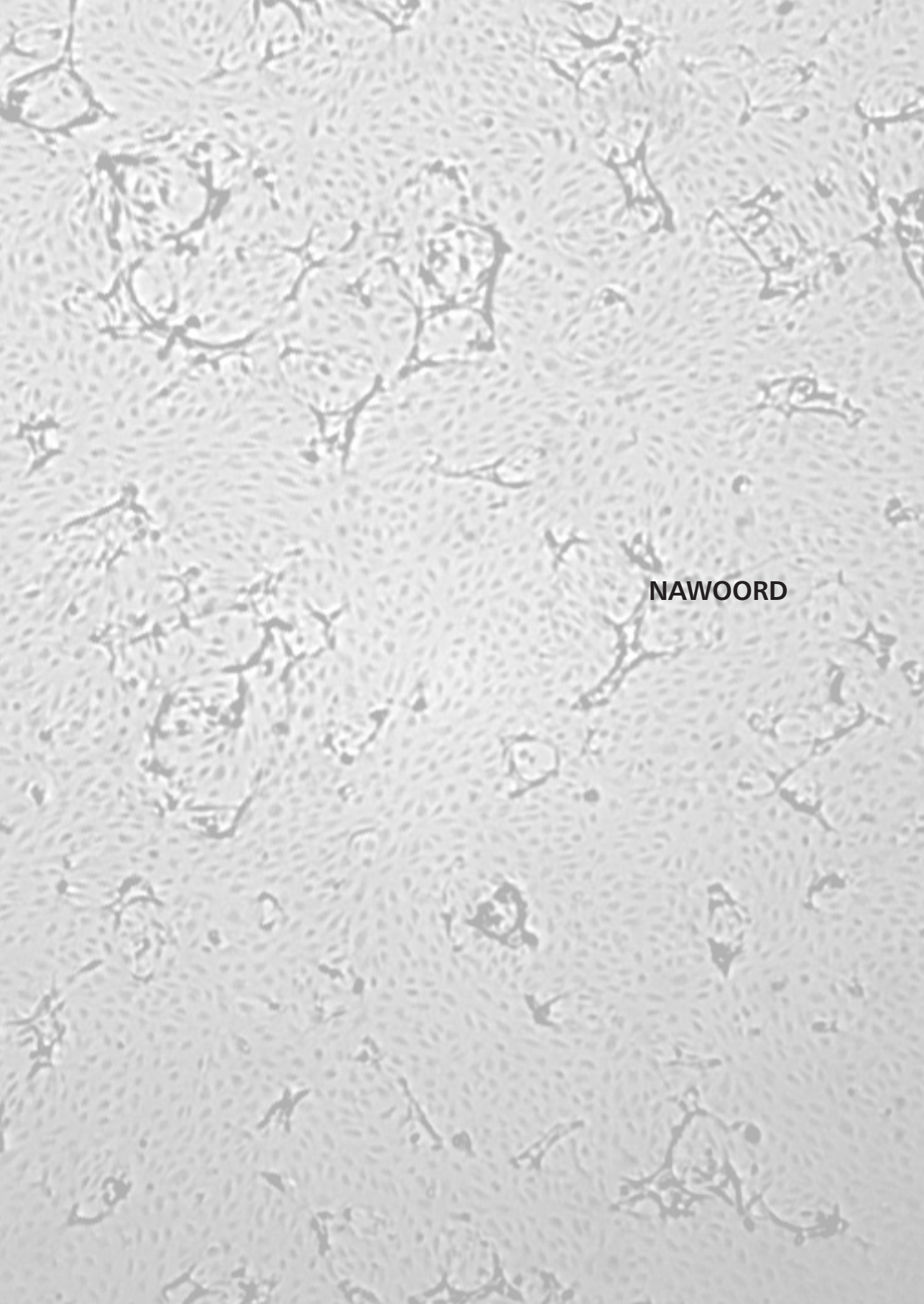
Kapiteijn K., Koolwijk P., Kaijzel R.L., van Gool A.J., Helmerhorst F.M., van Hinsbergh V.W.M. Effects of ovarian steroids on human endometrial endothelial and stromal cells. Evidence for paracrine regulation of angiogenesis. *Submitted.*

Kapiteijn K., de Bruijn C.S., de Boer E., de Craen A.J.M., Burger C.W., van Leeuwen F.E., Helmerhorst F.M. Does subfertility explain the risk of poor perinatal outcome after IVF and ovarian hyperstimulation? *Submitted.*

ABSTRACTS *(oral presentations abroad)*

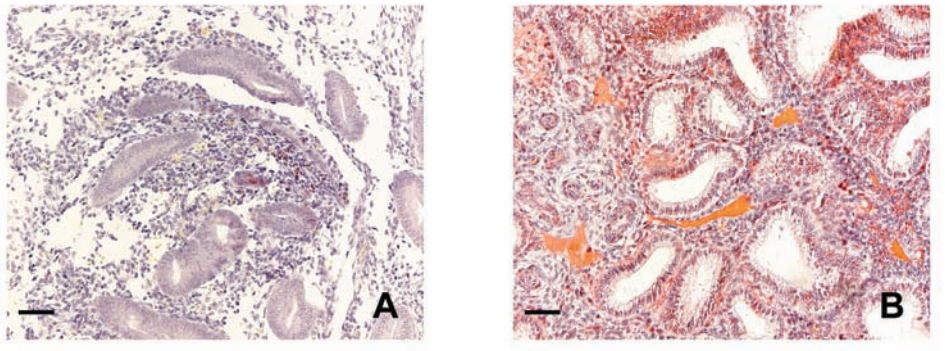
Kapiteijn K., Koolwijk P., van der Weiden R.M.F., van Hinsbergh V.W.M., Helmerhorst F.M. Human endometrial endothelial cells have a high angiogenic capacity, which depends on urokinase and matrix metalloproteinases (MMPs). Society for gynaecologic investigation 49th annual meeting, 2002, Los Angeles, USA.

Kapiteijn K., van der Weiden R.M.F., Koolwijk P., van Hinsbergh V.W.M., Helmerhorst F.M. Conditioned medium of human embryo and trophoblast cell line stimulate *in vitro* endometrial angiogenesis. Society for gynaecologic investigation 50th annual meeting, 2003, Washington, USA.


NAWOORD

Als laatste wil ik iedereen bedanken die op welke wijze dan ook een bijdrage heeft geleverd aan dit proefschrift. Een aantal mensen wil ik in het bijzonder noemen.
Bea en Erna, dank voor al jullie gezelligheid, steun en geduld om mij het "kweken" en de rest te leren. De angiogenese groep; Marielle, Annemie, Marten, Nancy, Eric, Catherine en Margreet, dank voor jullie hulp, troost en gezelligheid. Mede dankzij jullie ging ik nooit met tegenzin naar TNO. Paul, Roeland, Teake en Sjef, dank voor jullie bijdrage. Ik heb het als zeer prettig ervaren dat de drempel voor overleg altijd zo laag was. Geerten en Ton, dank voor alle (statische) spoedhulp. Alle TNO (oud-)medewerkers; Margreet, Martine, Annemiek, Karin, Daniël, Abbey, Vincent, Linda, Ria, Marian, Natascha, Erik, Lars, Bart, Gerrit, Anton, Adri, Kees, Jan Willem, Robert, Aad, Prof. Brakman, Jolanda, Bart en Elly, dank voor alle tips, hulp maar bovenal voor alle gezelligheid. Zonder jullie had ik 3 jaar in een lab niet vol gehouden. Collega's, dank voor de luisterende oren. Wil en Bea, onder het genot van een snoepje kon ik altijd mijn ei kwijt, dank. Jos, dank voor al je tijd, aandacht en vriendschap, je bent geweldig. Mariska, dank voor alle steun. Ton, Anneke en Carole, dank voor alle hulp aan de zijlijn. Ellen, grote zus, zoals al vaker heb ik op allerlei fronten weer enorm veel aan je gehad, dank dat je er altijd voor me bent. Ivo, een betere zwager is er niet, dank. Pap en mam, dank voor alles, dankzij jullie ben ik waar ik ben. Eef en Naut, de mooiste "producten" van angiogenese in het endometrium, jullie maken het compleet. Raymond, jou kan ik niet genoeg bedanken. In jou vind ik mijn rust, mijn thuis. Ik hou van je, zooooooooooooooooooooooooooooooooooooooooooooooooooooooooooooooooveel!

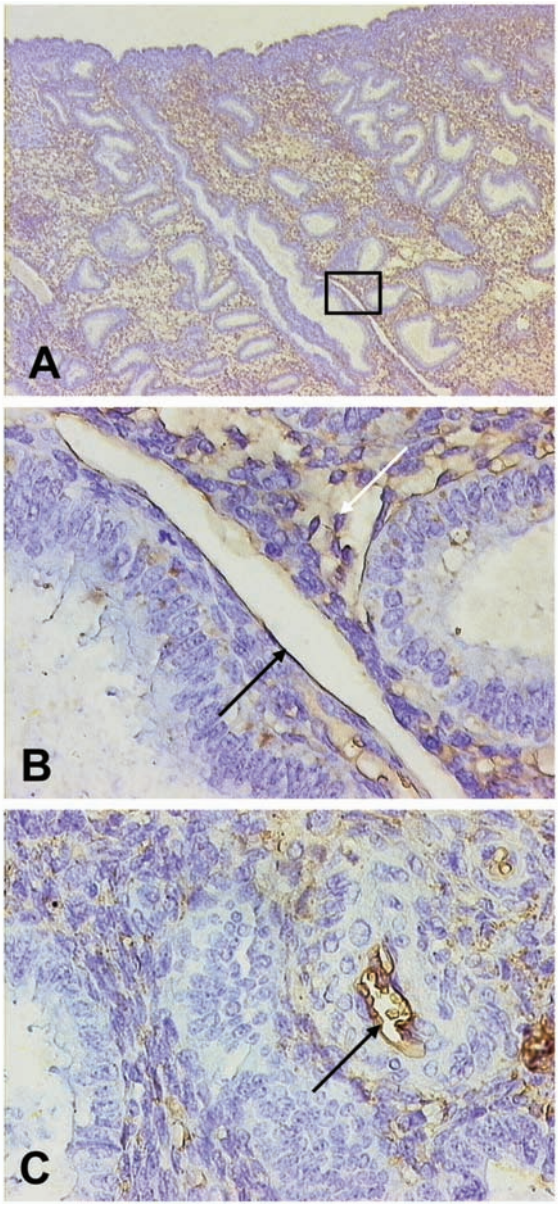
APPENDIX: COLOR FIGURES



Chapter 1, page 17

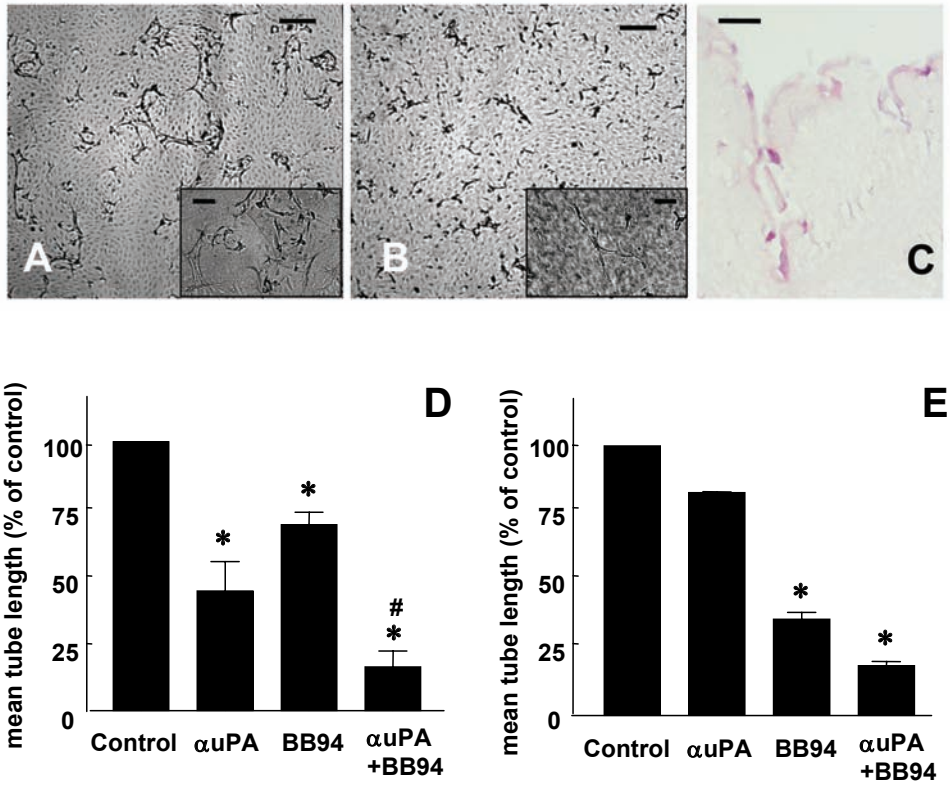
Figure 6. Fibrin staining in secretory endometrium.

In vivo the ECM of the endometrium consists of a number of proteins, such as laminin, fibrin, collagen type I, II, IV and VI, fibronectin and heparan sulphate proteogycan. The composition of the ECM varies during the cycle, as is shown here on paraffin sections of late-proliferative (A) and secretory (B) endometrium of two patients with a Martius Scarlet Blue staining. This staining stains fibrin red, collagen blue and erythrocytes yellow. During the secretory phase an increase of fibrin deposition is seen in the ECM of the endometrium. Bar = 100 μM.



Chapter 4, page 65

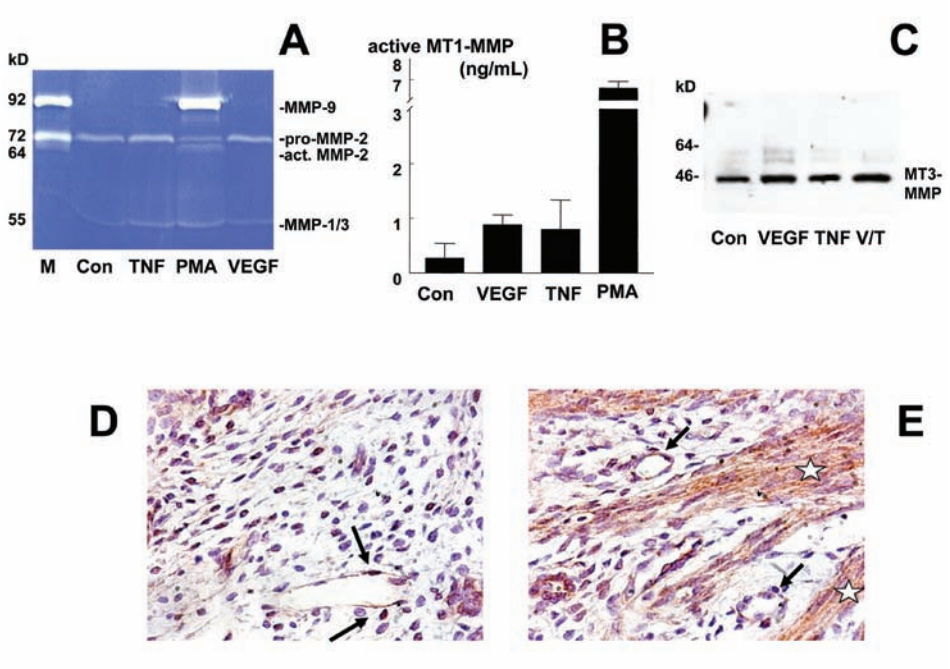
Figure 5. Expression of u-PA in blood vessels in human secretory phase endometrial tissue. Immunohistochemistry was performed with labeled antibody to u-PA on paraffin sections of human endometrium, as described in *Materials and Methods*. A, Brown staining shows accumulation of u-PA in the stromal endometrium; the surface and glandular epithelium are negative for the u-PA antigen (20x magnification). The *rectangle area* is enlarged in B. B and C, *Black arrows* indicate examples of positive endothelial cells, and a *white arrowhead* indicate positive stromal cells (200x magnification). The same results were obtained using proliferative phase endometrium.



Chapter 5, page 80

Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.

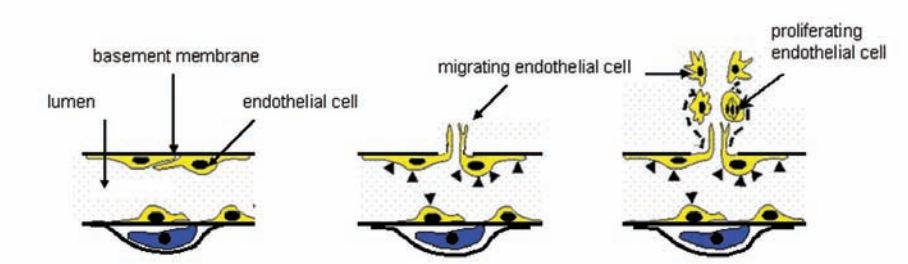
HEMVEC were cultured on top of a three-dimensional fibrin matrix (A,C,D) or 50-50% fibrin/collagen-type-1 matrix (B,E) and stimulated with VEGF-A (10 ng/mL). A and B: Micrographs taken after 4 days of culturing; *insets* in A and B show details of capillary-like structures. *Bar* = 300 μm, *Bar insets* = 100 μm. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (*bar* = 50 μm). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (αuPA, 100 μg/mL), BB94 (5 μg/mL) or a combination of BB94 and anti-u-PA. After 3-5 days of culturing, mean tube length was measured by image analysis. The data in panel D are expressed as a percentage of VEGF-A-induced tube formation ±SEM of 6 independent experiments of duplicate wells performed with 3 different hEMVEC isolations. Panel E represents 3 experiments. *: $p<0.05$ *vs*. control, #: $p<0.05$ *vs*. αuPA.



Chapter 5, page 82

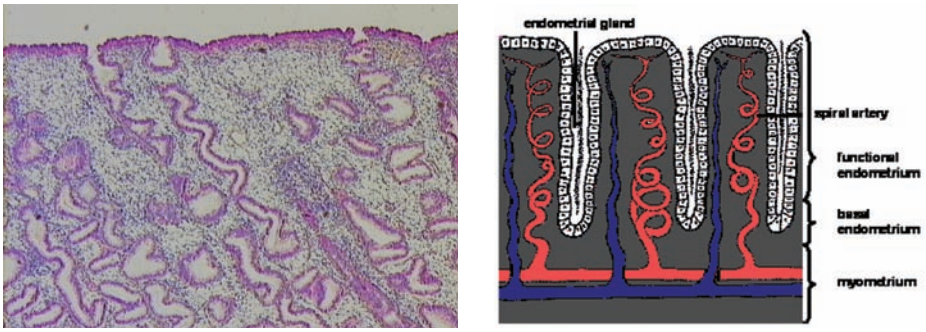
Figure 2. HEMVEC express various MMPs and MT-MMPs.

HEMVEC were cultured for 24 h in M199 supplemented with 0.5% HSA (A) or 20% HS (B,C) and were not stimulated (control) or stimulated with TNFα (2.5 ng/mL), VEGF-A (10 ng/mL) or PMA (10^{-8} M), as indicated. A: Gelatin zymography of 24 h conditioned medium. (M = ladder) B: MT1-MMP activity in cell lysates (mean ± range of two experiments performed in duplicate wells with two different isolations; detection limit of the assay 0.2 ng/mL). C: Western blot of MT3-MMP in 24 h conditioned medium. D and E: Immunohistochemical analysis of MT3-MMP in endometrial tissue shows the presence of MT3-MMP in endothelial cells (D, *arrows*) and myometrium (E, *stars*). Similar results were obtained in the tissue of three other donors.



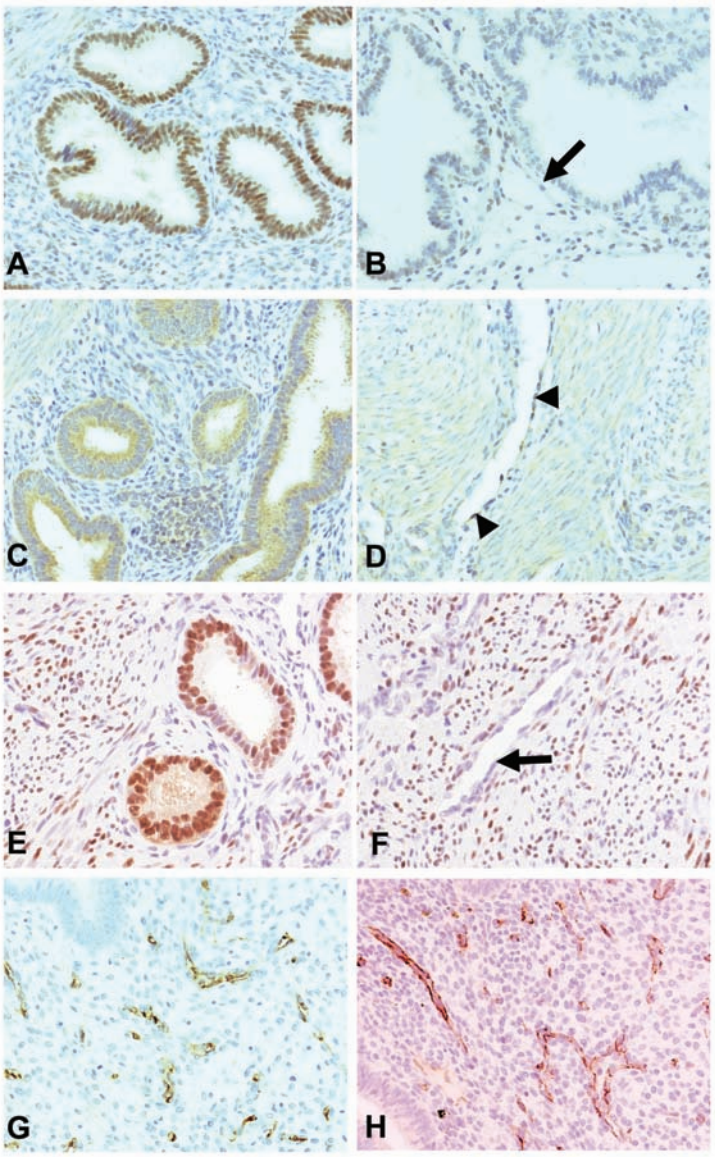
Chapter 6, page 95

Figure 1. In mature (non-growing) capillaries the vessel wall is composed of an endothelial cell lining and a basement membrane, in which pericytes (blue) usually are present. Angiogenic factors (▲) bind to endothelial cell receptors and initiate angiogenesis. When the endothelial cells are stimulated by angiogenic growth factors, they secrete proteolytic enzymes like metallo proteinases (MMPs) and enzymes of the plasminogen activator (PA) system, which degrade the basement membrane surrounding the vessel. The junctions between endothelial cells are loosened, the cells migrate through the space created, and the newly formed sprouts migrate and proliferate.*]*



Chapter 6, page 96

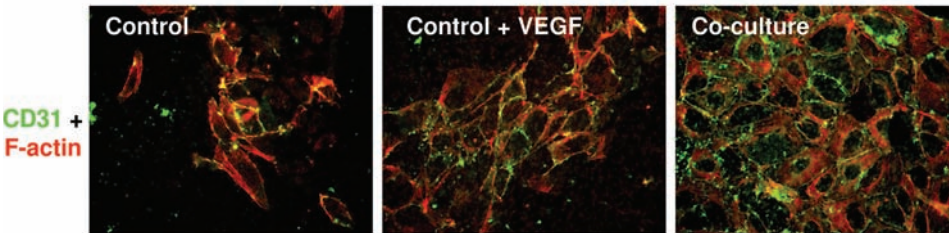
Figure 3. After menstruation from the basal endometrium a new functional endometrium grows. The basal arteries give rise to new blood vessels, which will form spiral arteries and a subepithelial capillary complex. Together with stromal and epithelial growth and differentiation, and increased vascular permeability, an edematous, thick receptive endometrium is prepared for implantation.



Chapter 7, page 120

Figure 1. Expression of ERα, ERβ and PR in human endometrial tissue.

Immunohistochemistry was performed with labeled antibodies to ERα, ERβ and PR on paraffin sections of human endometrium, as described in the methods section. Panel A and B; brown staining shows ERα in the epithelium and in the stromal compartment, the endothelium is negative for the ERα. C and D; endometrial stroma, epithelium and endothelium show positive staining for ERβ. E and F; PR staining is seen in the epithelium and in the stroma, the endothelium stains negative for the PR. G and H; von Willebrand and CD31 staining were used to indicate the endothelial cells in the endometrium. Black arrow heads indicate an example of positive endothelial cells, and black arrows indicate negative endothelial cells.



Chapter 7, page 127

Figure 7. HESC and VEGF contribute to maintenance of hEMVEC monolayers.

Cultures of hEMVEC and hESC were detached and seeded on the surface of a filter (hEMVEC) or dish (hESC) of a transwell™ system (Costar). The next day the hEMVEC-covered filters were transferred into the wells in which hESC had been grown or wells without cells (control). To half of the control conditions VEGF-A (10 ng/mL) was added. HEMVEC were immunostained for CD 31 (green) and F-actin was visualized by rhodamine-falloidin (red). HEMVEC monolayers remained intact in co-culture, but showed holes in control cells. The addition of VEGF-A improved the quality of the monolayers. *[See appendix: color figures]*