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Angiogenesis, proteases and angiogenic factors during the inception of pregnancy. Crucial contributors or trivial bystanders?

Plaisier, G.M.

Citation

Plaisier, G. M. (2008, May 7). *Angiogenesis, proteases and angiogenic factors during the inception of pregnancy. Crucial contributors or trivial bystanders?*. Retrieved from <https://hdl.handle.net/1887/12861>

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**ANGIOGENESIS, PROTEASES AND ANGIOGENIC
FACTORS DURING THE INCEPTION OF PREGNANCY.**
Crucial contributors or trivial bystanders?

ISBN: 978-90-8559-373-7

Cover design: Quirine Reijman, De Zagerij Ontwerpbureau, Den Haag

Lay-out: Quirine Reijman, De Zagerij Ontwerpbureau, Den Haag en Optima Grafische Communicatie bv, Rotterdam.

Printed by: Optima Grafische Communicatie bv, Rotterdam

Financial support for the publication of this thesis was provided by the Bronovo Research Fonds, the J.E Juriaanse stichting, Serono Benelux bv (an affiliate of Merck Serono SA) and Wyeth Pharmaceuticals bv.

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ANGIOGENESIS, PROTEASES AND ANGIOGENIC FACTORS DURING THE INCEPTION OF PREGNANCY

Crucial contributors or trivial bystanders?

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus Prof. Mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 7 mei 2008

klokke 15.00 uur

door

Geertruida Maria Plaisier

geboren te Dordrecht in 1975

Promotiecommissie

Promotores	Prof. Dr. F.M. Helmerhorst Prof. Dr. V.W.M van Hinsbergh	VUMC Amsterdam
Co-promotor	Dr. P. Koolwijk	VUMC Amsterdam
Referent	Dr. S.D. Charnock-Jones	University of Cambridge
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“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

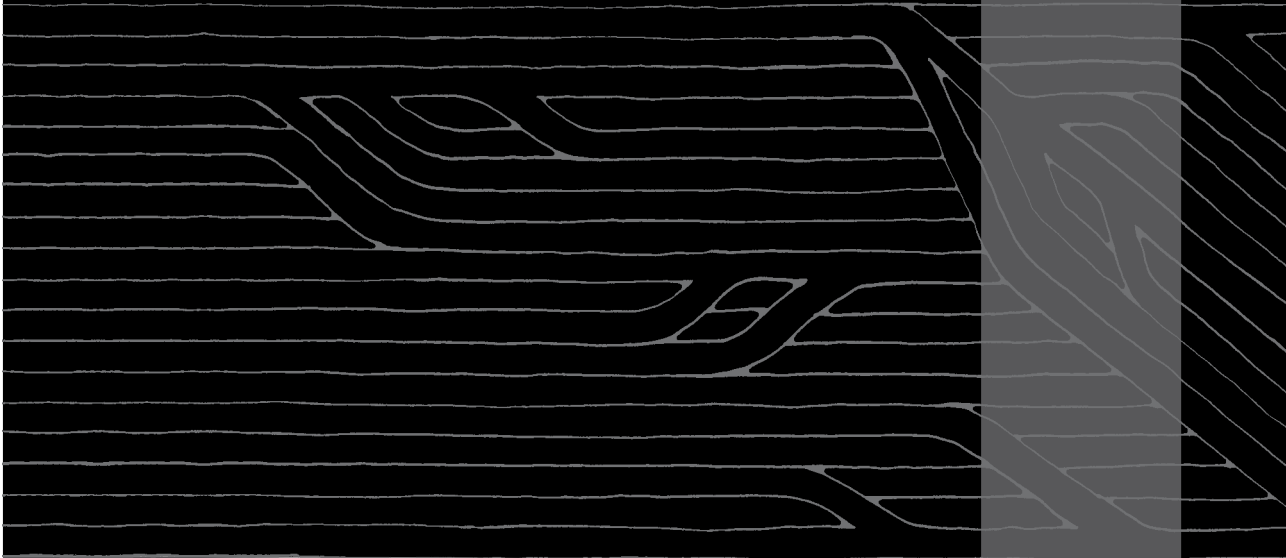
By Marie Curie (1867 - 1934)

CONTENTS

Chapter 1	General Introduction	9
Chapter 2	Involvement of membrane-type matrix metalloproteinases in capillary tube formation by human endometrial microvascular endothelial cells. Role of MT ₃ -MMP. <i>Journal of Clinical Endocrinology and Metabolism</i> 2004; 89: 5828-5836.	25
Chapter 3	Membrane-type matrix metalloproteinases (MT-MMPs) and vascularisation in human endometrium during the menstrual cycle. <i>Molecular Human Reproduction</i> 2006; 12:11-18.	43
Chapter 4	Human embryo-conditioned medium stimulates in vitro endometrial angiogenesis. <i>Fertility and Sterility</i> 2006; 85: S1: 1232-1239.	59
Chapter 5	Different degrees of vascularisation and their relation to the expression of VEGF, PlGF, Angiopoietins and their receptors in first-trimester decidual tissues. <i>Fertility and Sterility</i> 2007; 88: 176-187.	73
Chapter 6	Pericellular-acting proteases in human first-trimester decidua. <i>Molecular Human Reproduction</i> 2008; 14: 41-51.	91
Chapter 7	Decidual vascularisation and the expression of angiogenic growth factors and proteases in first-trimester spontaneous abortions. <i>Submitted to Human Reproduction</i> 2007.	111
Chapter 8	Preliminary Report: Angiogenic factors and their receptors in first-trimester human decidua of pregnancies further complicated by pre-eclampsia or foetal growth restriction. <i>Accepted for publication in Reproductive Sciences</i> 2008.	137
Chapter 9	General Discussion	147
Chapter 10	Conclusions	161
Chapter 11	Summary & Samenvatting	165
	References	175
	Abbreviations	193
	Authors & Affiliations	197
	Curriculum Vitae	201
	Acknowledgements	205
	Colour figures	211

Chapter 1

General Introduction



BACKGROUND

The maximum chance of a clinically recognised pregnancy given a menstrual cycle is only 10-15%, even when conditions are optimal. Increasing evidence points to implantation failure rather than conception failure as the reason for the relatively low fecundity observed in human species [Coulham 1991; Dickey *et al.*, 1994; Macklon *et al.*, 2002; Simpson *et al.*, 1994; Wang *et al.*, 2003; Wilcox *et al.*, 1988]. The success of implantation and of the embryo-maternal interaction depends on a well vascularised, receptive endometrium [Zygmunt *et al.*, 2003]. The generation of receptive endometrium starts during the secretory phase of the menstrual cycle, continues throughout the first-trimester and includes decidualisation, angiogenesis, and immune cell invasion. [Bulmer *et al.*, 1991; Giudice 1999; Lessey 2000; Salamonsen *et al.*, 2002/2003; Smith 2000]. The invasion of immune cells is enormous: from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. [Bulmer *et al.*, 1991]. Vascular adaptation includes (pseudo-) vasculogenesis, arterial remodelling, and angiogenesis, the formation of new vessels out of existing ones [Burton *et al.*, 1999, Pijnenborg *et al.*, 1983].

Disturbances in vascular development may play a role in the pathogenesis of pregnancy complications, such as miscarriage, pre-eclampsia, intrauterine growth restriction, and even during adulthood, i.e. cardiovascular disease [Barker *et al.*, 1993; Torry *et al.*, 2004; Vailhe *et al.*, 1999; Vuorela *et al.* 2000; Zygmunt *et al.*, 2003]. This thesis assessed the role of angiogenesis in cycling endometrium and during human implantation.

THE INCEPTION OF PREGNANCY

Endometrium

The uterine mucosa, or endometrium, goes through cyclic breakdown and regeneration throughout reproductive life. It is composed of two distinct layers; the functionalis layer, the upper two third layer which is shed and renewed monthly during reproductive life, and the basalis layer, the lower one third layer representing the germinal layer from which renewal occurs [Li *et al.*, 1994].

The cyclical process is regulated by changes in circulating levels of oestradiol and progesterone. Morphologic alterations are particularly evident in the functionalis layer and only minimal in the basalis layer [Mutter, Ferenczy 2002]. After menstruation, the functionalis layer is regenerated by proliferation of endometrial glands, stromal and endothelial cells in response to oestradiol. This phase is called the proliferation phase and will last for approximately 14 days. Once the ovarian follicle has matured, ovulation occurs and this preludes the post ovulation period or secretory phase. Progesterone, mainly produced by the corpus luteum, will cause precisely controlled changes in the oestradiol -primed

endometrium in preparation for blastocyst implantation. For instance, the glands display increased lumen and change secrete production. In addition, the stromal compartment becomes more prominent and is characterised by oedema, invasion of leucocytes, and cuffing of vessels. These morphological changes are known as pre-decidualisation and even occur in the absence of fertilisation [Aplin 2000; Lessey 2003]. When fertilisation fails, the corpus luteum degenerates and progesterone levels diminish. This causes breakdown and shedding of the functionalis layer during the menstrual phase [Li *et al.*, 1994; Mutter, Ferenczy 2002].

Decidua

Additional decidualisation occurs in the presence of pregnancy and slowly converts secretory endometrium into decidua. Several subtypes of decidua are described in the first-trimester. Decidual secretory endometrium (DSE) is only pre-decidualised and will develop into decidua parietalis (DP) under influence of pregnancy-induced hormones, i.e. progesterone, oestradiol and hCG. Decidua basalis (DB) will arise in the additional presence of the extravillous trophoblast (EVT, Figure 1).

The additional decidualisation results in further tissue remodelling, increased vascular permeability, oedema, proliferation and differentiation of stromal cells, invasion of leukocytes, and vascular remodelling [Aplin 2000; Salamonsen *et al.*, 2003]. Stromal cells transform from small spindle-shaped cells into large decidual round cells and display an increased production of secreted proteins and extra cellular matrix proteins. This may function in facilitating migration of EVTs towards spiral arteries [King 2000; Trundley, Moffett 2004]. Epithelial glands decrease in density, generate smaller amounts of secrete and have a more silent appearance. Furthermore, arteries become extensively remodelled and the length and size increase because of proliferation of endothelial and elongation rather than conventional sprouting angiogenesis [King 2000; Trundley, Moffett 2004].

Implantation and trophoblast invasion

Implantation is only facilitated during the narrow window of 7 to 10 days after ovulation, the “implantation window”. Initiation of implantation is due to an active biochemical process that requires interaction between the implanting blastocyst and the endometrial epithelium [Aplin 2000]. A variety of different molecules, e.g. prostaglandins, proteases, cytokines and growth factors, secreted by human trophoblast as well as endometrial cells regulate this “crosstalk” and allow apposition, attachment and invasion of the blastocyst [Guidice 1999; Krussel *et al.*, 2003; Nardo *et al.*, 2003; Salamonsen 2002; van der Weiden *et al.*, 1991]. Moreover, hCG production by the conceptus announces the presence of fertilisation to the maternal system [Reshef *et al.*, 1990].

Several days after fertilisation the embryo differentiates into a blastocyst, which contains an inner cell mass that will form the embryo and an outer trophoblast that will become placenta and chorion. Attachment of the blastocyst to the uterine wall triggers the differentiation of trophoblast into two layers: an inner cytotrophoblast layer and an outer syncytiotrophoblast layer. After attachment, the cytotrophoblast proliferates into buds which protrude through the syncytium. These protruding cytotrophoblasts become either villous or extra-villous trophoblasts (EVT). The first covers the chorionic villi and interacts with maternal blood in the intervillous space thus providing an exchange barrier between mother and foetus. The later invades into the decidua. Some EVT's, called endovascular trophoblasts, migrate into maternal capillaries to replace the endothelium [Burrows *et al.*, 1996; Norwitz *et al.*, 2001; Red-Horse *et al.*, 2004]. EVTs invade up to the myometrium; far enough to access a viable maternal blood supply but not so far that the mother is endangered.

Immune cell invasion

Immune cells infiltrate post-ovulatory endometrium. In the absence of pregnancy their number declines during menstruation, whereas in the presence of fertilisation, their number increases up to the 20th week of gestation [van den Heuvel *et al.*, 2005a]. In the

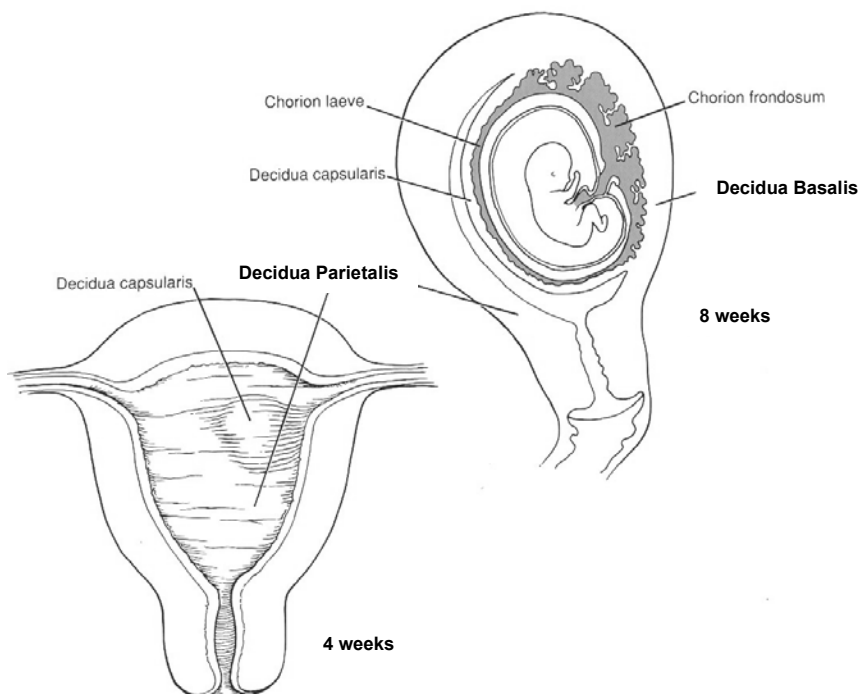


Figure 1. Schematic representation of the different types of decidua in first-trimester pregnancy.

first-trimester 30% of stromal cells are leucocytes; 75% of these leucocytes are uterine natural killer (uNK) cells and 10% are macrophages [Bulmer *et al.*, 1991].

Migration of uterine natural killer (uNK) cells is thought to be regulated both directly and indirectly by hormones, via oestrogen receptor ER β 1 and via chemo-attractants produced by hormone-stimulated stromal cells [Henderson 2003; King *et al.*, 1996]. Various growth factors and cytokines are expressed by uterine natural killer (uNK) cells, including angiogenic factors and proteases [Al-Atrash *et al.*, 2001; Albertsson *et al.*, 2000; Hanna *et al.*, 2006; Lash *et al.*, 2006; Li *et al.*, 2001a].

Possible functions of uterine natural killer (uNK) cells include controlling the invasion of trophoblast cells, regulate vessel stability (via IFN γ), decidualisation of endometrium, induce EVT apoptosis, regulate angiogenesis and immunomodulation [Bulmer, Lash 2005; Dosiou, Giudice 2005; Hanna *et al.*, 2006; King *et al.*, 1998; Quenby, Farquharson 2006]. A correlation with failure of implantation is inconsistently described. Women with recurrent miscarriages were shown to have elevated numbers of uterine natural killer (uNK) cells in peri-implantation endometrium compared to controls [Clifford *et al.*, 1999; Quenby *et al.*, 1999; Tuckerman *et al.*, 2007]. However, others have shown comparable numbers of uterine natural killer (uNK) cells in the same setting and in decidua of missed abortions compared to controls [Michimata *et al.*, 2002; Shimada *et al.*, 2004/2006].

Macrophages are immunosuppressive cells in human first-trimester decidua, probably mediated by prostaglandin E2 production [Parhar *et al.*, 1989] and play a role in controlling placenta growth. Their content of lysosomal enzymes could indicate phagocytic functions [Bulmer *et al.*, 1988].

Miscarriage

Only 30-50% of conceptions result in the birth of a child. Most pregnancies fail even before the next menstrual date is due [Macklon *et al.*, 2002; Rai, Regan 2006]. About 10-15% of the recognised pregnancies end in a miscarriage, 90-95% of which will occur before foetal cardiac activity has been detected [Kavalier 2005; Wang *et al.*, 2003]. Approximately half of all miscarriages will evacuate spontaneously as a spontaneous abortion. The other miscarriages remain in utero until noticed by ultra sound, the so-called missed abortions. Aetiological categories of miscarriages can be divided in embryo-related and maternal-related causes. The most likely cause of the first category are the chromosomal abnormalities. Maternal-related causes include uterine, endocrinological, immunological or thrombotic disorders [Coulham 1991; Kutteh 1999]. Few studies have addressed decidual and placental vascularisation in relation to miscarriage. Deficient villous vascularisation, differential decidual vascularisation, increased blood flow, and differential VEGF-A expression have been shown to be correlated with miscarriage [Greenwold *et al.*, 2003; Jauniaux, Burton 2005; Lisman *et al.*, 2004; Meegdes *et*

al., 1988; Vailhe *et al.*, 1999; Vuorela *et al.*, 2000]. Even abnormal uterine circulation in receptive phase of non-conception cycles has been shown in recurrent abortion patients [Habara *et al.*, 2002].

ANGIOGENESIS

General Angiogenesis

Under certain circumstances, the vascular network needs to adapt, expand and remodel to adjust to changing conditions. To that end, angiogenesis is induced. This process occurs only in few physiological conditions, i.e. the ovary, endometrium and placenta, and in various pathological conditions, such as tumour growth. Angiogenesis involves activation and proliferation of endothelial cells, degradation of their basal membrane, migration through the surrounding extracellular matrix (ECM), and finally stabilisation and maturation of vessels. Angiogenesis is a complex process, which is tightly regulated by angiogenic promoters and inhibitors. In quiescent tissue, promoters and inhibitors are in balance. During an episode of vessel growth, the balance tips in favour of the promoters.

Endometrial angiogenesis

Uterine blood supply is facilitated by the uterine arteries, which give rise to arcuate and radial arteries supplying the basal layer of the endometrium. Endometrial angiogenesis is mandatory to support the reconstruction of the endometrium after menstruation and to provide a vascularised, receptive endometrium for implantation and placentation [Gargett, Rogers 2001; Weston, Rogers 2000].

Endometrial angiogenesis appears spatially and transiently regulated during the cycle. Endothelium in the superficial layer of the endometrium shows cyclical variation in proliferation and angiogenic activity. However, due to the lack of correlation between vascular events, e.g. endothelial cell migration and proliferation, the timing of angiogenesis during the cycle remains unclear [Goodger, Rogers 1995]. Despite these reservations, previous studies have described the existence of three angiogenic episodes during the cycle.

The first episode occurs during the early proliferative phase representing post-menstrual repair; the second occurs during the late-proliferative phase under the influence of oestrogen; and the third during the secretory phase under the influence of progesterone [Ferenczy *et al.*, 1979; Goodger, Rogers 1995; Maas *et al.*, 2001; Rogers, Gargett 1998; Smith 2000]. However, no significant differences in endothelial cell proliferation were detected throughout the cycle [Goodger, Rogers 1994]. Furthermore, proliferating endothelial cells were mainly present in existing vessels rather than in vascular sprouts.

These findings suggest that endometrial angiogenesis is a continuing process throughout the cycle and that it proceeds by elongation and intussusceptions rather than by classical angiogenesis via sprout formation (Figure 2) [Gambino *et al.*, 2002; Rogers, Gargett 1998].

Although the overall control of endometrial growth and regression is regulated primarily by oestrogen and progesterone, the role of sex steroids in endometrial angiogenesis is less clear. Several reports describe the expression of progesterone and oestrogen receptors in endometrial cells, including endothelial cells, but their conclusions have not been decisive [Critchley *et al.*, 2001; Iruela-Arispe *et al.*, 1999; Krikun *et al.*, 2005; Rey *et al.*, 1998]. The general idea is that oestradiol and progesterone are able to regulate endometrial angiogenesis probably both directly and indirectly via locally produced angiogenic factors [Bausero *et al.*, 1998; Iruela-Arispe *et al.*, 1999; Kapiteijn *et al.*, 2001; Kayisli *et al.*, 2004; Perrot-Applanat *et al.*, 2000; Salamonsen 1994; Shifren *et al.*, 1996].

Overall, neither the timing of vascular growth during the menstrual cycle nor the mechanisms by which endometrial vessels are formed are currently understood, thus placing major limitations on our understanding of how angiogenesis promoters and inhibitors may act in the endometrium [Rogers, Gargett 1998].

Decidual angiogenesis

Successful pregnancy requires the development of a complex network that facilitates the maternal-foetal exchange. Human maternal vascular adaptation to implantation starts during the secretory phase and continues throughout the first-trimester. This process includes the induction of angiogenesis, vasculogenesis, vascular permeability, and arterial remodelling [Burton *et al.*, 1999; Pijnenborg *et al.*, 1983; Smith 2000]. These complex processes involve various cell types, including immune cells and stromal fibroblasts, which locally regulate the expression of mitogenic and angiogenic growth factors and cytokines [Sherer, Abulafia 2001]. Furthermore, oestradiol, progesterone and hCG also play a role. For example, hCG produced by the blastocyst has been shown to be an angiogenic factor itself [Zygmunt *et al.*, 2002].

Arterial remodelling involves swelling of endothelial cells, arterial dilatation and remodelling of the muscular walls. Regulation of arterial remodelling is poorly understood. Craven *et al.*, described these modifications to be a maternal response to pregnancy, since this remodelling occurred independently of the presence of trophoblast invasion, [Craven *et al.*, 1998]. In contrast, others have stated that the true physiological change, which involves medial necrosis and replacement with fibrinoid material, only occurs in the presence of interstitial trophoblast, thus in the deciduas basalis [Kam *et al.*, 1999; Pijnenborg 1998]. In addition, veins appear to be remodelled as well during early pregnancy, resulting in dilatation and intravenous fibrin depositions in association with trophoblasts [Craven *et al.*, 2002].

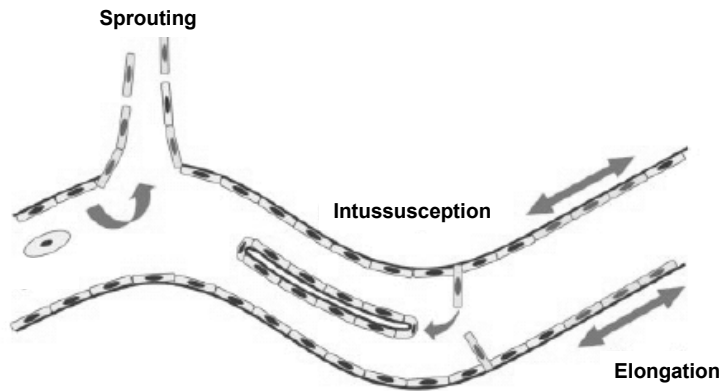


Figure 2. Endometrial angiogenesis proceeds by elongation and intussusception rather than by classical sprout formation. Adapted from Rogers 1998.

See colour figures supplement

An important feature during vascular development in the first-trimester is the invasion of maternal vessels by endovascular trophoblasts. This invasion results in replacement of maternal endothelial cells and plugging of spiral arteries [Pijnenborg *et al.*, 1983]. Partly because of these plugs, the maternal circulation to the placenta is restricted before the 8th week of pregnancy. Maternal blood flow will gradually extend over the next few weeks and by the 12th week blood flow to the inter-villous space is completely established [Burton *et al.*, 1999; Jaffe, Woods 1993; Jauniaux *et al.*, 2000]. Before the 10th week the uterine glands provide nutrition to the embryo. As a result, the human uteroplacental unit development takes place in a relatively low-oxygen environment during most of the first-trimester. Also a burst of oxidative stress occurs after establishment of maternal circulation [Burton *et al.*, 1999; Charnock-Jones *et al.*, 2004; Jauniaux *et al.*, 2000]. Moreover, the arterial plugging may protect the embryo from forceful maternal blood flows and oxygen overload [Burton *et al.*, 1999; Jaffe, Woods 1993; Jauniaux *et al.*, 2000; Kingdom, Kaufmann 1997].

Placental and uterine oxygen levels are spatially regulated as gestation progresses and regulate placental and decidual vascularisation by influencing the production of angiogenic factors [Charnock-Jones *et al.*, 2004; Jauniaux *et al.*, 2000; Sharkey *et al.*, 2000; Shore *et al.*, 1997]. Several reports state that first-trimester is characterised by both vasculogenesis and branching angiogenesis, while the second-trimester mainly displays branching angiogenesis and the third-trimester non-branching angiogenesis and that these series of events are probably regulated by oxygen [Charnock-Jones *et al.*, 2004; Geva *et al.*, 2002].

The low resistance arteriolar system results in pouch-like vessels, which are unresponsive to maternal vasomotor control. The lack of autoregulation of placental blood flow

allows dramatic increase in blood supply required to serve the growing demands of the foetus [Brosens *et al.*, 1967; Greiss *et al.*, 1976]. Limitations of this blood supply may have adverse clinical effects, like intra uterine growth restriction and pre-eclampsia [Khong *et al.*, 1986].

ANGIOGENIC FACTORS

VEGF family

The best known group of angiogenic factors is the vascular endothelial growth factor (VEGF) family, which consists of five mammalian members: placental growth factor (PlGF) and VEGF-A, VEGF-B, VEGF-C, VEGF-D. VEGF-A and PlGF are the most interesting factors of this family with regard to endometrium and decidua (Figure 3).

VEGF-A modulates the expression of many genes, enhances vascular permeability, induces endothelial cell proliferation, regulates apoptosis and plays an important role in the regulation of angiogenesis [Ferrara 2004; Hoeben *et al.*, 2004]. There are two receptors for VEGF-A; VEGF-R1 (flt-1) and VEGF-R2 (KDR) (Figure 3). Binding of VEGF-A to KDR induces proliferation and migration of endothelial whereas binding to flt-1 causes migration but not proliferation. In this way, VEGF-A induced endothelial proliferation and apoptosis can be regulated by changes in endothelial expression levels of KDR and flt-1 [Hoeben *et al.*, 2004].

Regulators of VEGF-A expression are the steroid hormones oestrogen and progesterone [Classen-Linke *et al.*, 2000; Hyder, Stancel 1999; Perrot-Applanat *et al.*, 2000]. Especially VEGF-A mRNA expression by endometrial carcinoma cell lines and stromal cells were found to be sensitive to steroidal stimulation [Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996]. Another stimulator of VEGF expression is hypoxia [Ferrara 2004; Sharkey *et al.*, 2000].

VEGF-A expression has been studied recurrently in endometrium. Various splice variants were detected, namely VEGF₂₀₆, VEGF₁₈₉, VEGF₁₈₃, VEGF₁₆₅, VEGF₁₄₅ and VEGF₁₂₁, of which VEGF₁₆₅, VEGF₁₄₅ and VEGF₁₂₁ are dominantly present in endometrium (Figure 3) [Krikun *et al.*, 2004a; Sherer, Abulafia 2001]. Data regarding the cyclical expression of VEGF-A were not always in agreement [Smith 1998]. Several reports describe an increased glandular expression in the secretory phase and an increased stromal expression during the proliferative phase [Charnock-Jones *et al.*, 1993; Moller *et al.*, 2001; Shifren *et al.*, 1996]. Others did not detect variations in epithelial or stromal VEGF expression or in VEGF-A secretion by endometrial explants throughout the cycle [Gargett *et al.*, 1999; Sugino *et al.*, 2002]. Strongest immunoreactivity of VEGF-A on endothelial cells was detected in late proliferative and secretory phases and correlated with the presence of KDR and flt-1 [Bausero *et al.*, 1998].

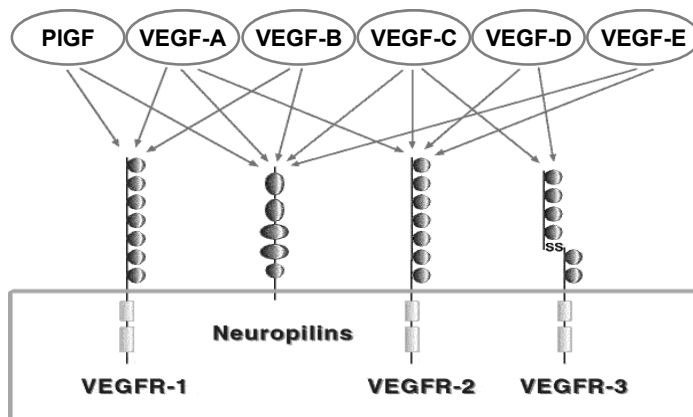


Figure 3. The VEGF family members and their receptors. Adapted from <http://ethesis.helsinki.fi/julkaisut/mat/bioti/vk/jeltsch/14revie7.html>.

KDR and flt-1 were mainly found in endometrial endothelial cells and KDR also in glands throughout the cycle [Krussel *et al.*, 1999; Meduri *et al.*, 2000; Moller *et al.*, 2001; Sugino *et al.*, 2002]. Although the abundant VEGF-A expression and the endothelial expression of VEGF-A receptors suggest a role in endometrial angiogenesis, the relation between VEGF, receptor activation and endothelial cell proliferation during the cycle is poorly understood.

Several studies have reported expression of VEGF-A and its receptors in early pregnancy decidua. Abundant levels of VEGF mRNA were detected at the site of implantation and lower levels elsewhere in the decidua [Clark *et al.*, 1996; Sharkey *et al.*, 1993]. VEGF-A, flt-1 and KDR proteins have been detected in maternal decidual, epithelial and endothelial cells [Clark *et al.*, 1996/1998a; Sharkey *et al.*, 1993; Sugino *et al.*, 2002]. VEGF-A proteins were also expressed by maternal macrophages, (syn)cytotrophoblast, and extravillous trophoblasts [Clark *et al.*, 1996; Cooper *et al.*, 1995; Jackson *et al.*, 1994]. VEGF, via its receptor flt-1, appears to play an active role in trophoblast invasion and angiogenesis during human and rhesus monkey implantation [Ahmed *et al.*, 1995; Clark *et al.*, 1996; Cooper *et al.*, 1995; Krikun *et al.*, 2004a; Sharkey *et al.*, 1993; Sengupta *et al.*, 2007; Sugino *et al.*, 2002; Torry, Torry 1997].

PlGF shares biochemical and functional features with VEGF and interacts with VEGFR-1 (Flt-1). PlGF and VEGF-A have synergistic effects regarding angiogenesis, but PlGF-induced vessels are more mature and stable than VEGF-induced vessels [Carmeliet *et al.*, 2001; Luttun *et al.*, 2002]. In contrast with VEGF, low oxygen tension results in reduced PlGF expression in trophoblasts *in vitro* [Shore *et al.*, 1997].

PlGF is abundantly expressed in human placenta, rising from the first-trimester to the late second-trimester and subsequently declining from 30 weeks of gestation to delivery

[Torry *et al.*, 1998]. PlGF mRNA is expressed in villous and extra-villous trophoblast cells while the protein is detected in vascular endothelium of term placental tissue [Clark *et al.*, 1998a; Jackson *et al.*, 1994; Vuorela *et al.*, 1997]. In addition, intense staining for PlGF antigens is detected in decidual stromal cells [Khalig *et al.*, 1996]. The receptor of PlGF, flt-1, is expressed on endothelial cells, perivascular smooth muscle cells and (extravillous) trophoblast during pregnancy [Athanasziades, Lala 1998; Torry *et al.*, 2004; Vuorela *et al.*, 1997]. PlGF may, via flt-1, act as a regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Khalig *et al.*, 1996; Sherer, Abulafia 2001; Torry *et al.*, 2004].

Angiopoietin family

Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and their receptor TIE-2 are known for their involvement in angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Ang-1 maintains vessel integrity, decreases vascular permeability and plays a role in endothelial and vascular maturation after VEGF-induced neovascularisation [Geva, Jaffe 2000]. Transgenic overexpression of Ang-1 in mice results in the development of more complex vascular networks [Suri *et al.*, 1998]. Ang-2 is a functional antagonist of Ang-1 and is only expressed at sites of vascular remodelling. Ang-2 leads to loosening of cell/cell interactions and allows access to angiogenic inducers like VEGF [Maisonpierre *et al.*, 1997]. Co-expression of VEGF and Ang-2 induces angiogenesis and increased vascular permeability, but Ang-2 results in vascular regression in the absence of angiogenic signals [Asahara *et al.*, 1998]. Hypoxia regulates both Ang-1 and Ang-2, i.e. upregulates Ang-2 and destabilises Ang-1 [Geva, Jaffe 2000; Zhang *et al.*, 2001].

Ang-1 is widely expressed in the adult, whereas Ang-2 is selectively expressed at sites of active angiogenesis, like the uterus and placenta [Maisonpierre *et al.*, 1997]. In endometrium, both Ang-1 and Ang-2 were detected in glands, stromal cells, and endothelium [Hewett *et al.*, 2002; Krikun *et al.*, 2000]. A significant upregulation in the late secretory phase has been described for Ang-1, whereas Ang-2 and TIE-2 showed only minor variations during the cycle [Hirchenhain *et al.*, 2003]. TIE-2 was mainly detected in endothelium and glands and only small amounts were found in stromal cells [Hewett *et al.*, 2002; Krikun *et al.*, 2000].

Ang-1 and -2 and TIE-2 are detected in human first-trimester decidua; TIE-2 mainly in maternal endothelial cells, endovascular trophoblasts, and (syn-) cytotrophoblasts and Ang-1 and -2 mainly in the latter. These findings suggest an additional role for angiopoietins, besides their role in angiogenesis, in regulating trophoblast behaviour in the development of uteroplacental circulation [Dunk *et al.*, 2000; Goldman-Wohl *et al.*, 2000; Zhou *et al.*, 2003]. The angiopoietins are regulated as gestation progresses: Ang-2 is maximally present in the first-trimester and declines thereafter, whereas Ang-1 increases from first- to third-trimester. This suggests that Ang-2 is mainly involved in first-trimester

vasculogenesis and branching angiogenesis and Ang-1 in third-trimester non-branching angiogenesis [Geva *et al.*, 2002]. An association of the angiopoietins with miscarriage has not been described but reduced endothelial TIE-2 expression has been linked to the occurrence of miscarriage [Vuorela *et al.*, 2000].

PERICELLULAR PROTEASES

Proteolysis plays a pivotal role in the regulation of angiogenesis and placental development [Heymans *et al.*, 1999; Pepper 2001a/2001b; Salamonsen 1999; Solberg *et al.*, 2003; Stetler-Stevenson 1999]. Key players in pericellular proteolysis are the uPA/plasminogen system and the membrane-type matrix metalloproteinases (MT-MMPs) [Alfano *et al.*, 2005; Kindzelskii *et al.*, 2004; Koolwijk *et al.*, 2001; Reuning *et al.*, 2003].

uPA/plasminogen system

The plasminogen activator (PA) system is based on the protease plasmin, which cleaves most ECM components. The circulating plasminogen is converted into the active protease plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). tPA is mainly involved in clot dissolution, whereas uPA mediates pericellular proteolysis during cell migration, tissue remodelling and angiogenesis [van Hinsbergh *et al.*, 2006]. uPA binds a specific cell-surface receptor, uPAR, which restricts the uPA-activity to the cell environment and enables activation of plasmin directly on the cell surface.

The activity of uPA is regulated by at least two specific serine proteinase inhibitors, plasminogen activator inhibitor type-1 and -2 (PAI-1 and 2), of which PAI-1 is expressed on endothelium and PAI-2 on monocytes and trophoblasts [Spengers, Kluft 1987]. Furthermore, the presence of uPA is not only determined by the ability of the cells to produce uPA, but also by their content of uPAR, which binds and internalises uPA in complex with its inhibitor PAI-1 [Blasi, Carmeliet 2002; Kroon *et al.*, 1999].

The role of uPA mediated plasminogen activation in cell migration has been studied for a variety of cells and for endothelial cells, leucocytes, and trophoblasts in particular [Blasi *et al.*, 1987; Heymans *et al.*, 1999; Hu *et al.*, 1999; Pepper 2001a; Reuning *et al.*, 2003; Salamonsen *et al.*, 2003]. The uPA expression is low in resting endothelial cells, whereas its expression is more abundant during angiogenesis and inflammation. This may explain the rich expression of uPA in endometrium endothelial cells [Koolwijk *et al.*, 2001]. Furthermore, also stromal cells contained uPA antigen, but glandular epithelium did not [Koolwijk *et al.*, 2001]. Both uPA and uPAR expression has been detected in the invasive trophoblast cells, which indicates a role for uPA and uPAR play in trophoblast

invasion [Floridon *et al.*, 1999; Hofmann *et al.*, 1994; Hu *et al.*, 1999; Multhaupt *et al.*, 1994; Pierleoni *et al.*, 1998; Salamonsen 1999].

Membrane-type Matrix metalloproteinases

The matrix metalloproteinases are a still expanding family of zinc-requiring enzymes that play a role in matrix remodelling and cell-matrix interactions. The membrane-type MMPs consist of six proteolytic enzymes which are particularly suited to function in pericellular proteolysis because of their membrane-associated localization [Hotary *et al.*, 2000; Visse, Nagase 2003]. The MT-MMPs incorporated unique domains which anchor them into the cell membrane. Four MT-MMPs are transmembrane proteins, namely MT1-, MT2-, MT3- and MT5-MMP (MMP-14, -15, -16 and 24) and two are GPI-anchored, MT4- and MT6-MMP (MMP-17 and -25) [Visse, Nagase 2003]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24), since these MT-MMPs induce capillary tube formation and their GPI-anchored counterpart MT4- and MT6-MMP was unable to do so [Hotary *et al.*, 2000].

MT1-MMP is the best known MT-MMP and is involved in degradation of ECM components, cell migration, and generating bioavailability of growth factors [Collen *et al.*, 2003; Galvez *et al.*, 2001; Hiraoka *et al.*, 1998]. MT1-MMP facilitates MMP activity by activation of pro-MMP-2 and pro-MMP-13. MT1-MMP has received considerable attention as being involved in tumour angiogenesis and has been shown to be able to induce angiogenesis [Hotary *et al.*, 2000; Seiki, Yana 2003; Sounni *et al.*, 2002]. MT2-MMP and MT3-MMP are also involved in cell migration and invasion. Furthermore, their overexpression in endothelial cells can induce capillary-tube formation, similar to MT1-MMP. MT1-, MT2- and MT3-MMP mRNA levels increase during tube formation *in vitro* [Hotary *et al.*, 2000; Lafleur *et al.*, 2002; Shofuda *et al.*, 2001]. The function of MT5-MMP is less well studied. MT5-MMP has a gelatinolytic effect and influences axonal growth and embryonic brain development [Llano *et al.*, 1999; Pei *et al.*, 1999].

MMP activity is modulated by growth factors, cytokines, plasmin, steroid hormones and several activated MMPs [Visse, Nagase 2003]. Specific inhibitors are four tissue inhibitors of metalloproteinases (TIMPs) [Gomez *et al.*, 1997; Greene *et al.*, 1996]. TIMP-1 inhibits the activity of all MMPs except MT1-MMP. TIMP-2 acts specifically on MMP-2 and its expression usually follows that of MMP-2. TIMP-3 is anchored in the matrix and inhibits all MMPs, including MT1-MMP, and has additional properties of stimulating cell growth and inducing apoptosis [Will *et al.*, 1996; Woessner 2001]. Finally, TIMP-4 functions in a more tissue specific manner as it is highly expressed in cardiac tissue [Greene *et al.*, 1996]. TIMP-1, -2 and -3 mRNA has been detected in humane endometrium, cytotrophoblasts and decidual endothelium and glandular epithelium during the first-trimester [Hurskainen *et al.*, 1996; Maatta *et al.*, 2000].

Members of the MMP family are widely expressed in the cycling endometrium and their expression and activity are sensitive to regulation by cytokines and to downregulation by progesterone [Freitas *et al.*, 1999; Lockwood *et al.*, 1998; Salamonson *et al.*, 1997; Singer *et al.*, 1999; Zhang *et al.*, 2000]. Progesterone withdrawal during the late secretory phase leads to increased MMP levels, focal tissue degradation and menstrual bleeding [Lockwood *et al.*, 1998; Salamonson *et al.*, 1997; Zhang *et al.*, 2000; Zhang, Salamonson 2002]. The expression, regulation and function of MT-MMPs are studied in less detail. The expression of MT1-, MT2- and MT3-MMP mRNAs and proteins in endometrial tissue has been described in various cycle phases [Chung *et al.*, 2002; Goffin *et al.*, 2003; Maatta *et al.*, 2000; Nakano *et al.*, 2001; Zhang *et al.*, 2000]. Suggested functions of endometrial MT-MMPs include tissue remodelling during the proliferative phase and tissue degradation during menstruation [Curry, Osteen 2003]. The involvement of MT-MMPs in regulating endometrial angiogenesis is not clear, although the expression of MT2-MMP, and to a lesser extent of MT1-MMP, has been described in endometrium endothelium *in vivo* [Maatta *et al.*, 2000; Zhang *et al.*, 2000].

With regard to MT-MMPs in decidua, only MT1- and MT2-MMP have been studied. MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, stromal cells, and the extra-villous trophoblast [Bjorn *et al.*, 1997/2000; Hurskainen *et al.*, 1998; Nakano *et al.*, 2001; Nawrocki *et al.*, 1996]. These MT-MMPs are assumed to regulate trophoblast invasion during implantation. Whether migration of other cell types, e.g. immune and endothelial cells, is also regulated by MT-MMPs remains to be seen [Salamonsen 1999].

OUTLINE OF THIS THESIS

The principal aim of this thesis is to assess the role of angiogenesis in cycling endometrium and during implantation. We hypothesise that angiogenesis is of paramount importance for the development of a receptive endometrium for implantation and therefore is a crucial contributor to the inception of pregnancy.

Pericellular proteolysis plays an important role in angiogenesis being required for endothelial cell migration, invasion and tube formation. **Chapter 2** focussed on regulation of *in vitro* endometrial angiogenesis by pericellular proteases, in particular MT1-MMP and MT3-MMP because of their demonstrated role in angiogenesis. We studied the expression of proteases by human endometrial microvascular endothelial cells (hEMVEC) and their involvement in the formation of capillary tubes. In **Chapter 3** we analysed the presence of MT-MMPs in human endometrium *in vivo* and their correlation with endometrial neovascularisation.

These first chapters provide data on angiogenesis in human endometrium, whereas the following chapters focus on angiogenesis in human decidua. The involvement of cytokines and angiogenic growth factors in angiogenesis during implantation was studied in **Chapter 4**, by determining the effect of IVF supernatants on angiogenesis by endometrial endothelial cells (hEMVEC) *in vitro*.

The vascularisation pattern and the decidual expression of angiogenic factors in human first-trimester pregnancies *in vivo* are described in **Chapter 5**. This study was performed in three first-trimester decidual tissues: decidual secretory endometrium (DSE), decidua basalis (DB) and parietalis (DP). By comparing these decidual tissues within subjects, the influences on vascularisation and expression of growth factors that occur independently of trophoblast invasion, i.e. induced by pregnancy-induced hormones, were separated from the influence of the invasive extra-villous trophoblast.

The presence of pericellular proteases in human first-trimester decidua was studied in **Chapter 6**. This study, like **Chapter 5**, is performed in three decidual tissues, DSE, DP and DB, to be able to determine the regulation of decidual pericellular proteases as well.

The hypothesized involvement of angiogenesis in the pathogenesis of a miscarriage was addressed in **Chapter 7**. First-trimester decidual tissues of miscarriages and matched controls were compared with regard to vascularisation and the expression of angiogenic growth factors and proteases.

Chapter 8, a preliminary report, focused on the role of first-trimester angiogenesis in the pathogenesis of pre-eclampsia and/or intrauterine growth retardation. First-trimester decidua obtained during chorion villous biopsies. The included patients were followed throughout their pregnancy to be able to relate the expression of the angiogenic factors to the pregnancy outcome, ie uncomplicated, pre-eclampsia or intrauterine growth retardation.

In **Chapter 9**, results from above mentioned studies are discussed and future perspectives presented. **Chapter 10** provides the conclusions of the thesis. Finally, **Chapter 11** summarises all presented studies and provides a dutch summary.

Chapter 2

**Involvement of membrane-type matrix metalloproteinases
in capillary tube formation by human endometrial
microvascular endothelial cells. Role of MT₃-MMP.**

M. Plaisier
K. Kapiteijn
P. Koolwijk
C. Fijten
R. Hanemaaijer

J.M. Grimbergen
A. Mulder-Stapel
P.H.A. Quax
F.M. Helmerhorst
V.W.M. van Hinsbergh

ABSTRACT

Objective: In the endometrium angiogenesis is a physiologically process, while in most adult tissues angiogenesis is initiated only during tissue-repair or pathological conditions. Pericellular proteolysis plays an important role in angiogenesis being required for endothelial cell migration, invasion and tube formation.

Materials and Methods: We studied the expression of proteases by human endometrial microvascular endothelial cells (hEMVEC) and their involvement in the formation of capillary tubes and compared these requirements with those of foreskin MVEC (hFMVEC).

Results: Inhibition of urokinase and matrix-metalloproteinase (MMPs) both reduced tube formation in a fibrin or fibrin/collagen matrix. hEMVEC expressed various MMPs mRNAs and proteins; in particular MMP-1, MMP-2, MT1-, MT3- and MT4-MMP. MT3- and MT4-MMP mRNA expressions were significantly higher in hEMVEC than in hFMVEC. Other MT-MMP mRNAs and MMP-9 were hardly detectable. Immunohistochemistry 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, while 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 IgG.

Conclusion: Thus, in contrast to tube formation by hFMVEC, which largely depends on MT1-MMP, capillary-like tube formation by hEMVEC is, at least in part, regulated by MT3-MMP.

INTRODUCTION

In the adult, angiogenesis plays a role in many pathological conditions, such as the growth of solid tumours, diabetic retinopathy, rheumatoid arthritis, and wound healing [Carmeliet 2003; Folkman 1995]. Physiological angiogenesis during adulthood is limited to the female reproductive tissue, namely in the ovary and endometrium. Endometrial angiogenesis plays a role in endometrial remodelling during the menstrual cycle and after conception during the implantation of the embryo [Bacharach *et al.*, 1992; Rogers, Gargett 1998; Smith 1998].

Angiogenesis is initiated by a shift in the balance between pro-angiogenic and anti-angiogenic factors [Bergers, Benjamin 2003; Hanahan, Folkman 1996]. It involves the sprouting of new capillary-like structures from existing vasculature and may involve blood-born cells that intussuscept in and around the new vascular structures [Carmeliet 2003]. These newly formed tubes are subsequently stabilised, 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 [Chi *et al.*, 2003], many of which are conserved *in vitro* [Chi *et al.*, 2003; Defilippi *et al.*, 1991; Koolwijk *et al.*, 2001]. 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 (VEGF) and form capillary tubules after exposure to VEGF-A, 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 TNF α [Koolwijk *et al.*, 1996; Koolwijk *et al.*, 2001; Kroon *et al.*, 1999].

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 [Pepper 2001a]. 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) [Bacharach *et al.*, 1992; Collen *et al.*, 2003; Galvez *et al.*, 2001; Hotary *et al.*, 2000; Koolwijk *et al.*, 1996; Kroon *et al.*, 1999; Lafleur *et al.*, 2002; Pepper 2001a; Pepper *et al.*, 1990; Pepper 1997; Werb 1997]. Initial data on the formation of tubular structures by hEMVEC indicated that cell-bound u-PA and plasmin contribute to this process [Koolwijk *et al.*, 2001]. In addition to the u-PA/plasmin cascade, the rapidly expanding family of MMPs [Visse, Nagase 2003] plays an important role in cell migration and invasion, and in angiogenesis *in vivo* [Heymans *et al.*, 1999; Pepper *et al.*, 1990; Stetler-Stevenson 1999]. MMPs

are widely expressed in the endometrium and play a role in tissue degradation and menstrual bleeding [Salamonsen, Woodley 1996]. Furthermore, a number of them are also detected during the proliferative and early secretory phase [Tabibzadeh, Babaknia 1996], which suggests a role in endometrial remodelling and angiogenesis [Rodgers *et al.*, 1994; Smith 2001]. 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 [Hotary *et al.*, 2000/2002; Zhou *et al.*, 2000]. The membrane-associated localisation of membrane-type MMPs (MT-MMPs) makes this group of MMPs particularly suited to function in pericellular proteolysis [Hotary *et al.*, 2000]. 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 [Collen *et al.*, 2003; Galvez *et al.*, 2001; Lafleur *et al.*, 2002]. 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 [Deryugina *et al.*, 2000; Galvez *et al.*, 2001; Sounni *et al.*, 2002; Zhou *et al.*, 2000]. MT1-MMP as well as MMP-2 are able to stimulate angiogenesis [Taraboletti *et al.*, 2002; Vagnoni *et al.*, 1998]. In hFMVEC, MT1-MMP becomes a key factor in capillary tube formation when collagen is present in the fibrinous matrix [Collen *et al.*, 2003; Hotary *et al.*, 2000]. MT2-MMP (MMP-15) and MT3-MMP (MMP-16) are also involved in cell migration and invasion, depending on the cell type [Hotary *et al.*, 2000; Shofuda *et al.*, 2001]. Their overexpression in endothelial cells can induce capillary-tube formation, similar to MT1-MMP [Hotary *et al.*, 2002]. 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 [Chung *et al.*, 2002; Goffin *et al.*, 2003; Maatta *et al.*, 2000; Zhang *et al.*, 2000]. It is generally believed that these MMPs also play a role in endometrial angiogenesis [Salamonsen 1994], but except for the expression and immunolocalisation 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 four 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 [Woessner 2001]. 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 [Li *et al.*, 2001b]. Furthermore, TIMP-3 can induce apoptosis in various cell types [Woessner 2001].

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 Island, 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 previously [Maciag *et al.*, 1979]. Heparin and thrombin were obtained from Leo Pharmaceuticals Products (Weesp, the Netherlands). Human fibrinogen was obtained from Chromogenics AB (Mölnådal, 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 (CLB, 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 tumour necrosis factor alpha (TNF α) was a gift from Dr. J. Tavernier (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 [Lamfers *et al.*, 2002; Quax *et al.*, 2001; Van der Laan *et al.*, 2003]. 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 in our laboratory. Mouse-anti-human monoclonal antibody against MT3-MMP was obtained from Oncogene Research Products (Boston, USA). Human recombinant MT1-MMP (pro-domain-catalytic domain-hemopexin

domain) was purchased from Chemicon (Temecula, CA, USA) and recombinant pro-MMP-9 from Invitex (Berlin, Germany). PBS/T concentrate was obtained from Organon Teknika (Boxtel, Holland). GAPDH control reagents (VIC-labeled) were purchased from Applied Biosystems (Nieuwerkerk a/d 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 (Pierce, St. Augustin, Germany) and the luminescent image workstation (Roche Diagnostics, Almere, the Netherlands) were used for visualisation.

Cells

Human endometrial microvascular endothelial cells (hEMVEC) were isolated from endometrial tissue from pre-menopausal women and cultured and characterised as previously described [Koolwijk *et al.*, 2001]. hEMVEC were maintained in hEMVEC culture medium: M199 without phenol-red supplemented with 20 mM HEPES (pH 7.3), 20% HS, 10% NBS, 150 μ g/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% CO₂ / 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. Human foreskin microvascular endothelial cells (hFMVEC) were isolated, characterised and cultured as previously described [Defilippi *et al.*, 1991; Van Hinsbergh *et al.*, 1987].

In vitro capillary-like tube formation assay

Human fibrin matrices were prepared as described before [Koolwijk *et al.*, 2001]. For the collagen gels, 7 volumes of rat tail collagen type-I (3 mg/mL) were mixed with 1 volume of 10x M199 with phenol red and 2 volumes of 2% (w/v) Na₂CO₃ (final pH 7.4). 300 μ l Aliquots were added to each well of a 48-wells plate and allowed to gelate at 37 °C in the absence of CO₂.

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 analysed by phase contrast microscopy. The total length of the structures formed was measured in 6 randomly chosen microscopic fields (7.3 mm²/field) by computer-equipped Optimas image analysis software (Bioscan, Demons, WA) connected to a monochrome CCD camera (MX5) and expressed as mm/cm².

Gelatin zymography

Gelatinolytic activities of MMPs secreted by hEMVEC were analysed by zymography on gelatin-containing polyacrylamide gels as described [Birkedal-Hansen, Taylor 1982]. Using this technique both active and latent species can be visualised. Samples were applied to a 10% (w/v) acrylamide gel co-polymerised 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 , 1 $\mu\text{mol/L}$ 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 , 1 $\mu\text{mol/L}$ ZnCl_2 and incubated overnight at 37°C. 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 using a monoclonal mouse anti-MT3-MMP and a horseradish peroxidase-conjugated horse-anti-mouse antibody. Specificity of the immunohistochemical reaction was verified by omission of the first antibody as well as using normal mouse serum in stead of the first antibody.

Western blotting

Total cellular extracts were prepared in the presence of protease inhibitors and applied to SDS-PAGE electrophoresis essentially as described [Kleemann *et al.*, 2003]. 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 previously [Chomczynski, Sacchi 1987]. Reverse transcription (RT) was carried out in 20 μL volumes using random primers and a cDNA synthesis kit purchased from Promega. MMP and MT3-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-labelled 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 [Collen *et al.*, 2003]. All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene GAPDH (VIC-labelled) 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 promotor) 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 [Quax *et al.*, 2001]. 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 activity assays (Biotrak; Amersham, Biosciences, Buckinghamshire, UK) as previously indicated [Collen *et al.*, 2003; Hanemaaijer *et al.*, 1998]. 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 $\pm$ 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 [Koolwijk *et al.*, 2001], hEMVEC form spontaneously capillary-like tubular structures in a fibrin matrix, a process that is markedly enhanced by VEGF-A (Figure 1A, C). When hEMVEC were seeded on top of matrices

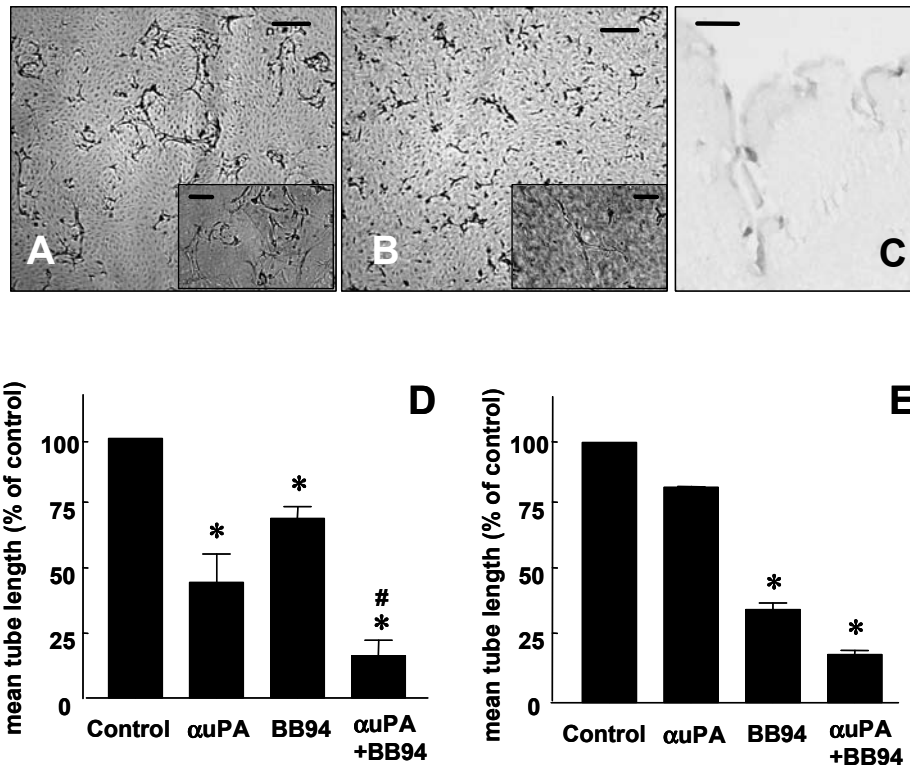


Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.

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 μ m, Bar insets = 100 μ m. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (bar = 50 μ m). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (α uPA, 100 μ g/mL), BB94 (5 μ g/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 $\pm$ 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. α uPA.

(see colour figures supplement)

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 $55 \pm 4\%$ under basal conditions ($n = 3$, not shown) and $53 \pm 2\%$ in the presence of VEGF-A (Figure 1B) as compared to the tube formation in a pure fibrin matrix (Figure 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 (Figure 1C and D). The VEGF-A-enhanced tube formation in a fibrin matrix was reduced by $55 \pm 11\%$ by u-PA-blocking antibodies (Figure 1D), and by $54 \pm 7\%$ by the plasmin inhibitor aprotinin (data not shown). In a matrix consisting of an equal mixture of fibrin and collagen anti-u-PA antibodies reduced tube formation only by $17 \pm 0\%$ (Figure 1E). The inhibiting effect of BB94 was increased by adding collagen, since tube formation in pure fibrin was inhibited by $31 \pm 5\%$ and in collagen-fibrin matrices by $64 \pm 3\%$. An almost complete inhibition ($84 \pm 6\%$ and $82 \pm 2\%$, respectively) of capillary-like structure formation was seen after the simultaneous addition of BB94 and anti-u-PA antibodies (Figure 1D and E).

Table 1. mRNA level of MT-MMPs in hEMVEC and hFMVEC.					
		hEMVEC		hFMVEC	
		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	$7.4 \pm 0.4^*$	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	$7.3 \pm 0.3^*$	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

Table 1. Analysis of MT-MMP mRNA expression in VEGF-A-stimulated hEMVEC and hFMVEC. 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 ($CT \pm SEM$). 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 $dCT \pm SEM$. 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.

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 express 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 ($\pm$ 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 ($\pm$ SEM)). No mRNA of MMP-3, MMP-7, MMP-8, MMP-12 and MMP-13 was detected in

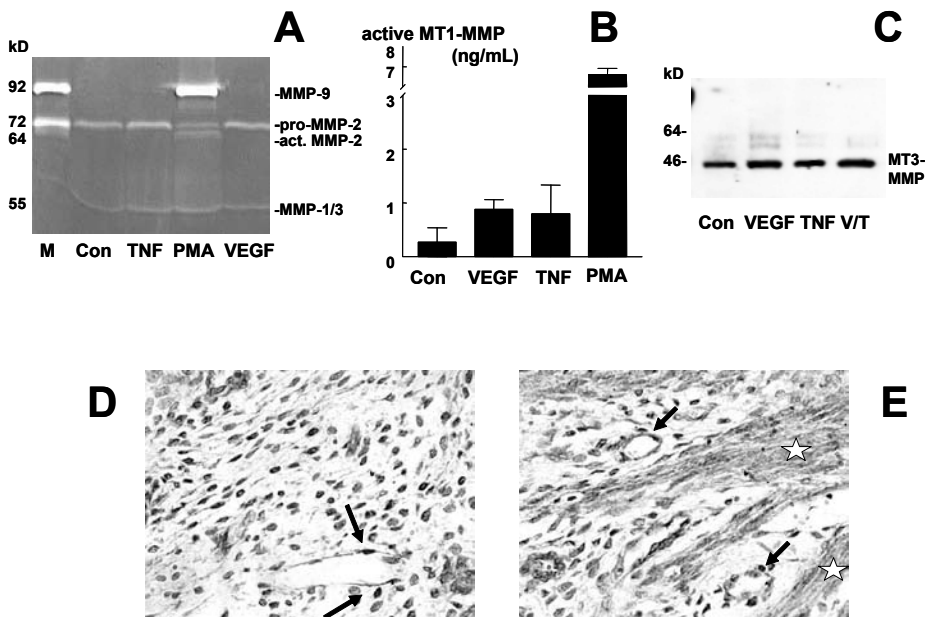


Figure 2. hEMVEC express various MMPs and MT-MMPs.

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 TNF α (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 $\pm$ 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).

(see colour figures supplement)

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, Figure 2A). The presence of MT1-MMP was demonstrated by activity assay (Figure 2B). Both VEGF-A and TNF α exposure doubled the activity of MT1-MMP, while phorbol ester caused a dramatic increase in MT1-MMP activity in hEMVEC (Figure 2B). The presence of MT3-MMP protein was confirmed by Western blotting. The production of MT3-MMP was not affected by TNF α and increased slightly after VEGF-A exposure (Figure 2C). MT3-MMP was detectable in endothelial cells of proliferative human endometrial tissue, as well as in endometrial epithelial cells and myometrial cells (Figure 2D and E).

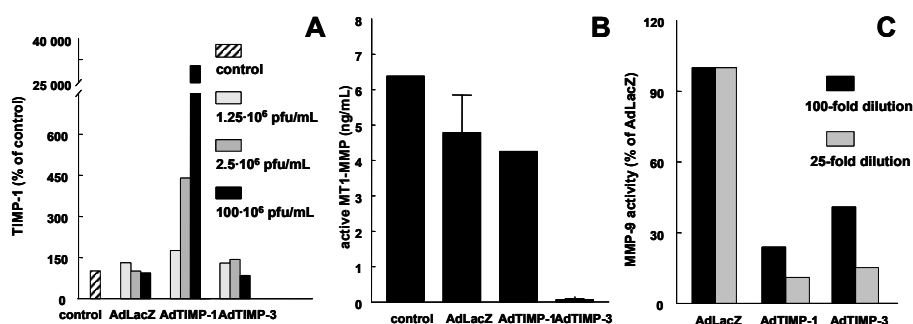


Figure 3. hEMVEC overexpress active TIMP-1 and -3 after transduction.

Confluent hEMVEC were transduced with $1.25 \cdot 10^6$, $2.5 \cdot 10^6$ and $1.0 \cdot 10^8$ 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 analysed as indicated in the Methods section. C: MMP-9 activity was assayed using a bioactivity assay as described in the Material and Methods. The ability of TIMP-1 and TIMP-3 to inhibit MMP-9 activity was measured in 100- (black bars) and 25-fold (gray bars) dilutions of the conditioned media of hEMVEC overexpressing TIMP-1 or TIMP-3.

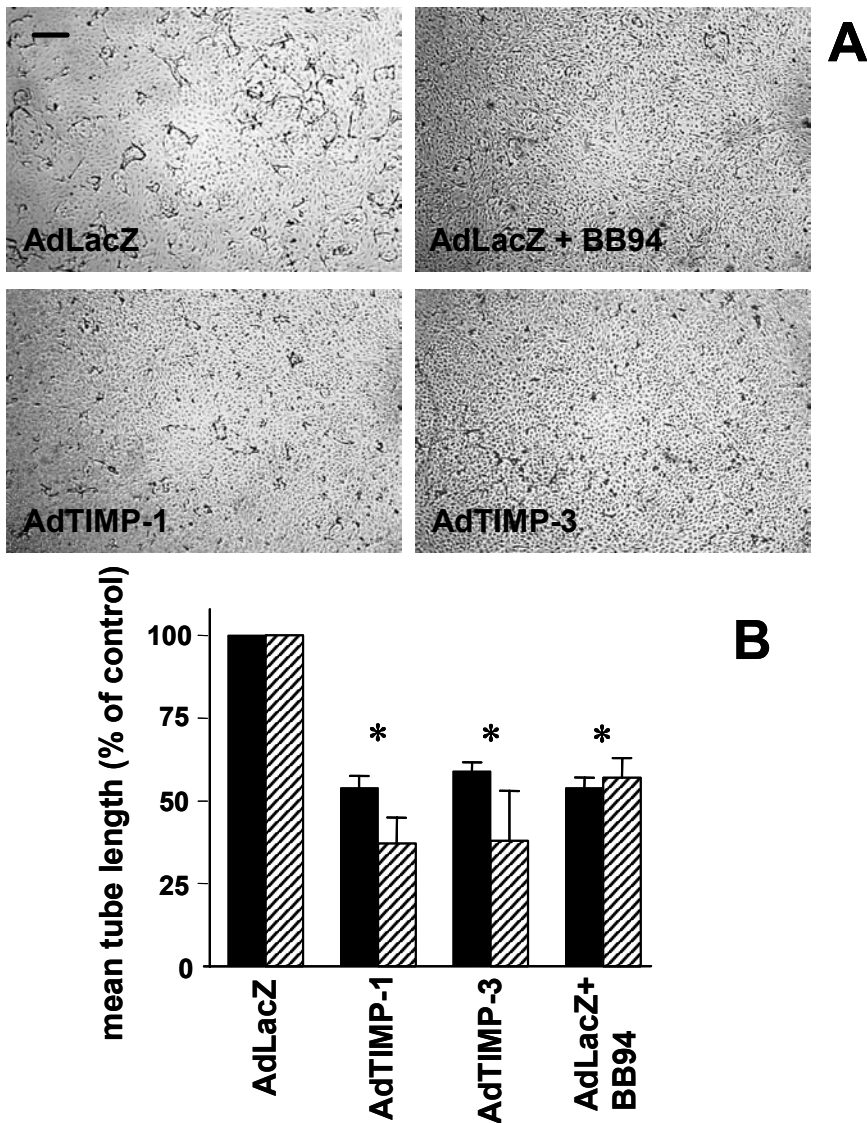


Figure 4. Both TIMP-1 and TIMP-3 inhibit capillary-like tube formation by hEMVEC.

hEMVEC were transduced with $2.5 \cdot 10^6$ 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 μ g/mL). A: Phase contrast micrographs after 3 days of culturing showing tube formation in the fibrin matrix, Bar = 300 μ m. B: Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells $\pm$ 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/cm² on the fibrin-collagen matrix. * $P < 0.03$ vs LacZ transduced cells.

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 2h 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 (Figure 3A). To verify whether the overexpressed TIMP-1 and -3 were func-

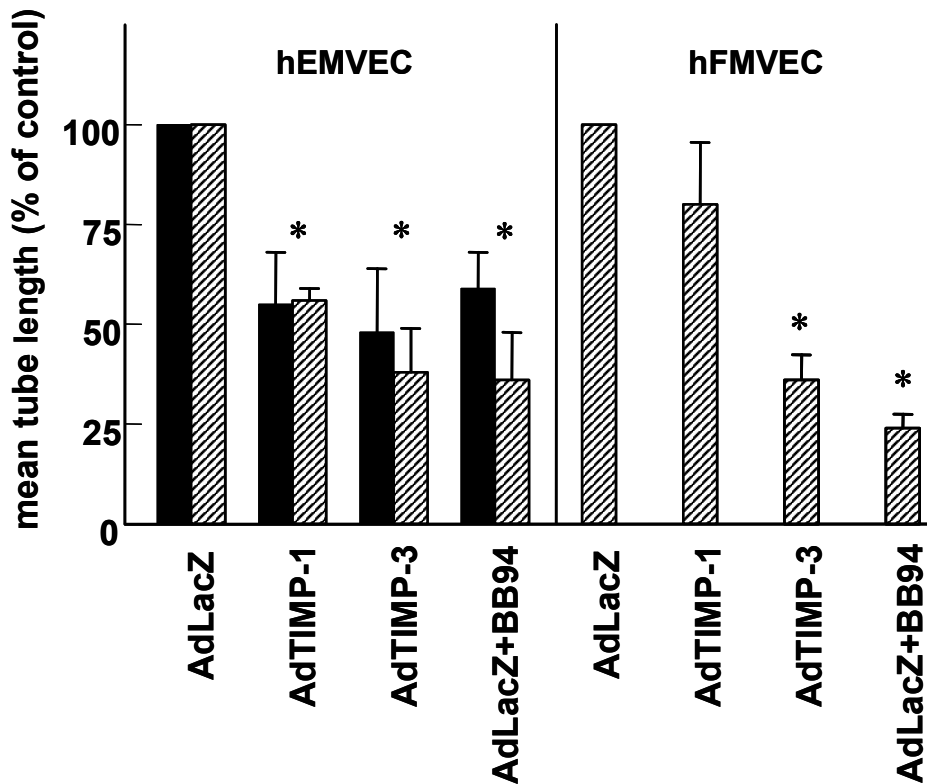


Figure 5. TIMP-1 inhibits capillary-like tube formation by hEMVEC but not by hFMVEC.

hEMVEC and hFMVEC were transduced with $2.5 \cdot 10^6$ 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 TNF α (10 ng/mL) with or without BB94 (5 μ g/mL). Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells $\pm$ 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/cm ^{2} on the fibrin-collagen matrix, * $P < 0.05$ vs. LacZ transduced cells.

tional and active, their effect on MT1-MMP and MMP-9 activity was analysed. 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 (Figure 3B). AdTIMP-1- and AdTIMP-3-transduced hEMVEC inhibited exogenous active MMP-9 comparably (Figure 3C).

Previous studies on HUVEC and hFMVEC have shown that TIMP-3 was a more potent inhibitor of capillary-tube formation than TIMP-1 [Lafleur *et al.*, 2002; Collen *et al.*, 2003; Anand-Apte *et al.*, 1997]. Unexpectedly, in hEMVEC both TIMP-1 and TIMP-3 overexpression inhibited VEGF-A-induced tube formation, to an extent similar as BB94 (Figure 4A and B). This was found both in fibrin and in fibrin-collagen matrices (Figure 4B). No apparent cell death or morphological changes were observed either in the AdTIMP-1- or AdTIMP-3-transduced hEMVEC.

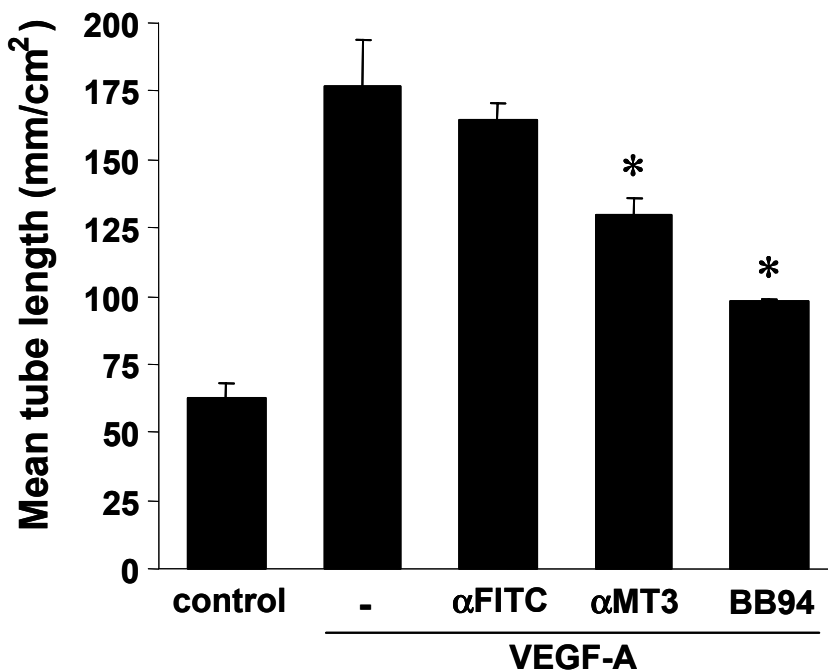


Figure 6. Inhibition of MT3-MMP reduces tube formation by hEMVEC.

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 (αMT3; 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 of 2 experiments performed in duplicate wells. * $P < 0.02$ vs VEGF stimulated hEMVEC; $P < 0.05$ vs VEGF /anti-FITC IgG treated 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/TNF α -stimulated hFMVEC [Collen *et al.*, 2003], we compared the effects of TIMP-1 and TIMP-3 overexpression on capillary-like tube formation by hEMVEC and hFMVEC under identical culture conditions. Both cells types were grown on a fibrin-10% collagen matrix and stimulated by the simultaneous addition of VEGF and TNF α , which is required to induce tubules by hFMVEC [Koolwijk *et al.*, 1996]. Like in VEGF-stimulated hEMVEC, both TIMP-1 and TIMP-3 reduced capillary-like tube formation in VEGF/TNF α -stimulated hEMVEC to the same extent as BB94 (Figure 5, striped bars). In contrast, only TIMP-3 inhibited tube formation by hFMVEC to a significant extent. Similar data were obtained with fibrin matrices (Figure 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/TNF α or TNF α 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 other MMPs 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 (Figure 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 in-

volved in the regulation of tube formation by hEMVEC, because tube formation by hEMVEC was inhibited by anti-MT3-MMP IgG *in vitro*, and MT3-MMP was encountered in endothelial cells of proliferative endometrium *in vivo*.

Our data on the expression of MMPs by hEMVEC *in vitro* are in agreement with observations reported from immunohistochemical studies in endometrial tissue sections. Freitas *et al* found MMP-1, MMP-2, MMP-3 and MMP-9 in endometrial vascular structures, which might include endothelial cells, and MMP-2 was demonstrated in newly formed capillary strands [Freitas *et al.*, 1999]. Skinner *et al* only found MMP-9 on endometrial endothelial cells after exposure to high progestagen levels [Skinner *et al.*, 1999]. MT1-MMP was detected at low levels on endothelial cells in proliferative and secretory endometrium [Maatta *et al.*, 2000; Zhang *et al.*, 2000]. MT2-MMP was observed at a constant low level throughout the menstrual cycle [Goffin *et al.*, 2003; Zhang *et al.*, 2000]. In addition, TIMP-1, -2, and -3 were demonstrated in endometrial endothelial cells by *in situ* hybridization [Chegini *et al.*, 2003; Maatta *et al.*, 2000; Rodgers *et al.*, 1994]. 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 [Goffin *et al.*, 2003]. 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 localisation that enables them to regulate localised 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 [Hotary *et al.*, 2002]. MT1-MMP and MT3-MMP are involved in the migration and invasion of various mesenchymal cells, such as fibroblasts and smooth muscle cells [Vernon *et al.*, 1995], while other cells, such as leukocytes and trophoblasts, use MT2-MMP [Eeckhout, Vaes 1977; Giavazzi *et al.*, 2000]. 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 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 [Anand-Apte *et al.*, 1997; Collen *et al.*, 2003; Galvez *et al.*, 2001; Hotary *et al.*, 2002; Lafleur *et al.*, 2002]. 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 [Hotary *et al.*, 2002]. 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 [Goffin *et al.*, 2003]. 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 summarise, 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.

Chapter 3

**Membrane-type matrix metalloproteinases (MT-MMPs)
and vascularisation in human endometrium during the
menstrual cycle.**

M. Plaisier
P. Koolwijk
R. Hanemaaijer
R.A. Verwey
R.M.F. van der Weiden

E.K.J. Risse
C. Jungerius
F.M. Helmerhorst
V.W.M. van Hinsbergh

ABSTRACT

Objective: Endometrial angiogenesis is necessary for the formation of a vascularized receptive endometrium. Previously we described that membrane-type-3 metalloproteinase (MT3-MMP) is associated with endometrial capillary-like tube formation *in vitro*. However, the association of MT-MMPs with endometrial angiogenesis *in vivo* is unknown. Therefore, this study analysed the presence of MT-MMPs in human endometrium and their correlation with endometrial neovascularisation.

Materials & Methods: Expressions of the six MT-MMPs were determined in cultured human endometrial endothelial, epithelial and stromal cells by immunohistochemistry and RT-PCR. Vascularisation parameters and MT-MMP expressions *in vivo* were evaluated by immunohistochemistry in serial sections from human endometrium samples.

Results: Mainly MT1-, MT2-, MT3- and MT4-MMP antigens were expressed in cultured endometrial endothelial cells. All MT-MMP antigens were expressed in various cell types of the endometrium, with decreased expression levels during the early secretory phase. Only MT2-, MT3- and MT4-MMP antigens were expressed by endothelial cells during the early-late proliferative and mid-late secretory phase. Strikingly, these phases also showed elevated vascularisation parameters; elevated total vascular surface in proliferative phases, elevated number of vessels in proliferative and late secretory phases and increased luminal surface in the proliferative phases.

Conclusions: All MT-MMP antigens are expressed in endometrium in a cycle-dependent pattern. The vascular expression of MT2- MT3- and MT4-MMP correlated with angiogenic episodes of the cycle. Since MT2- and MT3-MMP are known to regulate capillary-like tube formation, these findings support earlier *in vitro* data on the regulatory role of MT3-MMP in endometrial angiogenesis. Additionally, MT2-MMP appears to be associated with endometrial neovascularisation as well.

INTRODUCTION

Angiogenesis, the sprouting of new capillaries from existing vasculature, is essential to support endometrial reconstruction after menstruation and to provide a vascularized, receptive endometrium for implantation. Endometrial angiogenesis occurs periodically at three separate episodes during each cycle; during the early proliferative phase representing post-menstrual repair, during the mid-proliferative phase under the influence of oestrogen and during the secretory phase under the influence of mainly progesterone [Ferency *et al.*, 1979; Goodger, Rogers, 1995; Rogers, Gargett, 1998; Smith, 2001]. Most work to date concerning the regulation of endometrial angiogenesis has focused on steroidal hormones and vascular endothelial growth factor (VEGF). The steroidal hormones regulate endometrial growth and probably also endometrial angiogenesis either directly or indirectly through locally produced factors like VEGF and proteases [Bausero *et al.*, 1998; Iruela-Arispe *et al.*, 1999; Perrot-applanat *et al.*, 2000; Shifren *et al.*, 1996].

Angiogenesis is facilitated by proteolysis, since endothelial cells require proteolytic activity to be able to degrade their basal membrane, to migrate, and to invade the underlying extra-cellular matrix [Heymans *et al.*, 1999; Pepper, 2001a; Stetler-Stevenson, 1999]. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs) and in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs). Their membrane-associated localisation makes them particularly suited to functioning in pericellular proteolysis [Hotary *et al.*, 2000; Visse, Nagase 2003]. MT1-, MT2-, MT3- and MT5-MMPs are trans-membrane proteins, whereas MT4- and MT6-MMP are GPI-anchored. MT-MMPs are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs); TIMP-1 inhibits all MT-MMPs, except MT1-MMP, while TIMP-2 and -3 inhibit all MT-MMPs [Hernandez-Barrantes *et al.*, 2002; Visse, Nagase 2003]. MT1-MMP is also inhibited by TIMP-4 [Bigg *et al.*, 2001].

Previous reports showed that MT-MMPs play an important role in angiogenesis [Hotary *et al.*, 2000/2002; Zhou *et al.*, 2000]. MT1-MMP has received considerable attention as being involved in tumour angiogenesis [Seiki, Yana, 2003; Sounni *et al.*, 2002]. Furthermore, MT1-, MT2- and MT3-MMP have been shown to be able to induce capillary-like tube formation *in vitro* [Collen *et al.*, 2003; Galvez *et al.*, 2001; Hiraoka *et al.*, 1998; Hotary *et al.*, 2000/2002; Lafleur *et al.*, 2002]. Capillary formation by human foreskin microvascular endothelial cells is mediated by MT1-MMP [Collen *et al.*, 2003; Plaisier *et al.*, 2004]. However, we recently showed that capillary-like tube formation by human endometrial microvascular endothelial cells (hEMVEC) is mediated by MT3-MMP, suggesting that MT3-MMP may be a potential regulator of endometrial angiogenesis [Plaisier *et al.*, 2004].

Whether MT-MMPs are associated with endothelial cells during endometrial angiogenesis *in vivo* is not clear. Furthermore, also the role of MT-MMPs in endometrial receptivity and remodelling is still indistinct. Several MT-MMPs have been demonstrated in whole endometrial extracts at mRNA level [Chung *et al.*, 2002; Goffin *et al.*, 2003] and MT1- and MT2-MMP antigens have been demonstrated in various endometrial cell types [Määttä *et al.*, 2000; Zhang *et al.*, 2000]. However, little information is available regarding the expression of the six MT-MMPs in endothelium and their relationship to endometrial angiogenesis. Therefore this study aims to analyse the expression of MT-MMPs by endometrial cells, including endothelium, to assess their endometrial expression during the menstrual cycle, and to correlate these findings to the angiogenic status of the cycling endometrium.

MATERIALS AND METHODS

Collection of human endometrium samples

Endometrial samples were collected from 26 pre-menopausal and 5 post-menopausal women undergoing hysterectomy for benign gynecological pathologies (leiomyoma (n=23), uterine prolapse (n=1), dysmenorrhoea (n=1), menorrhagia (n=2)). Tissue was collected according to the guidelines of the Institutional Review Board and informed consent was obtained from each patient. None of the subjects had received steroid hormone therapy, i.e. OAC, IUD or HRT, for at least three months prior to surgery. Total donors with an irregular cycle n=4; n=1 in EP, n=1 in LP, n=1 in ES, n=1 in LS. Endometrial tissue (0.5-1.0 grams) was scraped off the *fundus uteri*, using a surgical knife, immediately after surgery. When leiomyoma was present, tissue was obtained from the opposite site of the uterus. Tissue was either stored at 4°C or fixed in formaldehyde.

All pre-menopausal samples were dated by an independent pathologist using the endometrial dating criteria of Noyes *et al* [Noyes *et al.*, 1975]. The histological appearance of the endometrium was classified as menstrual (day 28-3; M; n=2), early proliferative (day 4-10; EP; n=5), late proliferative (day 11-14; LP; n=7), early secretory (day 15-20; ES; n=6), and late secretory (day 21-28; LS; n=6) phase. Samples were excluded when containing endometritis, leiomyoma, adenomyosis or when dating was inconsistent with known last day of the menstrual cycle. The mean age of the pre-menopausal group was 42.4 years (range 31-48) and of the post-menopausal group 59.7 years (range 57-63). Age, pre-operative diagnosis and menstrual irregularity were equally distributed among the groups M, EP, LP, ES, and LS.

Cell isolation and culture

Human endometrial microvascular endothelial cells (hEMVEC), epithelial (hEEC) and stromal (hESC) were isolated from pre-menopausal endometrium and cultured as previously described [Koolwijk *et al.*, 2001]. Cells were extracted from minced tissue using 0.2% collagenase and further separated using gravity sedimentation as follows. After setting for 5 minutes, the supernatant was separated from the epithelium-rich settled pellet. The heterogeneous supernatant, containing mainly stromal and endothelial cells, was further separated into hEMVEC and hESC by repeated selections with anti-CD31-coated Dynabeads (DynaL Biotech, Oslo, Norway). The pellet was resuspended in M199 with 10% new born calf serum (NBCS) and placed in culture dishes. During the following 15 minutes, remaining stromal and endothelial cells attached to the dish, while epithelial structures remained in the media. By transferring the media and repeating this, an epithelial cell culture was derived. Cells were characterised by morphology and immunohistochemical markers; hEMVEC were positive for CD31 and von Willebrand factor and negative for cytokeratin-8 and -18. hEEC were positive for cytokeratin-8 and -18, and hESC were negative for these markers.

hEMVEC were cultured in M199 with 20% human serum (HS), 10% NBCS, 37.5 µg/ml endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin and 100 mg/ml streptomycin. hESC and hEEC were cultured in M199 with 10% HS, 10% NBCS, 75 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% CO₂/95% air atmosphere. Human recombinant VEGF-A (5 ng/ml, RELIatech, Germany) was added to the culture of primary isolates of endothelial cells to facilitate initial growth.

Reverse transcription and real-time quantitative RT-PCR

Total RNA from cultured cells was isolated as described previously [Chomzynski, Sacchi 1987]. RNA quantity and quality were determined in a spectrophotometer (Nanodrop ND-1000, OD₂₆₀/OD₂₈₀ between 1.8 and 2.0) Reverse transcription was performed with 1 µg total RNA, random primers and a cDNA synthesis kit (Promega Corporations, Madison, USA).

RNA expression was quantified according to the Taqman real-time PCR method (Applied Biosystems, Perkin Elmer, Wellesley, USA) using primers combined with a specific (FAM/TAMRA) double-labelled probe. Sequences of the primers and probes used to detect MT-MMPs were described previously [Collen *et al.*, 2003; Plaisier *et al.*, 2004]. The endogenous reference gene GAPDH, (primers/VIC-labelled probe, Applied Biosystems, Foster City, USA) was used to correct for the total mRNA content of the samples. Other genes, β-actin and cyclophilin, were also used as reference genes and showed comparable results (data not shown). The primer efficiencies were verified by determining a standard curve using serial dilutions of hESC cDNA. Primers were considered efficient

when the calculated efficiency ranged between 90%-110%. RT-PCR reactions for MT-MMP/GAPDH pairs were performed in duplicate and expressed in cycle times (Ct), the number of cycles required for the PCR reaction to enter the logarithmic phase. ΔC_t 's were calculated as the difference in Ct of the target gene and the reference gene GAPDH. Water and "-RT samples", obtained by the omission of the RT-enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry on cultured cells

Cultured cells were fixed for 10 minutes with cold 2% para-formaldehyde followed by 1 minute with cold acetone. After three washes with PBS, the cells were incubated for 15 minutes with 5% human serum and 5% goat serum in PBS to reduce aspecific binding. Subsequently, the cells were incubated with a first antibody overnight at 4°C. The following commercially available first antibodies were used: monoclonal mouse anti-MT2-MMP, monoclonal mouse anti-MT3-MMP (162-22G5 and 117-10C6, Oncogene, San Diego, USA), polyclonal rabbit anti-MT4-MMP, polyclonal rabbit anti-MT5-MMP (M3684 and M6684, Sigma, St Louis, USA) and polyclonal rabbit anti-von Willebrand Factor (A0082, Dakocytomation, Glostrup, Denmark). Anti-MT1-MMP antibodies were produced and characterised as previously described [Collen *et al.*, 2003; Koolwijk *et al.*, 1996; Plaisier *et al.*, 2004; Van Boheemen *et al.*, 1995] and polyclonal goat anti-MT6-MMP was a generous gift from W. English (Cambridge, UK). After three washes with PBS, the cells were incubated with a FITC-labelled secondary antibody for 1 hour at room temperature and finally washed three times with PBS. The DAPI (Vector laboratories, Burlingame, USA) was added and cells were mounted and evaluated using a Zeiss Axiovert 200 M inverted microscope. To detect lysosomes, a acidic organelle-selective probe was used, which accumulates in lysosomes and is linked to a fluorescence label. This Lysotracker Red DND-991 μ M (L-7528, Molecular Probes, Invitrogen, Breda, Netherlands) was added to unfixed cultured cells and visualised.

Immunohistochemistry on paraffin sections

Tissue samples were processed into paraffin sections as previously described [Kroon *et al.*, 1999]. Besides the above described first antibodies, monoclonal mouse CD34 (QBenD/10, Novocastra, Newcastle upon Tyne, UK) was also used on the tissue samples. Endogenous peroxidase was quenched with 3% H_2O_2 /methanol and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in trypsin was used for the detection of MT2- and MT4-MMP. Primary antibodies were applied overnight at 4°C followed by incubation with a biotinylated secondary antibody. Antibody binding was visualised using avidin-biotin and streptavidin-horseradish peroxidase conjugates. Sections were counterstained with Mayer's hematoxylin. Specificity of the

reaction was verified by the omission of the first antibody or using mouse, goat or rabbit serum as first antibody.

Evaluation of the vascularisation pattern

CD34-stained sections were used to scan six fields, randomly taken throughout the stained sections, at x100 magnification. CD34-positive vessels were counted and their luminal surface was measured using image analysis software (Qwin) developed by Leica Microsystems. The total vascular surface (mm^2/mm^2), the number of vessels per mm^2 and the luminal surface ($\mu\text{m}^2/\text{vessel}$) were calculated using the same software.

Evaluation of immunohistochemical staining

Immunostaining in all samples was evaluated, at 100x magnification, by calculating a staining index according to the following equation: proportion of stained cells x staining intensity. The proportion of stained cells was expressed as 0, 1, 2 or 3 (positive staining signal in 0%, <10%, 10-50% or >50% of cells, respectively) and intensity of staining as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score was 9 [Nap *et al.*, 2004]. The average of the scores of two independent observers was used to calculate the mean staining index, which represents the protein expression per cell type, namely glandular epithelial cells, stromal cells, endothelial cells and perivascular smooth muscle cells (PSMCs). The inter-observer variability was 4% for MT1-MMP, 14 % for MT2-MMP, 8 % for MT3-MMP, 10 % for MT4-MMP, 9 % for MT5-MMP and 5 % for MT6-MMP (mean variability 8%). Total mean staining indices (Total SI) were calculated as the sum of mean staining indices in each tissue sample and represent the total protein expression per tissue.

Statistical analysis

The one-way analysis of variance (ANOVA) and the Bonferroni post hoc test were used to examine the MT-MMP expression levels in endometrial cells *in vitro*. The Kruskal-Wallis test, a non-parametrical test for more than two independent samples, was used to examine the differences between the stages of the menstrual cycle. Results were considered statistically significant when $p < 0.05$ (SPSS, version 11.5).

RESULTS

Vascularisation pattern in endometrium during the cycle

Analysis of the endometrial vascularisation parameters, using CD34-stained paraffin sections (Fig. 1A), showed that the total vascular surface was elevated in the late proliferative phase, $0.05 \text{ mm}^2/\text{mm}^2$, compared to the other phases ($p < 0.01$, Fig. 1B). Vessel

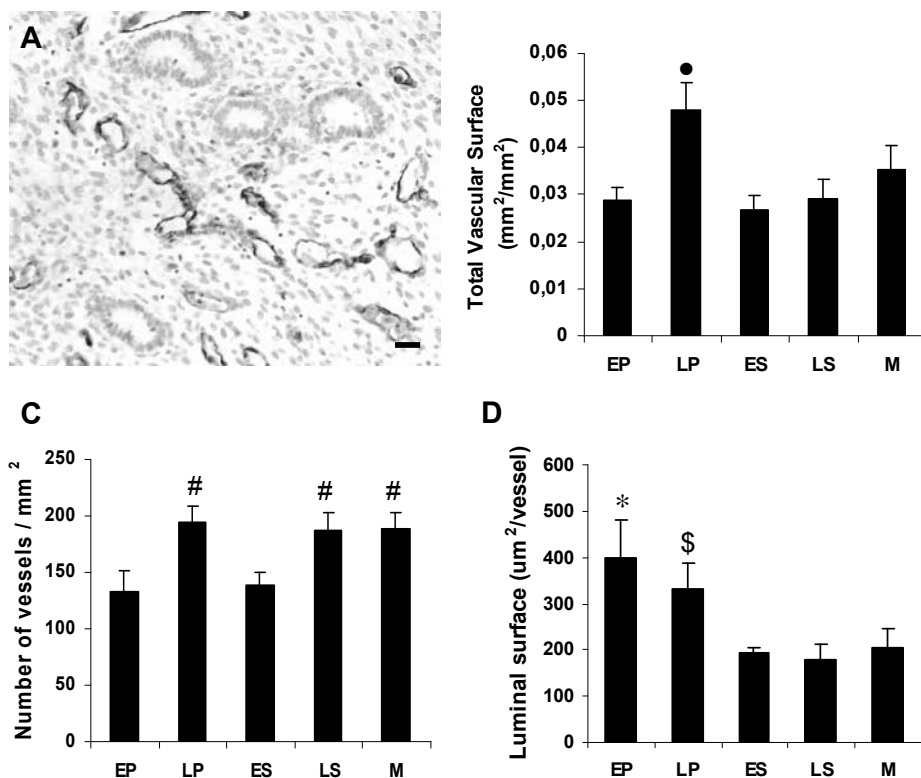


Figure 1. Vascularisation pattern in cycling human endometrium.

The vascularisation pattern in human endometrium was determined by image analysis of anti-CD34-stained sections. Six fields per biopsy were scanned at 100x magnification. **A** CD34 antigen expression in endothelial cells in late proliferative endometrium (bar = 50 µm). **B** The total vascular surface (mm²/mm²), **C** the number of vessels per mm² and **D** the luminal surface (µm²/vessel) were calculated for each sample and expressed as mean ± SEM. EP = Early proliferative phase (n=6), LP = Late proliferative phase (n=7), ES = Early secretory phase (n=6), LS = Late secretory phase (n=6), M = menstrual phase (n=2); • p<0.01 versus EP, ES and LS; # p<0.02 versus EP, ES; * p<0.01 versus ES, LS and M; and \$ p<0.05 versus ES, LS and M. (see colour figures supplement)

density ranged between 132 and 195 vessels per mm² (mean 168/mm²) during the cycle. LP, LS and M endometrium showed an increased number of vessels compared to EP and ES (p<0.02, Fig. 1C). Finally, the luminal surface was raised in the first half of the cycle, 331-398 µm²/vessel, compared to the second half, 178-193 µm²/vessel (p<0.05, Fig. 1D).

MT-MMP expression in endometrial cells in vitro

To screen which MT-MMPs are expressed by endometrial cells, we first determined the expression of the MT-MMPs by human endometrial microvascular endothelial (hEM-VEC), stromal (hESC) and epithelial (hEEC) cells in vitro using RT-PCR. hEMVEC mainly

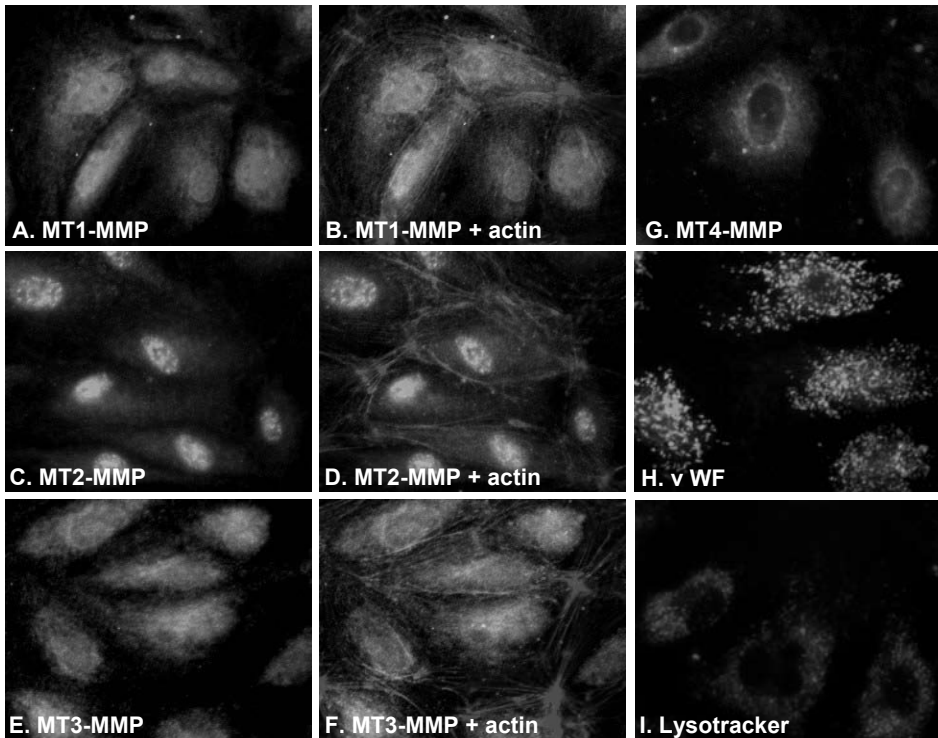


Figure 2. MT-MMP antigen expressions in hEMVEC.

Endothelial MT-MMP expression was determined by fluorescence microscopy (magnification 40x). The expression of MT1-MMP ($\pm$ actin **A, B**), MT2-MMP ($\pm$ actin **C, D**), MT3-MMP ($\pm$ actin **E, F**), MT4-MMP (**G**), von Willebrand Factor in Weibel Palade bodies (**H**) and lysotracker in lysosomes (**I**) in cultured endometrial endothelial cells (hEMVEC). The negative control only showed blue staining of the nuclei (not shown). Blue = DAPI in nuclei, green = target protein, red = actin or lysotracker.

(see colour figures supplement)

expressed MT1-, MT3-, and MT4-MMP mRNAs ($dCt < 9.0$, Table 1), whereas MT2-, MT5- and MT6-MMP mRNA were weakly expressed (Table 1).

Strikingly, MT1-MMP mRNA expression was considerably lower in endothelial cells compared to both stromal and epithelial cells (ΔdCt 6.1 and 6.1, $p < 0.01$). Epithelial cells expressed most MT-MMPs more abundant than the other cell types ($p < 0.01$, Table 1).

At protein level, all MT-MMPs were expressed by hEMVEC, but were rather located in the cell than on the cell membrane (Fig. 2). MT1-, MT2-, MT3- and MT4-MMP were clearly and consistently detectable (Fig. 2 A-G), whereas MT5- and MT6-MMP antigens were only weakly present (not shown). The MT-MMPs showed a perinuclear staining (compare its localisation with the cortical F-actin of the cells in Figs 2B, D and F), often in a granular pattern. This granular pattern was distinct of that of von Willebrand factor in Weibel Palade bodies (Fig 2H), but, in part, resembled the lysosomal compartment as

Table 1. MT-MMP mRNA expression in human endometrial cells *in vitro*.

	hEMVEC		hESC		hEEC	
	Ct	dCt	Ct	dCt	Ct	dCt
MT1-MMP	27.8±0.4	9.0±0.4	22.8±1.5	2.9±1.5*	22.7±0.5	2.9±0.1*
MT2-MMP	33.7±1.2	14.1±1.2	35.6±1.8	15.6±0.6	29.0±0.3	9.2±0.3*
MT3-MMP	26.4±0.2	7.4±0.4	30.5±0.4	10.6±1.2*	25.0±0.6	6.3±0.4
MT4-MMP	26.9±0.2	7.3±0.3	28.3±1.1	9.0±0.4*	29.8±0.1	10.1±0.1*
MT5-MMP	31.6±0.3	12.8±0.3	33.5±1.4	14.6±0.5*	27.7±0.6	7.9±0.4*
MT6-MMP	36.3±1.0	17.7±0.8	35.2±1.6	14.4±2.5	32.4±0.6	12.2±0.7

Table 1. RNA was isolated from cultured hEMVEC (3 donors), hESC (5 donors) and hEEC (3 donors) and cDNA was synthesised as described. Donors were pre-menopausal and of proliferative phases. RT-PCR for MT-MMP/GAPDH pairs were performed in duplicate and expressed as the number of cycles (Ct ± SEM). GAPDH was used to correct for the total mRNA content of the samples (mean GAPDH Ct 19.3 ± 0.3). The dCt values were calculated as the difference in the number of cycles required for the PCR reaction of the target gene and of the reference gene to enter the logarithmic phase and expressed as dCt ± SEM.

* $p < 0.01$ versus hEMVEC.

visualised by lysotracker, an acidic-organelle-selective fluorescent probe (Fig. 2I). Unexpectedly, MT2-MMP antigen was also encountered in the nuclei.

MT-MMP antigen expressions in cycling human endometrium

Subsequently, we investigated the presence of the six MT-MMP antigens in cycling endometrium by immunohistochemistry in serial paraffin sections of the samples that were also used for the evaluation of the vascularisation pattern.

MT-MMP antigen expressions in vascular cells

Endometrial endothelium exhibited weak expression of MT-MMP antigens with a maximal staining index of 2.2 (Fig. 3A, Table 2). MT2-MMP was most abundantly expressed by endothelium during the early proliferative, late proliferative and late secretory phase, in a similar way to MT4-MMP ($p > 0.05$, Fig. 3A, C and E). Vascular MT3-MMP antigens were only detected during the early and late proliferative phase (Fig. 3A and D). MT5- and MT6-MMP antigens were not detected in endothelium. Surprisingly, MT1-MMP was also not detected in endothelium, while it was clearly present in other cell types (Fig. 3B). MT1-MMP was also clearly present in cervix carcinoma endothelium in a parallel experiment (data not shown).

Perivascular smooth muscle cells (PSMCs) expressed all MT-MMPs abundantly throughout the cycle, with a mean staining index of 4.2 (range 1.0-7.9, Table 2, Fig. 3B).

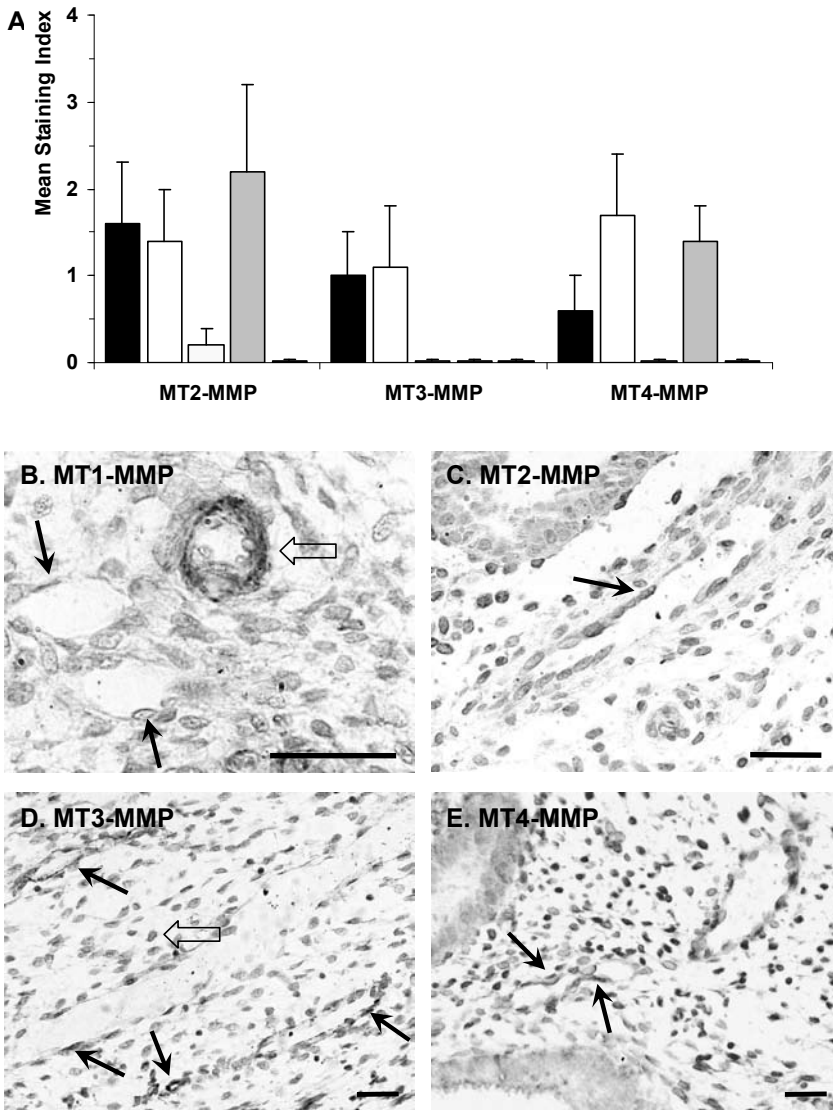


Figure 3. MT-MMP antigen expressions in endometrial endothelium and other cell types.

The expression of MT-MMP antigens by endometrial endothelial cells was determined by immunohistochemistry. **A.** MT-MMP antigen expressions in endothelial cells expressed per cycle phase as mean staining index $\pm$ SEM (all $p > 0.05$). The staining index is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). **B.** MT1-MMP antigen detected in PSMCs (open arrow) but not in endothelial cells (arrows) in late proliferative (LP) endometrium. **C.** MT2-MMP antigen expression in endothelium in LP endometrium (arrow). **D.** MT3-MMP expression in endothelial cells (arrows) and stromal cells (open arrow) in LP endometrium. **E.** MT4-MMP expression in endothelial cells (arrow) in LP endometrium. **F.** Negative control. Bar = 50 µm.

■ = Early proliferative phase (n=6), □ = Late proliferative phase (n=7), □ = Early secretory phase (n=6), □ = Late secretory phase (n=6), ▨ = menstrual phase (n=6).

(see colour figures supplement)

Table 2. MT-MMP antigen expression during the menstrual cycle.						
		EC	PSMC	GE	SC	Total SI
MT1-MMP	EP	0.0	4.1	5.2	0.6	9.9
	LP	0.0	5.0	5.0	1.3	11.3
	ES	0.0	4.2	4.8	2.2	11.2
	LS	0.0	5.6	4.0	1.8	11.4
	M	0.0	4.5	4.5	0.0	9.0
MT2-MMP	EP	1.6	7.0	4.8	5.2	18.6
	LP	1.6	5.7	7.3	4.9	19.6
	ES	0.2	5.8	4.4	3.6	14.0 [*]
	LS	2.2	6.2	5.0	5.8	19.2
	M	0.0	2.0	1.5	1.5	5.0
MT3-MMP	EP	1.0	5.3	4.4	2.2	12.9
	LP	1.0	4.6	3.6	1.9	11.0
	ES	0.0	3.0	1.3	1.3	5.5 [#]
	LS	0.0	4.0	3.4	1.8	9.2
	M	0.0	3.0	5.0	0.0	8.0
MT4-MMP	EP	0.6	5.0	3.6	1.8	11.0
	LP	1.7	4.9	5.3	3.7	15.6
	ES	0.0	5.0	5.3	1.3	11.5 [*]
	LS	1.4	7.8	4.2	5.4	18.8
	M	0.0	2.5	1.5	1.5	5.5
MT5-MMP	EP	0.0	1.0	1.8	0.2	3.0
	LP	0.0	4.7	3.1	0.7	8.6
	ES	0.0	3.0	3.5	0.3	6.8
	LS	0.0	3.6	3.0	0.8	7.4
	M	0.0	4.0	3.5	1.5	9.0
MT6-MMP	EP	0.0	3.0	2.0	0.5	5.5
	LP	0.0	3.9	3.9	1.0	8.7
	ES	0.0	3.0	3.0	0.8	6.8 ^{\$}
	LS	0.0	5.0	3.0	2.3	10.3
	M	0.0	0.0	3.0	0.0	3.0

Table 2. The MT-MMP antigen expressions are expressed as the mean staining indices per cell type per phase, which is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). The total staining indices (total SI) per phase is calculated as the sum of the mean staining indices of endothelial cells (=EC), perivascular smooth muscle cells (=PSMC), glandular epithelium (=GE) and stromal cells (=SC). EP = early proliferative (n=6), LP = late proliferative (n=7), ES = early secretory (n=6), LS = late secretory (n=6), M = menstruation (n=2), * = p<0.05 versus LP and LS, # = p<0.05 versus EP and LP, \$ = p<0.05 versus LS.

MT-MMP antigen expressions in endometrial cells other than vascular cells

All MT-MMPs were expressed by epithelial and to a lesser extent by stromal cells (Table 2). Both cell types expressed higher amounts of MT₁-, MT₂-, MT₃- and MT₄-MMP than of MT₅ and MT₆-MMP. All MT-MMPs were highly expressed in smooth muscle cells of the myometrium (data not shown).

MT-MMP regulation during the menstrual cycle and the post-menopause*MT-MMP antigen expressions during the menstrual cycle*

First, total MT-MMP antigen expressions in proliferative phase were compared to those in early secretory phase, showing that MT₂-, MT₃-, and MT₄-MMP expressions were markedly lower in ES endometrium (total SI differences 5.3, 8.8 and 4.3 respectively, $p < 0.05$, Table 2). The expression of MT₁-, MT₅- and MT₆-MMP only slightly decreased in the ES phase compared to the proliferative phases (Table 2).

Subsequently, early secretory phase were compared to the late secretory phase, demonstrating that total MT₂-, MT₃-, MT₄-, and MT₆-MMP expressions were elevated during the LS phase compared to the ES phases (MT₂-, MT₄- and MT₆-MMP $p < 0.05$, MT₃-MMP $p > 0.05$, Table 2). The total staining index of MT₁- and MT₅-MMP remained rather constant during the cycle phases (Table 2).

All MT-MMPs were expressed in considerable amounts during the menstrual phase. Unfortunately only two biopsies of menstrual phase endometrium were available and therefore the staining patterns can only be indicative.

MT-MMP antigen expressions in post-menopausal endometrium

Post-menopausal endometrial biopsies showed less abundant amounts of MT-MMP antigens as compared to the MT-MMP expressions in pre-menopausal endometrium. Nevertheless, all MT-MMPs, except MT₅-MMP, were present in postmenopausal endometrium. In post-menopausal tissues the total staining index (total SI) of MT₁-MMP was 4.3, that of MT₂-MMP was 4.5, that of MT₃-MMP 3.3, that of MT₄-MMP 3.0, that of MT₅-MMP 0.0, and that of MT₆-MMP was 1.0. Post-menopausal MT-MMPs were mainly expressed by epithelial cells and not by endothelium or PSMCs

DISCUSSION

The present study showed that all MT-MMPs are expressed in human endometrium, of which most expressions show a cycle-dependent pattern. Furthermore, vascular MT₂-, MT₃- and MT₄-MMP expression correlates with angiogenic episodes of the menstrual cycle. Since MT₂- and MT₃-MMP are known stimulators of angiogenesis in vitro [Hotary

et al., 2000; Plaisier *et al.*, 2004], these findings suggest that MT2- and MT3-MMPs are associated with endometrial angiogenesis.

MT-MMPs act at the cell surface where they can locally facilitate degradation of extra-cellular matrix, cell migration, invasion and angiogenesis. The abundance of all MT-MMP in cycling endometrium and the relatively weak MT-MMP expressions in the postmenopausal endometrium suggest that endometrial MT-MMPs play a role in remodelling of cycling endometrium.

A possible function of MT-MMPs may be associated with the shedding process, since their antigens were highly expressed during the late secretory and menstrual phase and other MMPs have been shown to be associated with menstruation as well [Lockwood *et al.*, 1998; Salamonsen, Woolley 1996; Zhang *et al.*, 2000]. Alternatively MT-MMPs may play a role in endometrial remodelling in preparation for implantation. Most MT-MMP expressions were reduced during the receptive window, probably under the influence of steroidal hormones. The low amount of MT-MMPs during the generation of receptive endometrium may be essential for the establishment of a stable environment in preparation for embryonic implantation. However, whether MT-MMPs play a role during implantation remains yet unresolved and this should be assessed in first-trimester decidua.

Another function of MT-MMPs may be the regulation of angiogenesis. Assuming the participation of MT-MMPs in angiogenesis, their antigens must be expressed by endothelium during phases of the menstrual cycle with high angiogenic activity. Therefore the present study did not only analyse the expression of MT-MMPs in cultured endometrial endothelial cells and in endometrial tissue samples but also determined their association with vascularisation in these samples.

MT2-, MT3-, and MT4-MMP were expressed by both *in vitro* and *in vivo* endothelial cells. Endometrial endothelium *in vivo* expressed MT3-MMP during the proliferative phase, whereas MT2- and MT4-MMP were expressed during both proliferative and late secretory phases. Strikingly, the endothelial MT-MMP expressions were expressed during phases with high angiogenic activity. The proliferative, and to a lesser extent the late secretory phase, showed an elevated endometrial angiogenesis, since respectively three and one vascularisation parameter(s) were raised in these phases. Previous studies have described these angiogenic episodes as well [Ferenczy *et al.*, 1979; Goodger, Rogers 1995; Rogers, Gargett 1998; Smith 2001; Morgan *et al.*, 1996].

MT1-MMP is expressed by endothelium *in vitro* whereas its endothelial expression *in vivo* was consistently not detectable. This difference may be explained by translational regulation or culture-induced changes in *in vitro* cell metabolism. MT1-MMP expression is regulated by several signal transduction pathways, including the MAPK and PI3K pathways, which are influenced by cytokines and growth factors [Boyd *et al.*, 2004; Chakraborti *et*

al., 2003; Galvez *et al.*, 2005]. Since human serum, ECGF (containing acidic FGF) and VEGF were used in the culture system, signalling pathways could have been activated and influenced MT1-MMP expression by endometrial cells [Plaisier *et al.*, 2004].

The endothelial expression of MT2-MMP in both phases and the consistent absence of endothelial MT1-MMP is in accordance with previous work by Zhang *et al* [Zhang *et al.*, 2000]. They showed that MT1-MMP antigens were present in all endometrial cell types, but were 'virtually absent' in endothelial cells. Määttä *et al* detected weak MT1-MMP mRNA expression in endothelium, but did not study MT1-MMP protein expression [Määttä *et al.*, 2000]. No data regarding the cellular expression of MT3- and MT4-MMP have been reported.

Although MT1-MMP is generally accepted to contribute to angiogenesis, MT2- and MT3-MMP are also described as stimulators of angiogenesis *in vitro* [Galvez *et al.*, 2001; Hotary *et al.*, 2000/2002; Lafleur *et al.*, 2002; Zhou *et al.*, 2000]. MT4-MMP is not able to induce capillary tube formation in 3D-matrices *in vitro* [Hotary *et al.*, 2002]. In a recent *in vitro*-study regarding endometrial angiogenesis, we showed that capillary-like tube formation by endometrial endothelial cells is not predominantly regulated by MT1-MMP but rather by MT3-MMP [Plaisier *et al.*, 2004]. These *in vitro* findings are supported by the absence of endothelial MT1-MMP *in vivo* and the presence of MT3-MMP antigens in endothelium *in vivo* during the angiogenic proliferative phase. Moreover, the detection of endothelial MT2-MMP expression during the angiogenic proliferative and late secretory phase suggests MT2-MMP to be an additional regulator of angiogenesis to consider [Hiraoka *et al.*, 1998; Hotary *et al.*, 2000; Lafleur *et al.*, 2002]. Future research should clarify their involvement in endometrial angiogenesis and the role of MT4-MMP in endothelium.

A remark should be made about the analysed endometrium samples, which were mainly obtained from women diagnosed with leiomyoma. Although the samples were obtained distant of the lesions and were microscopically "normal", it is noteworthy that recently a relation between MMPs and myoma has been described. An altered level of MMP-9 was found in uterine cavities of leiomyoma patients [Inagaki *et al.*, 2003a], but whether this, or myoma themselves, can affect MT-MMP production by endothelial or other endometrial cells, remains unclear.

The present study showed that all MT-MMPs were expressed at protein level in human endometrium in a cycle-dependent pattern, with decreased expression levels in hormone-dependent secretory phases. Furthermore, the expression of MT2- MT3- and MT4-MMP antigens by endometrial endothelium correlated with angiogenic episodes of the menstrual cycle. Since MT2- and MT3-MMP are known to regulate capillary-like tube formation, these findings support earlier *in vitro* data on the role of MT3-MMP in the

regulation of endometrial angiogenesis. In addition to MT₃-MMP, MT₂-MMP may be associated with vascular remodelling processes in cycling endometrium as well.

Chapter 4



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

ABSTRACT

Objective: Successful implantation and placentation depend on the interaction between the endometrium and the embryo. Angiogenesis is crucial at this time. Here, the direct influence of the human embryo on in vitro endometrial angiogenesis was investigated.

Materials & Methods: Human endometrial microvascular endothelial cells (hEMVEC) were cultured in an in vitro angiogenesis model. Conditioned media (CM) of human embryos and recombinant angiogenic factors were used to stimulate in vitro angiogenesis. VEGF-A was determined by enzyme-linked immunosorbent assay (ELISA).

Results: Conditioned media of human embryos, containing significant amounts of vascular endothelial growth factor (VEGF)-A, caused an increase in hEMVEC tube formation. This effect was prevented by soluble VEGFR-1, which quenches VEGF-A activity. Recombinant EGF alone and leukaemia inhibitory factor in combination with VEGF-A stimulated hEMVEC tube formation. None of the other tested recombinant mediators, which have been described as 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 TGF α), had an effect on tube formation by hEMVEC.

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

INTRODUCTION

Successful implantation and subsequent placentation depend on the interaction between a receptive decidualised 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 decidualised 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 optimises its implantation site [Van der Weiden *et al.*, 1996]. 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 foetal growth restriction [Ahmed, Perkins 2000].

Before the embryo and the endometrium can make physical contact, an interaction by signalling molecules must have been established [Norwitz *et al.*, 2001]. 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 [Armant *et al.*, 2000; Kimber 2000; Krussel *et al.*, 2000a; Salamsen *et al.*, 2000]. 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 $-\beta$, IL-6, IL-10, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) α and β , tumour 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), TGF α and β , TNF α , 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 [Ferrara 2001]. It induces angiogenesis and increases the permeability of blood vessels [Dvorak *et al.*, 1995].

Here, the influence of the embryo on endometrial angiogenesis was investigated 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 characterised [Koolwijk *et al.*, 2001]. 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 Sanguin (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* [Maciag *et al.*, 1979]. Heparin and thrombin were obtained from Leo Pharmaceuticals Products (Weesp, the Netherlands). Human fibrinogen was purchased from Chromogenics (Möln dal, 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). Tumour necrosis factor α was a gift from Dr. J. Traverrier (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 (Bethesda, MD). Recombinant human EGF, human CSF, IFN γ , IGF-I and -II, IL-1 α , -1 β , -6 and -10 and TGF α and - β were commercially obtained from PeproTech (Rocky Hill, NJ). Human chorionic gonadotrophin (Pregnyl) was obtained from Organon (Oss, the Netherlands). Recombi-

nant human LIF was purchased from Chemicon International (Temecula, CA, USA) 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 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, Jansen 1998], 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 [Koolwijk *et al.*, 2001] 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% CO₂ / 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.

Incorporation of ^3H -thymidine

Incorporation of ^3H -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, ^3H -thymidine was added and the cells were incubated for another 6 h period. Subsequently, the ^3H -labeled DNA was precipitated and counted in a liquid scintillation counter and the stimulation index was calculated as previously described [Koolwijk *et al.*, 2001].

In vitro angiogenesis model

Human fibrin matrices were prepared as described previously [Koolwijk *et al.*, 2001]. Confluent hEMVEC (passages 6 to 11 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 analysed by phase contrast microscopy. The total length of the tube-like structures was measured [Kroon *et al.*, 1999].

Enzyme-Linked Immunosorbent Assays (ELISA)

The VEGF-A antigen determinations were performed by the commercially available Duo-Set ELISA Development Kit for human VEGF-A (R&D system, Minneapolis, MN), which recognizes VEGF-A₁₆₅ and VEGF-A₁₂₁. Human recombinant VEGF-A₁₆₅ (R&D systems) was used as a standard.

Statistics

The data are expressed as the mean $\pm$ 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.

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 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 me-

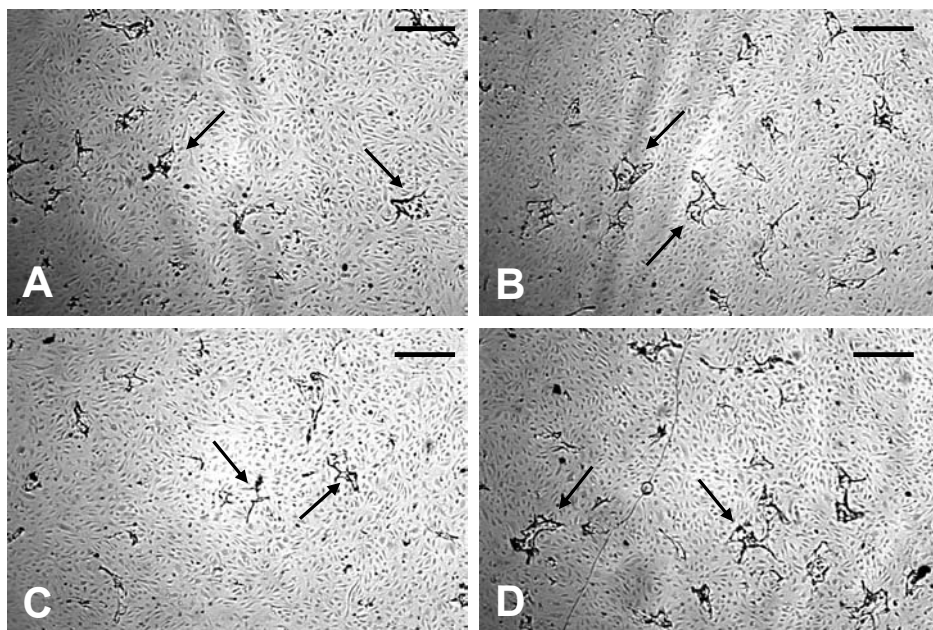


Figure 1. Conditioned medium of early stage embryos stimulate *in vitro* hEMVEC tube formation.

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 μ M.

dium 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 non-conditioned 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

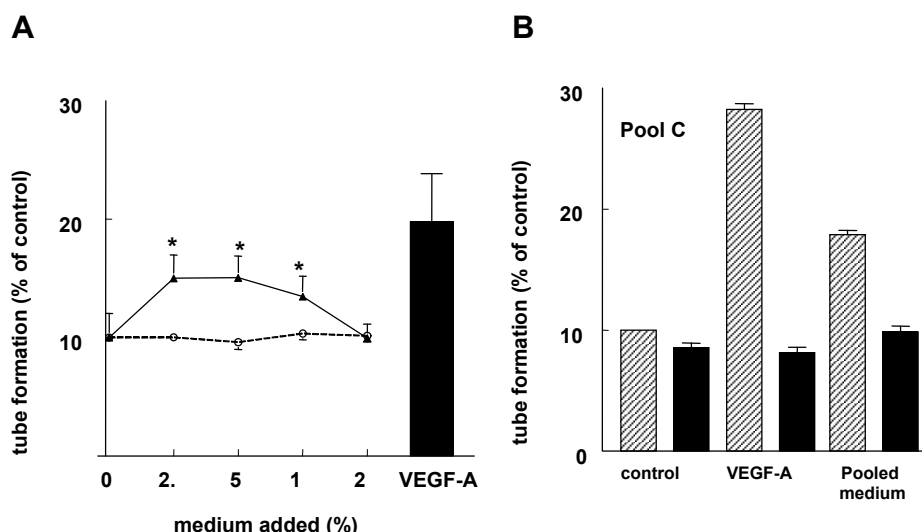


Figure 2. Conditioned medium of embryos stimulate *in vitro* hEMVEC tube formation, an effect which is VEGF-A-mediated.

A. hEMVEC, from passage 5-10 of one donor, were cultured on top of a 3D fibrin matrix and stimulated with 2.5, 5, 10, and 20% (v/v) of pooled medium (pool B,C, or D; $\circ$ 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 ($\circ$ with dotted line) or 10 ng/ml VEGF (solid bars). After 3-5 days of culturing, mean tube length was measured. Data are expressed as a percentage of the control and represent mean $\pm$ 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/cm². **B.** Addition of 0.5 μ g/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 $\pm$ range. Hatched bars: without sVEGFR-1, solid bars: with sVEGFR-1. The mean tube length of the controls was 77.4 mm/cm². The experiments were performed in duplicate wells.

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 ^3H -thymidine incorporation [Koolwijk *et al.*, 2001] 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-1 α and activin (only the highest concentration) significantly inhibited hEMVEC proliferation, whereas all the other tested cytokines (EGF, LIF, CSF-1, IFN γ , IGF-I, IGF-II, IL-1 β , IL-6, IL-10, PDGF, TGF α , TGF β , PLGF and hCG) did not have a significant effect. Only incubation with increasing concentrations of IL-1 α 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 TNF α , at concentrations of 1 and 2.5 ng/mL, but addition of 6.25 ng/mL VEGF-A prevented the TNF α -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.

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-1 α (Fig. 3A) and IL-1 β (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, TGF β , PLGF, hCG, CSF-1, IFN γ , IGF-I/-II, IL-6, PDGF, and TGF α in the indicated concentrations had no effect on tube formation (data not shown).

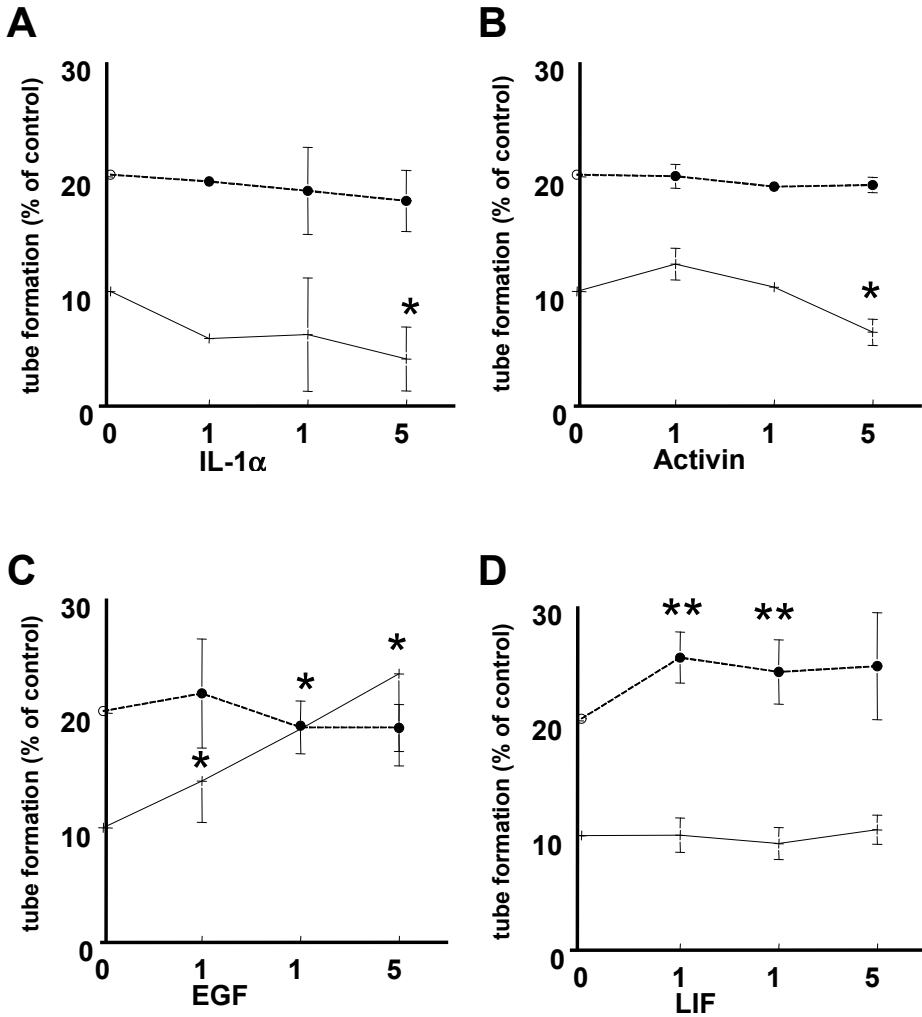


Figure 3. The influence of recombinant cytokines and hCG on the formation of capillary-like structures by HEMVEC.

HEMVEC were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 20% human serum and 10% NBGS 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 $\pm$ range of two independent experiments performed in duplicate wells. * = $p < 0.05$ compared to control condition, ** = $p < 0.05$ compared to VEGF-stimulated condition.

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 [Lindhard *et al.*, 2002; Lopata 1996; Norwitz *et al.*, 2001; Tabibzadeh, Babaknia 1995; Van der Weiden *et al.*, 1991]. Previously, Sakkas *et al.* described that the human blastocyst directly induces changes in endometrial epithelial cells [Sakkes *et al.*, 2003]. 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 [Geva *et al.*, 2002]. 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 [Abberton, Rogers 1995; Goodger, Rogers 1993]. 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.* 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) [Krüssel *et al.*, 2000b/2001]. 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 μ L vs. 10-30 μ L 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.* almost ruled out the possibility of paternal contamination by using embryos, which resulted from intracytoplasmic sperm injection [Krüssel *et al.*, 1999/2001]. They found that unfertilised oocytes did not express VEGF-A mRNA, which proved that the VEGF-A mRNA was truly embryonic [Krüssel *et al.*, 2000b].

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 may contribute to and explain the phenomenon [Hornung *et al.*, 1998].

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 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 extra-embryonic vessels [Carmeliet *et al.*, 1996/1997; Ferrara *et al.*, 1996]. 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. IL1 β , hCG), or its receptors in endometrial cells [Licht *et al.*, 2001; Sunder, Lenton 2000].

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 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 vascularised [Chen *et al.*, 1999; Stewart *et al.*, 1992; Vasse *et al.*, 1999]. 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 or stimulate endothelial cell proliferation and tube formation [Ferrara *et al.*, 1992; Gendron *et al.*, 2000; Hatzi *et al.*, 2002; Pepper *et al.*, 1995; Vasse *et al.*, 1999]. Also on the influence of LIF on the proteolytic potential of endothelial cells, an important phase in angiogenesis, opposite results were found [Pepper *et al.*, 1995; Sunder, Lenton 2000; Vasse *et al.*, 1999]. 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

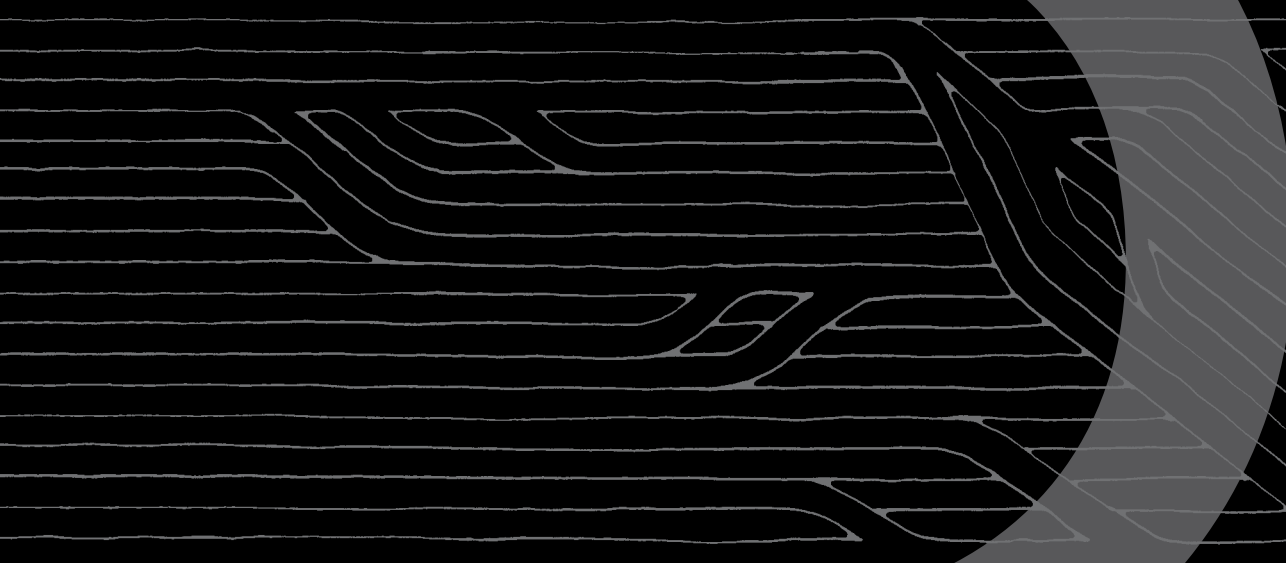
to angiogenic factors at the time of implantation, the embryo might very well cause an increased angiogenic response at the implantation site.

In conclusion, VEGF-A has been recognised as an important mediator in the process of endometrial angiogenesis during the menstrual cycle [Koolwijk *et al.*, 2001; Rogers, Gargett 1998; Smith 1998]. 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 tumour 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.

Chapter 5

Different degrees of vascularisation and their relationship to the expression of VEGF, PlGF, Angiopoietins and their receptors in first-trimester decidual tissues.



M. Plaisier
S. Rodrigues
F. Willems

P. Koolwijk
V.W.M. van Hinsbergh
F.M. Helmerhorst

ABSTRACT

Objective: To evaluate vascular adaptation to implantation by studying vascularisation and angiogenic factors in decidua basalis (DB), decidua parietalis (DP) and decidua secretory endometrium (DSE) of first-trimester pregnancies. Comparing these tissues provides information about the regulation of vascularisation by pregnancy-induced hormones and/or the extra-villous trophoblast (EVT).

Patients & Methods: Decidual samples from vacuum-aspiration were collected from women (n=32) undergoing voluntarily first-trimester termination of pregnancy. Vascularisation, determined by CD34-immunohistochemistry, and VEGF-A, PlGF, Flt-1, KDR, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and Tie-2 protein and mRNA expression, determined by RT-PCR and immunohistochemistry, were evaluated in serial paraffin sections.

Results: Pregnancy-induced hormones and EVT influence vascularisation by enhancing vascular and luminal surface and reducing vessel density at the implantation site. These changes correlate with differences in gene and protein expression. PlGF mRNA and PlGF and Flt-1 protein expressions were elevated in DB under influence of EVT. Also the angiopoietins were differentially expressed, in favor of Ang-2, in DB.

Conclusions: The EVT and pregnancy-induced hormones regulate vascularisation and the expression of angiogenic factors in decidua. The induction of PlGF, Flt-1, and Ang-2/Ang-1 ratio in DB suggests that these factors play a role in regulating angiogenesis at the implantation site.

INTRODUCTION

Vascularised, receptive endometrium is essential for implantation and for the success of the embryo-maternal interaction [Zygmunt *et al.*, 2003]. Disturbances in vascular development may play an important role in frequently occurring pathologies during pregnancy, such as early pregnancy wastage, pre-eclampsia and foetal growth restriction [Vailhe *et al.*, 1999; Vuorela *et al.*, 2000; Zygmunt *et al.*, 2003].

Endometrial adaptation to implantation starts during the menstrual cycle by means of stromal decidualisation and the induction of angiogenesis, the formation of new vessels from existing vasculature. These processes continue after conception under influence of oestradiol, progesterone, hCG, cytokines and growth factors derived from corpus luteum, endometrium and implanting embryo.

Angiogenic changes of the decidua include proliferation, migration and increased vascular permeability [Goodger, Rogers 1993; O'Shea *et al.*, 1983]. After the 6th week of gestation, the arterioles are remodelled from low-capacitance high-resistance to high-capacitance low-resistance vessels, and by the 12th week maternal blood flow to the intervillous space is established [Burton *et al.*, 1999; Pijnenborg *et al.*, 1983].

In general, angiogenesis is a multi-step process [Carmeliet 2003; Ferrara 2000; Plaisier *et al.*, 2004; Smith 2000]. Numerous factors are able to regulate angiogenesis, of which the vascular growth factors, Vascular Endothelial Growth Factor-A (VEGF-A) and Placental Growth Factor (PlGF), are probably the most well known. VEGF interacts with the VEGFR-1 (Flt-1) and VEGFR-2 (KDR) to promote endothelial cell proliferation, cell migration and vascular permeability. In rabbits, VEGF, together with its receptor Flt-1, plays an active role in the angiogenic process during implantation [Das *et al.*, 1997a].

PlGF shares biochemical and functional features with VEGF and interacts with VEGFR-1 (Flt-1). PlGF and VEGF have synergistic effects regarding angiogenesis, but vessels induced by PlGF are more mature and stable than vessels induced by VEGF [Carmeliet 2003; Luttun *et al.*, 2002]. PlGF is abundantly expressed in human placenta and may be an important paracrine regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Sherer, Abulafia 2001].

A second family of growth factors, the angiopoietins, and their receptor TIE-2 are also known for their regulating capacities regarding angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Angiopoietin-1 maintains vessel integrity and plays a role in the later stages of vascular remodelling [Geva, Jaffe 2000]. Transgenic overexpression of Ang-1 in mice results in the development of more complex vascular networks [Suri *et al.*, 1998].

Angiopoietin-2 is a functional antagonist of Ang-1 and leads to loosening of cell/cell interactions and allows access to angiogenic inducers like VEGF [Maisonpierre *et al.*, 1997]. Co-expression of VEGF and Ang-2 induces angiogenesis, but Ang-2 results in vascular regression in the absence of angiogenic signals [Asahara *et al.*, 1998]. Ang-1 is widely expressed in the adult, whereas Ang-2 is selectively expressed at sites of active angiogenesis, such as ovary, uterus and placenta [Davis *et al.*, 1996; Maisonpierre *et al.*, 1997].

Vascular adaptation to implantation and its regulation have been studied thoroughly with regard to arterial remodelling and placental vascularisation [Craven *et al.*, 1998; Kam *et al.*, 1999]. However, it is unknown how maternal decidual vascularisation is regulated and whether regulatory signals are originating from the extra-villous trophoblast and/or from pregnancy-induced hormones. Therefore, the present study was performed in three first-trimester decidual tissues: decidual secretory endometrium (DSE), decidua basalis (DB) and parietalis (DP).

By comparing these decidual tissues within subjects, the changes that occur independently of trophoblast invasion, i.e. pregnancy-induced hormones, can be separated from the influence of the invasive extra-villous trophoblast (EVT, Fig. 1). Vascularisation pattern and the expressions of VEGF-A, PlGF, Flt-1, KDR, Ang-1, Ang-2 and TIE-2 were determined in these three decidual tissues. In this way, decidual vascular adaptation to embryonic implantation can be studied in detail.

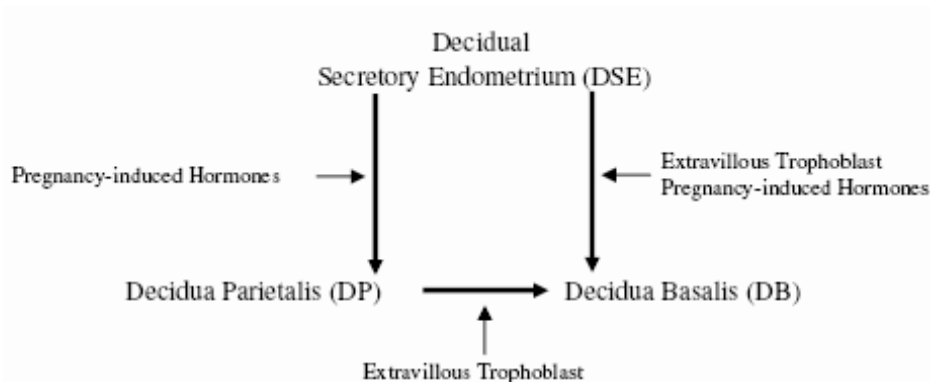


Figure 1. Schematic representation of the influences of extra-villous trophoblast and pregnancy-induced hormones on decidual tissues of first-trimester pregnancies.

MATERIALS AND METHODS

Materials

Lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% SDS), 20 mg/ml proteinase K (Life-Technologies Gibco BRL, Gaithersburg MD) and solution D (4 M guanidium isothiocyanate, 0.75 M Sodiumcitrate, 10% sarkosyl and 2-mercapto-ethanol) were used for RNA isolation. cDNA synthesis was performed using Ready-to-go You-Prime first strand beads (Amersham Biosciences, Buckinghamshire, UK). Primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R1 (Flt-1), VEGF-R2 (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2 and GAPDH (VIC-labelled) were purchased from Applied Biosystems (Foster City, CA USA).

The following first antibodies were used: broad spectrum polyclonal rabbit anti-cytokeratin (1:2000, Zo622, DAKO, Glostrup Denmark), monoclonal mouse anti-CD34 (1:1000, QBend/10, Novocastra, Newcastle upon Tyne, UK), polyclonal rabbit anti-VEGF-A (1:100, sc-152, Santa Cruz, CA, USA), polyclonal rabbit anti-VEGFR-1 (Flt-1, 1:50, sc-316, Santa Cruz, CA, USA), monoclonal anti-VEGFR-2 (KDR, 1:50, sc-6251, Santa Cruz, CA, USA), polyclonal goat anti-PlGF (1:200, sc-1880, Santa Cruz, CA, USA), polyclonal goat anti-Angiopoietin-1 (1:50, sc-6319, Santa Cruz, CA, USA), polyclonal goat anti-Angiopoietin-2 (1:50, sc-7016, Santa Cruz, CA, USA) and polyclonal rabbit anti-TIE-2 (1:100, sc-9026, Santa Cruz, CA, USA).

The following second antibodies were used: biotinylated horse anti-mouse antibody (1:300, BA-2000, Vector, Burlingame, USA), biotinylated donkey anti-rabbit antibody (1:300, RPN1004, Amersham Biosciences, Buckinghamshire, UK), biotinylated rabbit anti-goat (1:300, E-0466, DakoCytomation, Glostrup, Denmark). Immunohistochemical reactions were visualised using avidin-biotin complex (DakoCytomation, Glostrup, Denmark) and NovaRED (Vector, Burlingame, USA).

Study group

Decidua samples were obtained from women (n=32) with a healthy, viable intrauterine gravidity, undergoing vacuum aspiration due to a legal voluntarily abortion. The study was approved by the ethics committee of the Leiden University Medical Centre and informed consent was provided by all study subjects. Foetal cardiac activity and gestational age were confirmed by ultrasound. Women with symptoms of a missed abortion, such as vaginal bleeding, and women with underlying pathologies were excluded. Inconsistency between the ultrasound-determined gestational age and the known last day of menstruation also led to exclusion.

Two groups were formed based on gestational age (GA). We created two distinct groups with mean GA < 8-9 weeks and GA > 8-9 weeks. This cut-off of GA 8-9 weeks was chosen because evidence exists that this period is associated with changes in placental vascu-

Table 1. Characteristics of the study subjects.

	Group I (n=25) Mean $\pm$ SD	Group II (n=8) Mean $\pm$ SD
Maternal Age (years)	29.4 $\pm$ 6.9	29.6 $\pm$ 14.4
Gestational age (days)	45.3 $\pm$ 7.8	90.1 $\pm$ 12.6
Gestational age (weeks)	6.5 $\pm$ 1.1	12.6 $\pm$ 1.8
Number of pregnancies	1.3 $\pm$ 0.3	1.6 $\pm$ 0.5
Number of Miscarriages	0.2 $\pm$ 0.1	0.4 $\pm$ 0.3

larisation [Burton *et al.*, 1999]. After 8-9 weeks of GA, the intervillous space is gradually filled with maternal blood, causing oxygen level and oxidative stress to rise, thereby stimulating placental differentiation and vascularisation. This process is thought to be largely completed at 12 weeks gestation. This cut-off was the basis of the early first-trimester group (n=25) with a mean GA of 45.3 days ($\pm$ 7.8) and the late first-trimester group (n=8) with a mean GA of 90.1 days ($\pm$ 12.6). Maternal age and number of previous pregnancies and miscarriages did not differ significantly between the two groups. Patient characteristics of the study groups are given in Table 1.

Tissue Samples

Decidua samples were obtained from the aspirated tissue, fixed in formaldehyde and embedded in paraffin. A Hematoxylin Phloxin Safrane (HPS) staining was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium during the menstrual cycle. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (Fig. 2). Only subjects with a complete set of DSE, DB and DP were included. Serial sections of the paraffin embedded tissue samples were used for all experiments and all outcome measures were compared between tissues within subjects.

RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples based on methods described previously [Specht *et al.*, 2001]. In short, 5 μ m sections were mounted on RNase-free glass slides. The first and last sections were used to verify the presence of the tissues of interest. The other sections were deparaffinised and the tissues of interest, DSE, DP and DB (without villous tissue), were macro-dissected and dissolved in 190 μ l lysis buffer and 10 μ l proteinase K for 18 hours at 60°C. Subsequently, 400 μ l Solution D was added and RNA was isolated according to the Chomczynski method [Chomczynski, Sacchi 1987]. RNA quantity and quality was determined by measuring its absorbance in a

spectrophotometer (Nanodrop ND-1000) and reverse transcription was performed with 1 µg total RNA, random primers and a cDNA synthesis kit.

Real-time RT-PCR

The mRNA expression was quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R₁ (Flt-1), VEGF-R₂ (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene. Other genes, β-actin, β₂-microglobulin and cyclophilin, were also used as reference genes and showed comparable results (data not shown). RT-PCR reactions for target gene/GAPDH pairs were performed in duplicate, expressed in cycle threshold (Ct) and quantified into ng/µl using a standard curve of total RNA. The amount of RNA expression of DSE was set at 100% to compare DSE with DP and DB and the gene expression of DP was set at 100% to compare DP with DB. Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry

Serial sections were deparaffinised, endogenous peroxidase was quenched with 3% H₂O₂/methanol and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in trypsin was used for the detection of TIE-2, whereas detection of cytokeratin, Ang-2 and Ki67 required boiling in 0.1 M citrate buffer. Primary antibodies were applied overnight at 4°C followed by incubation with a biotinylated secondary antibody. Antibody binding was visualised using avidin-biotin and streptavidin-horseradish peroxidase conjugates. Sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reaction was verified by the omission of the first antibody as well as using normal mouse, goat or rabbit serum as first antibody. To evaluate the expression in extra-villous trophoblast, cytokeratin and target protein staining were performed on serial sections of 3 µm.

Evaluation of the vascularisation pattern

CD34-stained endometrial sections were used to scan sequential fields. Two to four samples of each type of tissue were analysed of each study subject and six fields per sample were scanned at 100x magnification. The number of vessels per mm², vascular surface per mm² (mm²/mm²) and luminal surface (µm²/vessel) were determined using image analysis software (Qwin, Leica Microsystems).

Evaluation of Immunohistochemical staining

Immunostaining was evaluated by calculating a staining index (SI): proportion of stained cells x staining intensity. The proportion of stained cells was expressed as 0, 1, 2 or 3, which marks positive staining signal in 0%, <10%, 10-50% or >50% of the cells of a particular cell population. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score 9 [Nap *et al.*, 2004]. The average score of two independent observers was used to calculate the mean and total staining index (Mean and Total SI). The Mean SI represents the protein expression per studied cell type. The Total SI represents the total protein expression per tissue and was calculated as the sum of the Mean SI of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (SC) in DSE and DP samples. The Mean SI of EVT was also included in the Total SI of DB samples.

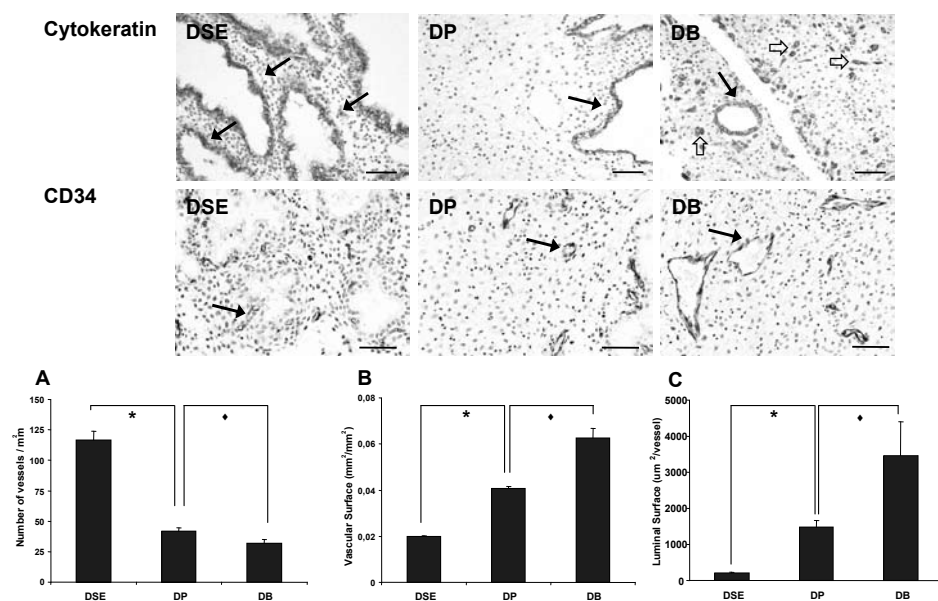


Figure 2. Cytokeratin expression and vascularisation in the three decidual tissues.

Differentiation between three decidual tissues was obtained by HPS and anti-cytokeratin staining (top panel). DSE and DP expressed cytokeratin only in glandular epithelial cells (closed arrows), whereas cytokeratin is also expressed in extra-villous trophoblasts of DB (open arrows). The vascularisation pattern in human decidual tissues was determined by image analysis of anti-CD34-stained sections (middle and bottom panel). CD34 antigen expression in endothelial cells (arrows) of DSE, DP and DB was compared within subjects. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were calculated and expressed as mean ± SEM. * $p < 0.0001$; ♦ $p < 0.04$. Bar = 100 μm. (see colour figures supplement)

Statistical analysis

All parameters were compared between DSE and DP, between DSE and DB and between DP and DB within subjects. A general linear model for repeated measurements, repeated measures ANOVA, was performed to analyse the double paired data within subjects of early first-trimester decidua as well as to compare data between the early and late first-trimester group (SPSS 11.5). Where appropriate we used Friedman's test for non-parametric investigations of correlated observations. The used statistical analyses are described in legends and table footnotes. P-values of < 0.05 were considered significant.

RESULTS

Vascularisation in early first-trimester decidua

Intense CD34 staining was observed in all vessels of the three decidual tissues, with no difference in staining intensity between the tissues.

The vessel density was significantly elevated in DSE (116.4 vessels/mm²) compared to DP (41.7 vessels/mm², $p < 0.001$) and DB (31.8 vessels/mm², $p < 0.0001$); vessel density in DP and DB also differed significantly ($p < 0.03$, Fig 2A). Since the stromal cells concentration differed between the tissues (434 cells/mm² in DSE, 208 cells/mm² in DB and DP, $p < 0.05$), vessel density was corrected for the stromal cell count. The corrected vessel density showed a similar pattern; density was elevated in DSE (0.28 vessels/stromal cells) compared to DP and DB and elevated in DP (0.20 vessels/stromal cells) compared to DB (0.15 vessels/stromal cells).

The vascular surface was significantly smaller in DSE (0.020 ± 0.0001 mm²/mm²) compared to DP (0.041 ± 0.001 mm²/mm²) and DB (0.063 ± 0.004 mm²/mm², all $p < 0.0001$, Fig. 2B). The vascular surface in DP differed significantly from that in DB ($p < 0.04$).

DSE showed a significant smaller luminal surface in comparison with DP (208 versus 1474 μ m²/vessel, $p < 0.0001$) and DB (3465 μ m²/vessel, $p < 0.001$); this parameter was also significantly smaller in DP compared to DB ($p < 0.05$, Fig 2C).

Thus, pregnancy-induced hormones and extra-villous trophoblasts influence vascularisation, since larger but fewer vessels were present in DP compared to DSE and in DB compared to DP (Fig. 1 and 2).

mRNA levels of angiogenic factors

The expression of several angiogenic growth factors and their receptors were evaluated at the RNA level (Table 2). All tested genes were expressed by the three decidual tissues. First, the mRNA levels of the angiogenic factors in DSE were compared with those in DP. Only TIE-2 was differentially expressed, reflected by a 49% reduction in DP compared

Table 2. Differential gene expression in early first-trimester decidua ^a.

	DSE ^b	DP	DSE ^b	DB	DP ^c	DB
PIGF	100 %	82 ± 21%	100 %	1286 ± 302%*	100 %	3000 ± 237% [§]
VEGF-A	100 %	85 ± 12%	100 %	96 ± 14%	100 %	134 ± 36%
KDR	100 %	105 ± 16%	100 %	74 ± 15%	100 %	76 ± 14%
Flt-1	100 %	105 ± 14%	100 %	130 ± 24%	100 %	131 ± 23%
Ang-1	100 %	92 ± 17%	100 %	39 ± 11%*	100 %	55 ± 15% [§]
Ang-2	100 %	115 ± 29%	100 %	34 ± 8%*	100 %	36 ± 6% [§]
TIE-2	100 %	51 ± 11% [#]	100 %	21 ± 5%*	100 %	43 ± 7% [§]

^a RNA levels were determined by RT-PCR in DSE, DP and DB of early first-trimester (n=25) and compared within subjects. The 'repeated measures ANOVA' was performed to analyse the data.

^b The RNA levels of DSE was set at 100% to compare mRNA levels in DSE with those in DP and DB.

^c The RNA levels of DP was set at 100% to compare DP with DB.

[#] p < 0.02 DP compared to DSE, * p < 0.02 DB compared to DSE, [§] p < 0.01 DB compared to DP.

to DSE (p<0.02). The other genes, VEGF-A, PIGF, Flt-1, KDR, Ang-1 and Ang-2, showed comparable mRNA levels in DP and DSE (Table 2).

Subsequently, the mRNA levels in DSE were compared to those in DB. This comparison showed a 1286 % induction of PIGF, 61% reduction of Ang-1, 66% reduction of Ang-2 and a 79% reduction of TIE-2 in DB compared to DSE (Table 2, p<0.02). The expressions of VEGF, Flt-1 and KDR did not differ significantly. Finally, mRNA expressions in DB were compared to that in DP to evaluate the influence of the extra-villous trophoblast (EVT). This comparison demonstrated a similar pattern as the comparison DSE to DB, namely an increased expression of PIGF (3000%) and reduced expressions of Ang-1 (45%), Ang-2 (64%) and TIE-2 (57%) in DB compared to DP (Table 2, all p<0.01).

The expression of angiogenic factors at protein level of early first-trimester decidua

The distribution of angiogenic factors was determined at the protein level in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues (Table 3).

The protein expressions in DSE were compared to those in DP, which only showed a higher total staining index for Flt-1 in DP (Table 3, p<0.05). In addition, when DSE and DB were compared, higher endothelial and total expressions of both PIGF and its receptor Flt-1 were demonstrated in DB. The total Ang-1 expression was lower in DB (Table 3, p<0.05). Thirdly, DB was compared to DP and this showed a similar pattern as the comparison DSE and DB, namely higher total and endothelial expressions of PIGF and Flt-1 in DB (Table 3, Fig. 3A, C and E, p<0.05). Also, the total Ang-1 expression was less abundant in DB (p<0.01), which resulted in a higher Ang-2/Ang-1 ratio in DB than in the other tissues (2.6 vs.1.4 and 1.2, p<0.01). The expressions of VEGF-A, KDR, Ang-2 and TIE-2 proteins were comparable in the three tissues.

Table 3. Protein expression of angiogenic factors in early first-trimester decidua ^a.

		EC ^b	PSMC ^b	GE ^b	SC ^b	EVT ^b	Total SI ^c
VEGF	DSE	0.1	3.5	5.3	1.7	NP	10.6
	DP	0.3	3.3	6.2	3.0	NP	13.1
	DB	0.0	2.5	4.9	2.6	3.3	13.3
PlGF	DSE	0.1	3.4	5.4	2.5	NP	11.4
	DP	0.1	2.4	4.8	3.0	NP	10.3
	DB	0.9 *	2.7	6.2	3.3	2.8	15.9 *
KDR	DSE	1.0	5.4	6.0	0.7	NP	13.1
	DP	0.6	3.5	4.7	2.2	NP	11.0
	DB	0.9	3.2	4.4	2.0	0.8	11.3
Flt-1	DSE	0.5	2.7	5.8	1.6	NP	10.6 &
	DP	0.4	3.3	5.6	3.3	NP	12.6
	DB	1.2 *	3.4	5.4	2.5	2.2	14.7 *
Ang-1	DSE	0.0	1.1	4.7	0.3	NP	6.1
	DP	0.0	1.3	4.4	0.9	NP	6.6
	DB	0.1	0.0	3.1	0.2	0.0	3.4 *
Ang-2	DSE	0.2	1.7	5.6	0.0	NP	7.5
	DP	0.6	1.1	6.2	1.7	NP	9.6
	DB	0.7	1.5	4.9	0.9	0.9	8.9
TIE-2	DSE	1.8	3.2	6.0	2.2	NP	13.2
	DP	2.3	3.5	5.1	4.1	NP	15.0
	DB	1.8	2.1	4.1	3.1	2.8	13.9

^a The protein expression of angiogenic factors in DSE, DP and DB were compared within subjects. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis.

^{b,c} The staining was expressed as mean staining indices per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue.

NP = not present. * $p < 0.05$ in DB versus DP and DSE, & $P < 0.05$ in DSE versus DP.

Endothelium displayed the expression of all angiogenic factors, except Ang-1 (Fig. 3A and F). VEGF-A was hardly detectable in endothelium (Fig. 3A and B). Ang-2 and KDR were mainly expressed by glandular epithelium and PSMCs, but also by endothelial cells (Fig.

3A, D and G). Moreover, TIE-2 showed the highest expression in endothelium (Table 3, Fig. 3A and H).

All angiogenic factors, except Ang-1, were also detected in extra-villous trophoblasts; PlGF, VEGF, Flt-1 and TIE-2 were moderately expressed, whereas KDR and Angiopoietin-2 expressions were weak (Table 3, Fig. 4).

Vascularisation and expression of angiogenic factors in late first-trimester decidua

The vascularisation parameters in late first-trimester decidua showed similarities with vascularisation in the early first-trimester; the vascular and luminal surface were larger and vessel density was smaller in DP and DB compared to DSE and in DB compared to DP (all $p < 0.05$, Fig. 5A, B and C). Comparison of the vascularisation patterns at both time points showed that the vessel density was lower in DB and DP of late first-trimester

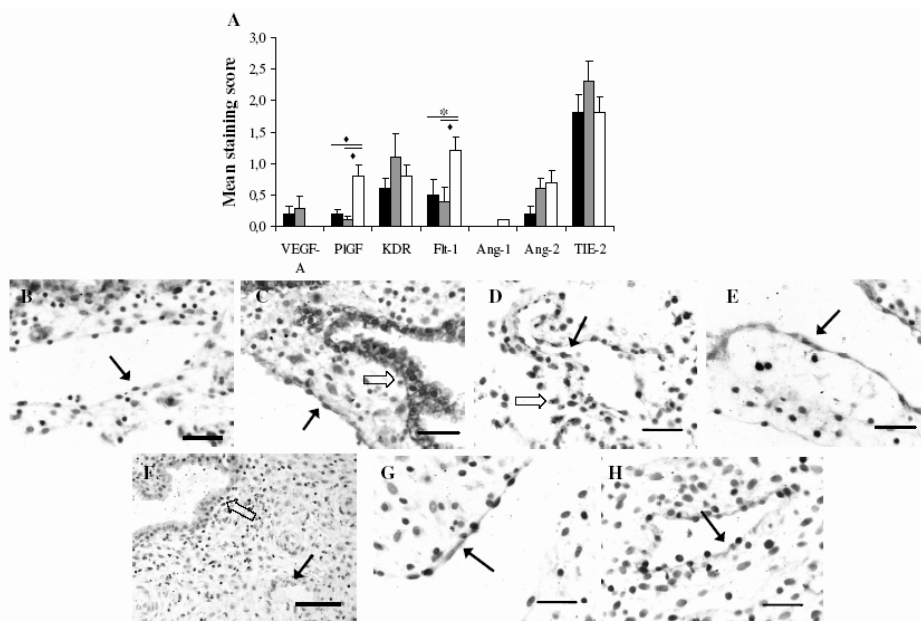


Figure 3. Protein expression of angiogenic factors in endothelium of early first-trimester decidua.

The protein expression of angiogenic factors was determined in DSE, DP and DB. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis. **A.** Protein expression of each angiogenic factor in endothelium was expressed as the mean staining index $\pm$ SEM and **B-H** show examples of these expressions. **B.** Endothelial VEGF expression was not detectable in DB. **C.** PlGF was expressed in epithelium (open arrow) and endothelium (closed arrow) of DB. **D.** Endothelial KDR expression (closed arrow) and stromal KDR expression (open arrow) in DB. **E.** Endothelial Flt-1 expression in DB (arrow). **F.** Angiopoietin-1 in DB was detected in epithelium (open arrow), but not in stromal and endothelial cells (closed arrow). **G.** Endothelial angiopoietin-2 expression in DB (arrow). **H.** Endothelial TIE-2 expression in DB (arrow). DSE, DP, DB. Bar = 100 μ m (except F = 50 μ m). (see colour figures supplement)

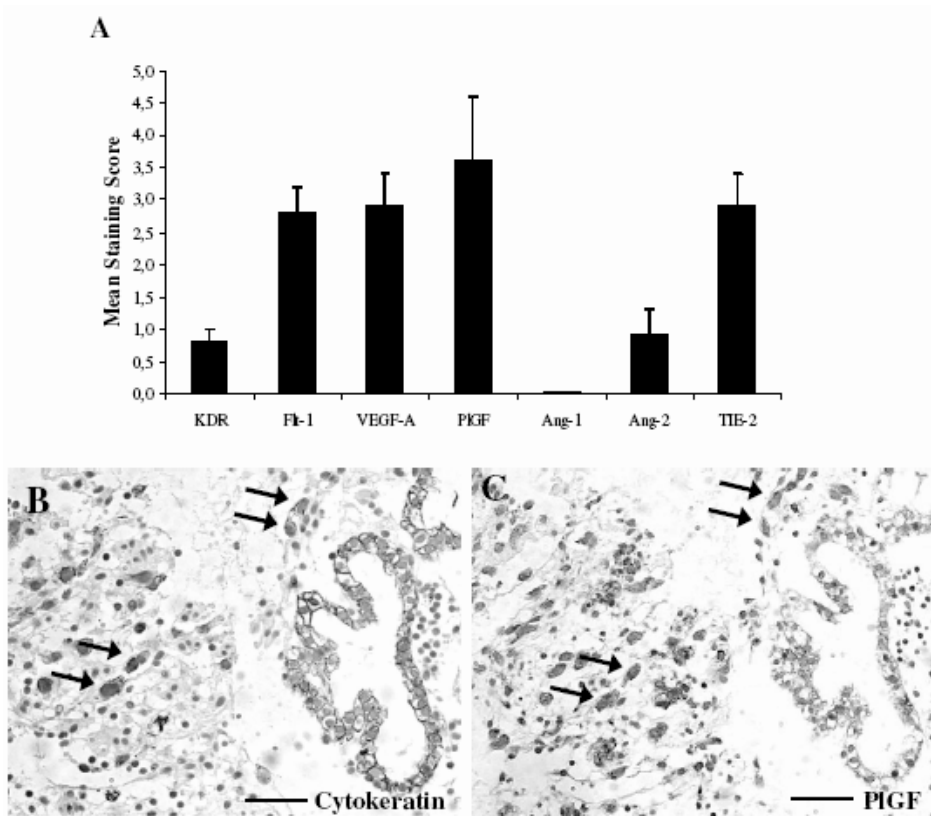


Figure 4. Protein expression of angiogenic factors in extra-villous trophoblasts (EVT) of early first-trimester decidua.

The protein expression of angiogenic factors in EVT in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Protein expression in EVT was expressed as the mean staining index $\pm$ SEM. **B.** Cytokeratin (arrows) and **C.** serial PIGF expression (arrows) in EVT. Bar = 50 μ m. (see colour figures supplement)

(17.8 vs. 31.8 vessels/ mm^2 in DB and 29.2 vs. 41.7 vessels/ mm^2 in DP, $p < 0.05$). The luminal surface was larger, although not significantly, in DB and DP of late first-trimester, namely 5447 vs. 3465 μm^2 /vessel in DB and 1829 vs. 1474 μm^2 /vessel in DP (Fig. 5B). The vascular surface was similar in decidua at the two time points (Fig. 5C).

Then, mRNA levels in early and late first-trimester were compared. VEGF-A and Ang-2 mRNA were both induced in late first-trimester DB and DP as compared to early first-trimester; the induction was 347% ($p = 0.08$) in DB and 288% ($p < 0.01$) in DP for VEGF-A and 257% ($p < 0.01$) in DB and 171% ($p < 0.01$) in DP for Ang-2. Genes in DSE showed no differential expression in time. At the protein level, total VEGF, Ang-1 and Ang-2 protein expressions were elevated in DB of late first-trimester compared to early first-trimester (165%, 288% and 174% respectively, $p < 0.002$). Interestingly, VEGF and PlGF proteins

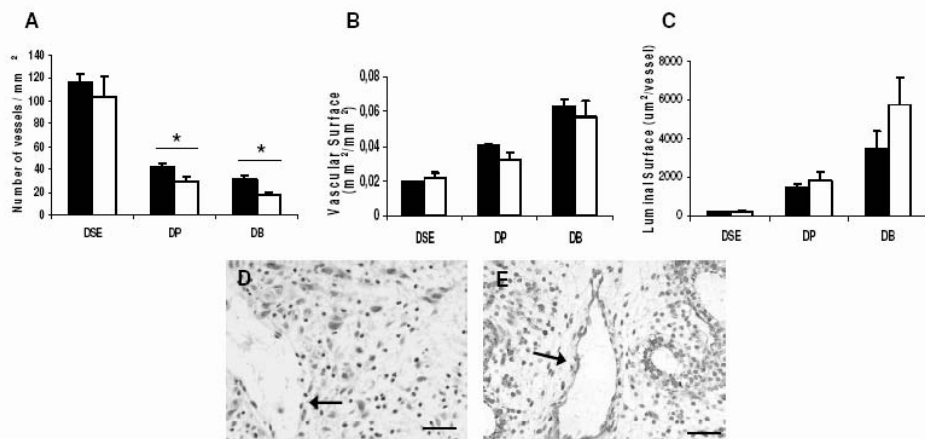


Figure 5. Vascularisation pattern and endothelial protein expression in early versus late first-trimester decidua.

The vascularisation pattern in human early (black bars) and late (white bars) first-trimester decidual tissues was determined by image analysis of anti-CD34-stained sections. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were expressed as mean ± SEM. VEGF expression was not detectable in early first-trimester endothelium of DB (arrow, **D**), whereas the expression was present in late first-trim. endothelium of DB (arrow, **E**). Early first-trimester and late first-trimester. Bar = 50 μm, * p<0.04. (see colour figures supplement)

were detected in higher amounts on the endothelium of late first-trimester decidua than in early first-trimester (Fig. 5D and E, p<0.05). Endothelial Flt-1, KDR, Ang-2 and TIE-2 expressions were comparable in early and late first-trimester.

DISCUSSION

The present study showed differences in vascularisation pattern between decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP). These vascular changes correlated with differences in gene expression. Furthermore, analysis of vascularisation in early and late first-trimester decidua suggests that decidual vascularisation is remodelled as gestation progresses to meet up with the increasing needs of the implanting embryo.

Maternal vascular adaptation to implantation and its regulation has been subject to discussion, but has only been studied thoroughly with regard to arterial remodelling and villous vascularisation [Craven *et al.*, 1998; Kam *et al.*, 1999]. The present study focused on decidual vascular adaptation to implantation and its regulation. The comparison of three decidual tissues, DSE, DB and DP, within subjects provides the opportunity

to separate the influence of the extra-villous trophoblast (EVT) from the changes that occur independently of trophoblast invasion (Fig. 1). Furthermore, all methods were performed on serial paraffin sections, which allowed studying vascularisation, RNA and protein expression in the same tissue.

Changes in vascularisation pattern, the increase of vascular and luminal surfaces and decrease of vessel density, were detected between the three decidual tissues DSE, DP and DB. The enlargement of the vascular surface per area in DB and DP has been reported previously and likely is functionally related to the increased blood flow during pregnancy [Kam *et al.*, 1999; Gibbons, Dzau 1994]. The increase in luminal diameter may represent an additional modulatory effect, although the possibility that this was due to vasodilatation cannot be excluded. Increased luminal diameter in DP and DB could also be induced by a relatively low oxygen levels, but whether oxygen levels in DP, DB and DSE differ is not known [Charnock-Jones 2002].

The vessel density in decidual secretory endometrium (116 vessels/mm²) is lower than the vessel density reported in cycling secretory endometrium, 186 vessels/mm² reported by Rogers *et al* and 195 vessels/mm² by Plaisier *et al* [Plaisier *et al.*, 2006; Rogers *et al.*, 1993]. Possibly, vascularisation in DSE is already affected by hormones, although the decidual morphology is not yet present. The decreased vessel density in DP and DB compared to DSE and DB compared to DP suggests fusion or degeneration of vascular structures. Vailhe *et al* reported a non-significant decreased vessel density in DB compared to DP, but did not study DSE [Vailhe *et al.*, 1999]. The vessel density reported in their study, ranging between 20 and 30 vessels/mm², is comparable with our findings. Overall, the vascular differences between the decidual tissues indicate a regulatory role in decidual vascularisation for both pregnancy-induced hormones and the EVT.

Both steroidal hormones and EVT are able to modulate vascularisation directly or indirectly via angiogenic factors like VEGF-A, PlGF, angiopoietins and hCG [Greb *et al.*, 1997; Hyder, Stancel 1999/2000; Krikun *et al.*, 2004b; Licht *et al.*, 2003; Simoncini *et al.*, 2003; Toth *et al.*, 1994/2001; Wulff *et al.*, 2000; Zygmunt *et al.*, 2002]. Therefore, we studied whether the changes in vascularisation in the decidual tissues correlated with a differential expression of angiogenic factors. First, gene and protein expressions in DSE and DP were compared. Although TIE-2 mRNA was the only differentially expressed mRNA, its protein expression was not significantly differing between DSE and DP. This suggests that TIE-2 protein expression is not influenced by pregnancy-induced hormones, which is in agreement with the finding that TIE-2 protein expression is not regulated during the menstrual cycle [Krikun *et al.*, 2000]. The presence of endothelial TIE-2 in all decidual tissues suggests that TIE-2 plays a rather general role in decidual vascularisation.

The differential expression of the studied angiogenic factors did not provide a satisfactory explanation for the vascular differences between DSE and DP. Possibly, steroidal hormones act through other angiogenic factors or exert direct effects on decidual endothelium.

Subsequently, the expressions of angiogenic factors in DB were compared to those in DSE and DP. Ang-1, Ang-2 and TIE-2 mRNA expressions were significantly lower in DB compared to both DSE and DP and therefore appear to be regulated by the EVT. The decreased TIE-2 expression might result from the decreased Ang-1 and/or Ang-2 levels, since mice lacking Ang-1 have reduced TIE-2 mRNA levels [Suri *et al.*, 1996]. At protein level, the Ang-2/Ang-1 ratio was significantly elevated in DB compared to DP and DSE, suggesting that vessel destabilization is favoured over vessel maturation in DB. The high amount of Ang-2, together with the abundant VEGF expression, may lead to more angiogenesis in DB which is in concordance with the increased vascular surface in DB [Asahara *et al.*, 1998].

Interestingly, all three decidual tissues showed Ang-2/Ang-1 ratios in favour of Ang-2. Embryonic implantation normally occurs under hypoxic conditions and oxygen levels in decidua are relatively low in the first-trimester, ranging between 50 and 75 mmHg [Jauniaux *et al.*, 2000]. Hypoxia enhances Ang-2 transcription and destabilizes Ang-1, which may result in the increased Ang-2/Ang-1 ratio [Zhang *et al.*, 2001]. Although implantation appears to favour Ang-2, a small amount of Ang-1 must be essential for implantation since disruption of the Ang-1 gene in mice results in embryonic lethality [Suri *et al.*, 1996].

Another striking observation was the abundant induction of PlGF mRNA and proteins in DB compared to DSE and DP. The increased PlGF protein expression was not only due to PlGF production by EVT, since all studied cell types, except PSMCs, showed increased PlGF protein expression in DB. Furthermore, uterine NK cells are known for their PlGF production as well and probably contribute to the production of angiogenic factors in decidua as well.

Interestingly, PlGF and its receptor, Flt-1, were mainly expressed on endothelium of DB. PlGF, together with Flt-1, is able to potently induce angiogenesis resulting in mature and stable vessels [Arroyo *et al.*, 2004; Carmeliet *et al.*, 2001; Khaliq *et al.*, 1996; Luttun *et al.*, 2002; Torry *et al.*, 1999]. The induction of PlGF and Flt-1 mRNAs and proteins in decidua basalis suggests that both factors play a specific role in regulating angiogenesis at the site of implantation and may, at least partially, account for the vascular changes noticed in decidua basalis.

VEGF mRNAs and proteins were abundantly, but not differentially, expressed in all decidual tissues and KDR, Flt-1 and VEGF proteins were expressed on endothelium in

DB and DP. These findings suggest a general role for VEGF in angiogenesis in human decidual tissues.

As gestation progresses, vascular adaptation continues which manifested itself in the present study by decreased vessel density in DB and DP. VEGF and Ang-2 mRNA and VEGF, Ang-1 and -2 protein expressions were increased in late first-trimester DB. These increased levels of the angiogenic factors suggest increased angiogenic activity at the implantation site as gestation progresses. This was further supported by the increased endothelial expression of PlGF and VEGF proteins in late first-trimester decidua.

The vascular adaptation to implantation is clearly important in the development of a healthy pregnancy. We have shown that vascular adaptation occurs under influence of both pregnancy-induced hormones and the extra-villous trophoblast, by enhancing the vascular surface, reducing vessel density and regulating the expression of angiogenic factors. VEGF-A, via KDR, probably plays a general role in supporting decidual vascularisation, whereas the angiopoietins, via TIE-2, and PlGF, via Flt-1, appear to be key regulators of the vascular adaptive process and may, partially, account for the vascular changes at the site of embryonic implantation in decidua basalis. Finally, studying vascularisation during mammalian implantation hopefully leads to new understandings of currently inexplicable events associated with pregnancy, such as pre-eclampsia and missed abortions.

Chapter 6

Pericellular-acting proteases in human first-trimester decidua.

M. Plaisier
P. Koolwijk
F. Willems
V.W.M. van Hinsbergh
F.M. Helmerhorst

ABSTRACT

Objective: Proteolysis is essential for decidual development during embryonic implantation, but little is known regarding the expression and functions of membrane-type matrix metalloproteinases (MT-MMPs) and urokinase and its receptor uPAR in decidua. Therefore, their protein and mRNA expressions were analysed in three first-trimester decidual tissues, decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB).

Methods: Decidua was obtained during first-trimester pregnancy termination. uPA, uPAR, and MT_{1/2/3/5}-MMP expression were studied by RT-PCR and immunohistochemistry, and CD56-positive uNK cells and CD68-positive macrophages were quantified in serial sections.

Results/conclusion: The mRNAs and antigens of all proteases and uPAR were detectable in the decidual tissues and extravillous trophoblasts (EVT). mRNA levels of all proteases and uPAR, except MT₅-MMP, were elevated in both DB and DP compared to DSE, being significant for MT₁-MMP and uPAR in DP. MT₂- and MT₃-MMP mRNAs in DB were 24- and 10-fold higher than in DSE, and 19- and 7-fold increased compared to DP. At the protein level uPA and uPAR were particularly elevated in DB, while pro-angiogenic MT₁- and MT₃-MMPs were elevated in both DB and DP compared to DSE. MT₂-MMP was prominent present in all conditions. The number of uNK cells was increased in DB and DP versus DSE, while a comparable increase in macrophages did not reach statistical significance. These data are consistent with a differential regulation of peri-cellular proteases in decidua by pregnancy-induced hormones, immune cells and EVT.

INTRODUCTION

Proteolysis is essential for decidual remodelling and vascularisation during implantation. Disturbances in decidual development may play a role in the pathogenesis of miscarriages and pre-eclampsia [Solberg *et al.*, 2003; Vailhe *et al.*, 1999; Vuorela *et al.*, 2000; Zygmont *et al.*, 2003]. Endometrial adaptation to implantation starts during the receptive secretory phase of the menstrual cycle and continues throughout the first-trimester. This adaptation includes decidualisation, tissue remodelling, angiogenesis, and immune cell invasion. The invasion of immune cells is enormous: from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70% of these leucocytes are uNK cells and 10% are macrophages [Bulmer *et al.*, 1991].

Decidual remodelling, cell invasion as well as angiogenesis are facilitated by proteolysis. Trophoblasts, uNK cells and endothelial cells require proteolytic activity to degrade their extra cellular matrix (ECM) proteins and to migrate [Al Atrash *et al.*, 2001; Heymans *et al.*, 1999; Pepper 2001; Stetler-Stevenson 1999; Salamonsen 1999]. These cells are able to generate proteolytic activity by either producing proteases or using proteases on neighbouring cells and/or ECM [Albertsson *et al.*, 2000; Kim *et al.*, 2000; Koolwijk *et al.*, 2001; Salamonsen 1999; Van den Heuvel *et al.*, 2005b]. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs), in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs), and to the plasmin/plasminogen system [Alfano *et al.*, 2005; Kindzelskii *et al.*, 2004; Reuning *et al.*, 2003].

The membrane-associated localisation of MT-MMPs makes them suited for pericellular proteolysis [Egeblad, Werb 2002; Hotary *et al.*, 2000; Seiki, Yana 2003; Van Hinsbergh *et al.*, 2006]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24). Only these MT-MMPs have the proteolytic potential to induce capillary tube formation, while the GPI-anchored MT4- and MT6-MMP were unable to do so [Hotary *et al.*, 2000]. MT-MMPs are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs); TIMP-1 inhibits all MT-MMPs, except MT1-MMP, while TIMP-2 and -3 inhibit all MT-MMPs [Hernandez-Barrantes *et al.*, 2002; Visse, Nagase 2003]. MT1-MMP is also inhibited by TIMP-4 [Bigg *et al.*, 2001]. MT1-MMP is the best known MT-MMP, which degrades ECM components and promotes cell migration, angiogenesis and tumour metastasis [Collen *et al.*, 2003; Galvez *et al.*, 2001; Hiroaka *et al.*, 1998; Lafleur *et al.*, 2002; Seiki, Yana 2003; Sounni *et al.*, 2002; Visse, Nagase 2003;]. MT2- and MT3-MMP are less well studied and are known to be involved in cell migration and invasion. MT1-, MT2- and MT3-MMP induce angiogenesis *in vitro* and MT2- and MT3-MMP may even be potential regulators of endometrial angiogenesis *in vivo* [Collen *et al.*, 2003; Galvez *et al.*, 2001; Hiroaka *et al.*, 1998; Hotary *et al.*, 2000; Hotary *et al.*, 2002; Lafleur *et al.*, 2002; Plaisier *et al.*, 2004; Plaisier *et al.*, 2006].

MT5-MMP is known for a gelatinolytic effect in the brain, which induces embryonic brain development and axonal growth [Llano *et al.*, 1999; Pei 1999].

With regard to MT-MMPs in decidua, only MT1- and MT2-MMP have been studied. MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, stromal cells, and the extra-villous trophoblast [Bai *et al.*, 2005; Bjorn *et al.*, 2000; Curry, Osteen 2003; Hurskainen *et al.*, 1998; Nakano *et al.*, 2001; Nawrocki *et al.*, 1996]. MT-MMPs are assumed to regulate trophoblast invasion during implantation [Salamonsen 1999]. Whether migration of other cell types, e.g. immune and endothelial cells, is also regulated by MT-MMPs remains to be seen.

The plasminogen activator (PA) system is based on the protease plasmin, which cleaves most ECM components. The circulating protein plasminogen is converted into the active protease plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). tPA is mainly involved in clot dissolution, whereas uPA mediates pericellular proteolysis during cell migration, tissue remodelling and angiogenesis [Van Hinsbergh *et al.*, 2006]. uPA binds a specific cell-surface receptor, uPAR, which restricts the uPA-activity to the cell environment and enables activation of plasmin directly on the cell surface. The activity of uPA is regulated by at least two specific serine proteinase inhibitors, plasminogen activator inhibitor type-1 and -2 (PAI-1/2) [Spengers, Kluft 1987].

The role of uPA mediated plasminogen activation in cell migration has been studied for a variety of cells and for endothelial cells, leucocytes, and trophoblasts in particular [Heymans *et al.*, 1999; Reuning *et al.*, 2003; Pepper 2001; Blasi *et al.*, 1987; Hu *et al.*, 1999; Salamonsen *et al.*, 2003]. Both uPA and uPAR expression has been detected in the invasive trophoblast cells, which indicates a role for uPA and uPAR play in trophoblast invasion [Hofmann *et al.*, 1994; Hu *et al.*, 1999; Multhaupt *et al.*, 1994; Pierleoni *et al.*, 1998; Salamonsen *et al.*, 1999].

Little information is available regarding the expression of MT-MMPs and uPA/uPAR in the various decidual tissues and cell types. Moreover, their regulation and involvement in decidual remodelling, vascularisation, and immune cell and trophoblast invasion is not well established.

Endometrial adaptation to pregnancy is induced by pregnancy-induced hormones, i.e. hCG, oestradiol and progesterone, immune cells, and the extra-villous trophoblasts (EVT). The differential presence of these factors results in the generation of various first-trimester decidual tissues. Decidual secretory endometrium (DSE) is only pre-decidualised and will develop into decidua parietalis (DP) under influence of pregnancy-induced hormones. Decidua basalis (DB) will arise in the additional presence of the EVT. The present study analyses the expression of MT-MMPs and uPA/uPAR at mRNA and pro-

tein level in decidual secretory endometrium (DSE), decidua parietalis (DP) and decidua basalis (DB). These parameters will be compared between tissues within subjects. In this way, MT-MMP and uPA/uPAR expression in various decidual cell types and decidual tissues can be determined. As the influx and activity of immune cells, in particular uNK cells and macrophages, may influence that production of proteases, we also compared the accumulation of these cells in the same decidual tissues. These data may shed light on the functions of the studied proteases in human decidua and might generate new hypotheses regarding the inception of pregnancy.

MATERIALS AND METHODS

Study group

Decidua samples were obtained from women (n=32) with a viable intrauterine gravidity, undergoing a legal voluntarily abortion. The study was approved by the Institutional Review Board, the ethics committee, of the Leiden University Medical Centre and informed consent was provided by all study subjects. Foetal cardiac activity and gestational age were confirmed by ultrasound. Women with symptoms of a missed abortion, such as vaginal bleeding, and women with underlying pathologies were excluded. Inconsistency between the ultrasound-determined gestational age and the known last day of menstruation also led to exclusion.

Two groups were formed based on a gestational age (GA) of <8-9 and of > 8-9 weeks. This cut off was chosen since after 8-9 weeks GA the intervillous space is gradually filled with maternal blood, causing the oxygen level and the oxidative stress to rise and thereby stimulating placental differentiation and vascularisation [Burton *et al.*, 1999]. This process is thought to be largely completed at 12 weeks gestation. The early group (n=25) has a mean GA of 45.3 days (6^{+3} weeks) and the late group (n=8) has a mean GA of 90.1 days (12^{+6} weeks). Maternal age and number of previous pregnancies and miscarriages did not differ significantly between the two groups. Patient characteristics of the study groups are given in Table 1.

Tissue Samples

Decidua samples were obtained from the aspirated tissue (vacuum aspiration), fixed in formaldehyde overnight and embedded in paraffin. Hematoxylin Phloxin Safrane (HPS) and anti-cytokeratin staining were performed. Hematoxylin (50 g potassium aluminium-sulfate, 1 g haematox, 500 mg citric acid, 25 g chloralhydrate, 200 mg NaJO_3 in 1000 ml aqua dest,) stains nuclei and calcium blue. Phloxin (0.25 g phloxin in 100 ml aquadest) stains erythrocytes, cytoplasm, fibrin and muscle red. Safrane (3 g safrane in 1000 ml 100% alcohol) stains calcium free bone, cartilage and collagen yellow. The HPS staining

Table 1. Characteristics of the study subjects.

	Early first- trimester group (n=25) Mean $\pm$ SD	Late first- trimester group (n=8) Mean $\pm$ SD
Maternal Age (years) ^a	29.4 $\pm$ 6.9	29.6 $\pm$ 14.4
Gestational age (days) ^a	45.3 $\pm$ 7.8	90.1 $\pm$ 12.6
Number of previous pregnancies ^a	1.3 $\pm$ 0.3	1.6 $\pm$ 0.5
Number of previous miscarriages ^a	0.2 $\pm$ 0.1	0.4 $\pm$ 0.3

^a p>0.05.

was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (see section immunohistochemistry, Figure 5).

DSE, DP and DB were obtained from the same curettement and therefore originate from the same depth and area of the uterine wall. Only subjects with at least two complete set of DSE, DB and DP were included. Serial sections of the paraffin embedded tissue samples were used for all experiments and all parameters were compared between tissues within subjects.

RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples [Plaisier *et al.*, 2007; Specht *et al.*, 2001]. Several samples per patient were used and these samples contained a proportionate amount of cells and cell types. In short, 5 μ m sections were mounted on RNase-free glass slides. The first and last sections were used to verify the presence of the tissues of interest. The other sections were deparaffinised and the tissues of interest, DSE, DP or DB (without villous tissue), were macro-dissected and dissolved in 190 μ l lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% SDS) and 10 μ l proteinase K (20 mg/ml proteinase K, Life-Technologies Gibco BRL, Gaithersburg) for 18 hours at 60°C. Subsequently, 400 μ l Solution D (4 M guanidium isothiocyanate, 0.75 M sodium citrate, 10% sarkosyl and 2-mercapto-ethanol) was added and RNA was isolated [Chomczynski, Sacchi 1987].

RNA quantity and quality were analysed in a spectrophotometer (Nanodrop ND-1000). Reverse transcription was performed with 1 μ g total RNA, random primers and cDNA synthesis kit according to the manufacturer's protocol and the obtained 32 μ l cDNA was diluted 1:3 (Ready-to-go You-Prime first strand beads, Amersham Biosciences, Buckinghamshire, UK).

Real-time RT-PCR

The mRNA levels were semi-quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for MT1-, MT2-, MT3-, and MT5-MMP, uPA and uPAR. glycerylaldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene (all primer/probe sets purchased from Applied Biosystems, Foster City, CA USA). Other genes, β -actin, β_2 -microglobulin and cyclophilin, were also used as reference genes and showed comparable results (data not shown).

RT-PCR reactions for target gene/GAPDH pairs were performed in 12.5 μ l reactions, containing 2.5 μ l cDNA solution, using mastermix (RT-QP2X-03, Eurogentec, Maastricht, The Netherlands), DNase free water and the above described primer/probe sets. Reactions were performed in duplicate, expressed in cycle threshold (Ct). A standard curve for each primer/probe pair was created by determining Ct values of various concentrations of total RNA (range 125-0.016 ng/ μ l). Then Ct values of the samples were quantified into ng/ μ l. RNA levels in DSE was set at 100% to compare DSE with DP and DB and the gene expression of DP was set at 100% to compare DP with DB. Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry

Serial sections were deparaffinised, endogenous peroxidase was quenched with 3% H₂O₂/methanol and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in a trypsin solution was used for detection of MT2- and MT5-MMP [Plaisier *et al.*, 2006]. Heat retrieval in citrate buffer (pH 6.0) was used for detection of CD56 and CD68.

The following first antibodies were used: broad spectrum polyclonal rabbit anti-cytokeratin (1:2000, Z0622, DAKO, Glostrup Denmark), monoclonal mouse anti-CD56 (IgG₁, 1:50, MONX 10844, clone 1B6, Monosan, Uden, the Netherlands), monoclonal mouse anti-CD68 (IgG₁, 1:200, Mo814, DAKO, Glostrup Denmark), monoclonal mouse anti-MT2-MMP (IgG₁, 1:250, 162-22G5, Oncogene Research Products, San Diego, USA), monoclonal mouse anti-MT3-MMP (IgG₁, 1:300, 117-10C6, Oncogene Research Products, San Diego, USA), polyclonal rabbit anti-MT5-MMP (1:200, M6684, Sigma-Aldrich, USA), monoclonal mouse anti-uPA (IgG₁, 1:50, 3689, American Diagnostica Inc, Greenwich, USA). Polyclonal rabbit anti-MT1-MMP (1:1000) and anti-uPAR (1:400) antibodies were produced and characterised as previously described [Collen *et al.*, 2003; Plaisier *et al.*, 2004; van Boheemen *et al.*, 1995; Koolwijk *et al.*, 1996]. The following secondary antibodies were used: biotinylated horse anti-mouse antibody (1:300, BA-2000, Vector, Burlingame, USA), biotinylated donkey anti-rabbit antibody (1:300, RPN1004, Amersham Biosciences, Buckinghamshire, UK).

Primary antibodies were applied overnight at 4°C followed by one hour incubation with biotinylated secondary antibody. Antibody binding was visualised using StreptABComplex/HRP, a streptavidin complexed with biotinylated peroxidase (K0377, DakoCytomation, Glostrup, Denmark) and NovaRED™ substrate (SK-4800, Vector, Burlingame, USA) according to the manufacturer's protocol. Only CD56 immunohistochemistry was stained with DAB (diaminobenzidine). All incubations were performed in 1% bovine serum albumin in PBS.

Sections were counterstained with Mayer's haematoxylin. Specificity of the immunohistochemical reaction was verified by the omission of the first antibody as well as using non-immune mouse IgG₁, concentration adjusted to primary IgG₁ concentration, and rabbit serum instead of first antibody. To evaluate the expression in extra-villous trophoblast, cytokeratin and target protein staining were performed on serial sections of 3 µm.

Evaluation of Immunohistochemical staining

Immunostaining of CD56+ and CD68+ cells was evaluated by counting the number of positive cells and the number of stromal cells in a 16 µm² grid (10 fields per tissue per patient). In this way, the mean percentage of positive cells per stromal cells and per µm² could be determined.

To limit the possible bias of subjectivity during the evaluation of the immunostaining, we have chosen to evaluate the immunostaining of the proteases and uPAR by calculating a staining index (SI): proportion of stained cells x staining intensity [Plaisier *et al.*, 2006; Plaisier *et al.*, 2007; Nap *et al.*, 2004]. The proportion of stained cells was expressed as 0, 1, 2 or 3, which marks positive staining signal in 0%, <10%, 10-50% or >50% of the cells of a particular cell type. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong, respectively). The minimum score was 0 and the maximum score 9. The average score of two independent observers was used to calculate the mean and total staining index (Mean and Total SI).

The Mean SI represents the protein expression per studied cell type. The Total SI represents the total protein expression per tissue and was calculated as the sum of the Mean SI of endothelial cells (EC), perivascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (SC) in DSE and DP samples. The Mean SI of EVT was also included in the Total SI of DB samples.

Statistical analysis

All parameters were compared between DSE and DP, between DSE and DB and between DP and DB within subjects. A general linear model for repeated measurements, repeated measures ANOVA, was performed to analyse the double paired data within subjects of early first-trimester decidua as well as to compare data between the early and late first-

trimester group (SPSS 11.5). Sphericity was corrected using the Greenhouse-Geisser correction. Where appropriate we used Friedman's test for non-parametric investigations of correlated observations. The used statistical analyses are described in legends and table footnotes. P-values of < 0.05 were considered significant.

RESULTS

mRNA levels of proteases in early first-trimester decidua

The expression of uPA, uPAR and the transmembrane spanning MT-MMPs were evaluated at the mRNA level. All these genes were expressed by the three decidual tissues (Table 2).

First, the amounts of specific mRNAs in decidual parietalis (DP) were compared to those in decidua secretory endometrium (DSE, 100%). mRNA levels of uPAR and all proteases, except MT5-MMP, were elevated in DP, but only MT1-MMP (171%, $p < 0.05$) and uPAR (185%, $p < 0.01$) were significantly induced. The mRNA levels of MT5-MMP, were comparable in DP and DSE (Table 2).

Subsequently, the mRNA levels in decidua basalis (DB) were compared to those DSE (100%). This comparison showed a reduction of MT5-MMP (84%, $p < 0.01$) and an induction of uPAR (208%, $p < 0.01$), MT1-MMP (198 %, $p < 0.05$), MT2-MMP (2329%, $p < 0.01$)

Table 2. Differential mRNA levels in early first-trimester pregnancies (percentage $\pm$ SD)^a.

	DSE ^b	DP	DB	DP ^c	DB
MT1-MMP	100 $\pm$ 14 %	171 $\pm$ 29 % [#]	198 $\pm$ 52 % [#]	100 %	155 $\pm$ 44 %
MT2-MMP	100 $\pm$ 33 %	308 $\pm$ 119 %	2329 $\pm$ 428 %*	100 %	1929 $\pm$ 316 %*
MT3-MMP	100 $\pm$ 30 %	150 $\pm$ 32 %	1023 $\pm$ 288 %*	100 %	768 $\pm$ 119 %*
MT5-MMP	100 $\pm$ 12 %	70 $\pm$ 8 %	16 $\pm$ 2 %*	100 %	25 $\pm$ 3 % [#]
uPA	100 $\pm$ 20 %	232 $\pm$ 74 %	125 $\pm$ 31 %	100 %	62 $\pm$ 16 % [#]
uPAR	100 $\pm$ 13 %	185 $\pm$ 22 %*	208 $\pm$ 27 %*	100 %	128 $\pm$ 19 %

^a mRNA levels were determined by RT-PCR in DSE, DP and DB of early 1st trimester (n=25) and compared within subjects. Mean cycle threshold (Ct) per gene: GAPDH 25-26, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29, uPA 31, uPAR 30. Cycle threshold were converted into ng/ μ l using a standard curve of total RNA and values were corrected for RNA input by calculating the ratio (ng/ μ l (target gene))/(ng/ μ l (GAPDH)). The 'repeated measures ANOVA' was performed to analyse the data.

^b The mRNA expression of DSE was set at 100% to compare mRNA levels in DSE with those in DP and DB. Fold induction was calculated as the ratio (DP)/(DSE) and (DB)/(DSE).

^c The mRNA expression of DP was set at 100% to compare DP with DB. Fold induction was calculated as the ratio (DB)/(DP).

[#] $p < 0.05$, * $p < 0.01$.

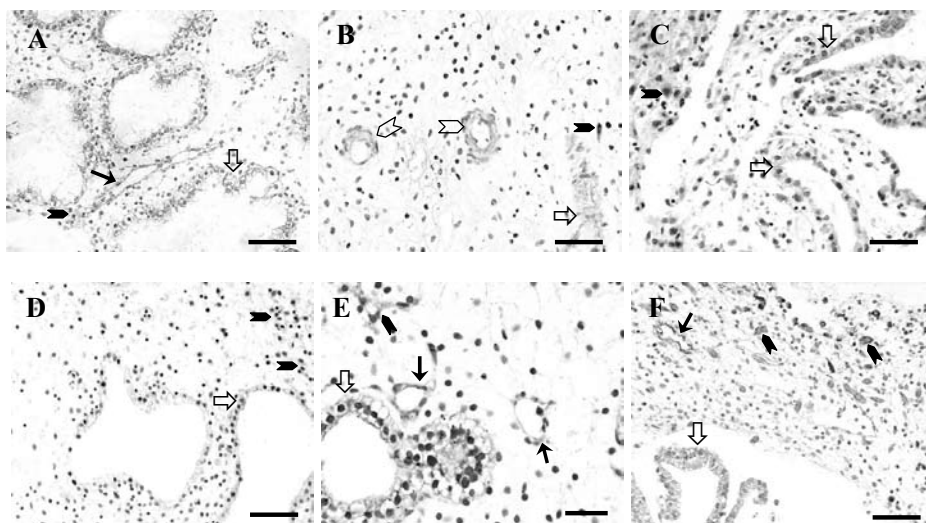


Figure 1. Protein levels of proteases in early first-trimester decidua.

The level of proteases was determined in DSE, DP and DB. **A.** MT1-MMP antigen was detectable in epithelial (open arrow), endothelium (closed arrow) and stromal cells (closed arrowhead) of DSE. **B.** MT2-MMP antigen in epithelium (open arrow), stromal cells (closed arrowhead) and pericytes (open arrowhead) of DB. **C.** Epithelial (open arrow) and stromal (closed arrowhead) MT3-MMP antigen in DB. Pericytes not present in this field. **D.** MT5-MMP antigen in epithelium (open arrow), and only dimly in stromal cells (closed arrowheads) of DP. **E.** uPA antigen in epithelium (open arrow), stromal cells (closed arrowhead) and endothelium (closed arrows) in DB. Pericytes not present in this field. **F.** Epithelial (open arrow), endothelial (closed arrows) and stromal cells (closed arrowheads) uPAR protein in DB. **A-D,F** Bar = 100 μm , **E.** Bar = 50 μm .

(see colour figures supplement)

and MT3-MMP (1023%, $p < 0.01$) in DB compared to DSE. The expression of uPA did not differ significantly (Table 2).

Finally, mRNA content in DB was compared to that in DP (100%). This comparison showed a reduced amount of MT5-MMP (75%, $p < 0.05$) and uPA (38%, $p < 0.05$) and markedly increased expression of MT2-MMP (1929%, $p < 0.01$) and MT3-MMP (768%, $p < 0.01$) in DB compared to DP. The concentrations of MT1-MMP and uPAR mRNA did not differ in DB compared to DP (Table 2).

Immunohistochemistry of proteases in early first-trimester decidua

The presence and cellular localisation of the six proteins involved in pericellular proteolysis were determined at the protein level in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues, and their presence was graded as mean staining indices (mean SI) per cell type and total staining indices (total SI) based on the sum of the cell types-dependent indices (Table 3, Figure 1, 2 and 3).

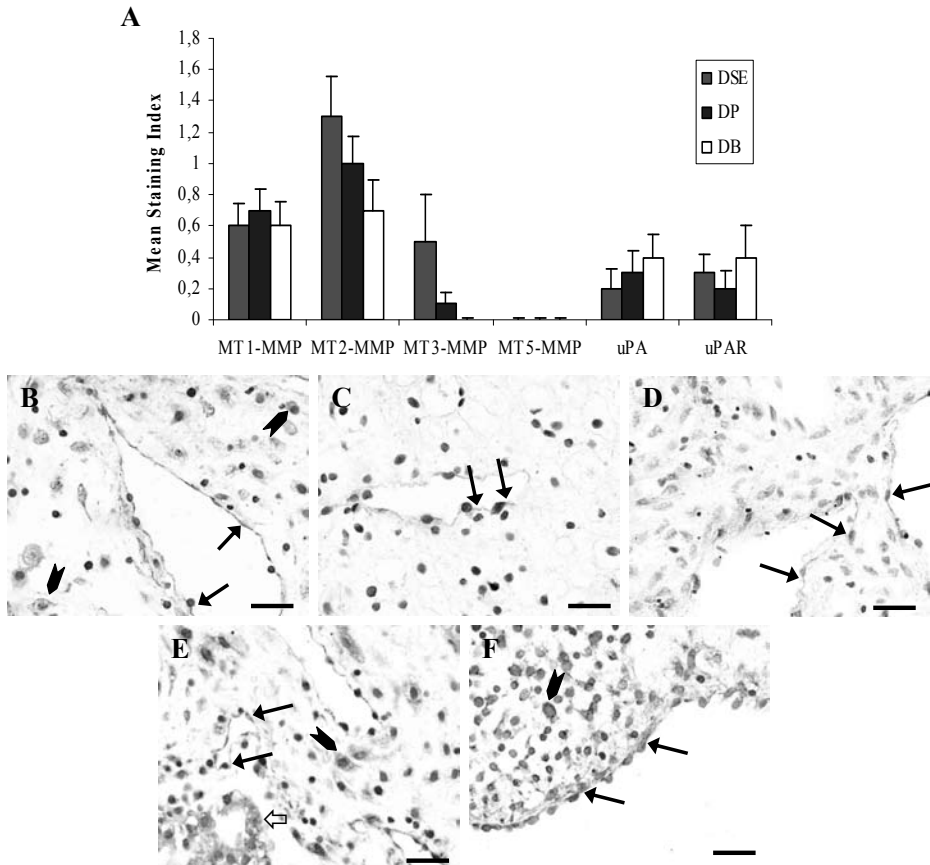


Figure 2. Protein levels of proteases in endothelium of early first-trimester decidua.

A. Presence of protease antigens in endothelium (EC) was expressed as the mean staining index $\pm$ SEM and **B-F** show examples of endothelial protease expression. **B.** Endothelial (arrows) and stromal (arrowheads) MT1-MMP was detectable in DB. **C.** MT2-MMP was detected in endothelium (arrows) of DB. **D.** Endothelial MT3-MMP antigen (arrows) in DSE. **E.** uPA antigen in endothelium (closed arrows), epithelium (open arrow) and stromal cells (arrowhead) in DB. **F.** uPAR in DB was detected in endothelial cells (arrows) and stromal cells (arrowheads). **B-F.** Bar = 50 μ m.

(see colour figures supplement)

The total staining indices in DSE were comparable to those in DP with only a higher total SI for MT1-MMP in DP (Table 3, $p < 0.05$). Secondly, when DSE and DB were compared, only an elevated total SI for uPA was found in DB compared to DSE (Table 3, $p < 0.05$). Thirdly, DB was compared to DP and this showed a similar pattern; only an elevated total SI for uPA in DB compared to DSE (Table 3, $p < 0.05$).

Analysis of the protein expression in the various cell types showed that the mean staining indices were most abundant in glandular epithelial cells, followed by perivascular smooth muscle and stromal cells (Table 3, Figure 1). Endothelium displayed weak ex-

pression of all proteins, except MT5-MMP. MT5-MMP was not detectable (Figure 2). Significant differences were not found between the three decidual tissues with regard to

Table 3. First-trimester protein expression in early versus late first-trimester pregnancies^a.

		EC ^b		PSMC ^b		GE ^b		SC ^b		EVT ^b		Total SI ^c	
		early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-
MT1	DSE	0.6	0.0*	1.8	0.3	4.0	2.0	0.6	0.2	NP	NP	7.0 [§]	2.5 [§]
	DP	0.7	0.0*	2.2	0.3	4.8	2.5	1.5	0.2	NP	NP	9.2	3.0*
	DB	0.6	0.0*	1.7	1.0	3.7	3.0	1.7	1.2	1.1	0.5	8.8	5.7
MT2	DSE	1.3	0.5	3.4	0.7	4.2	3.5	1.1	0.0	NP	NP	10.0	4.7*
	DP	1.0	0.3*	3.7	1.5	4.3	2.7	1.8	0.5	NP	NP	10.8	5.0*
	DB	0.7	0.4	2.3	1.0	3.7	3.8	1.7	2.2	1.0	1.3	9.4	8.7
MT3	DSE	0.5	0.0	2.2	0.7	4.8	1.0	0.3	0.0	NP	NP	7.8	1.7*
	DP	0.1	0.0	2.4	0.7	4.9	1.0	1.5	0.0	NP	NP	8.9	1.7*
	DB	0.0	0.0	1.6	1.0	5.2	1.0	1.7	0.0	1.2	0.0	8.8	2.0*
MT5	DSE	0.0	0.0	0.3	0.0	1.7	2.0	0.2	0.2	NP	NP	2.2	2.2
	DP	0.0	0.0	0.3	0.0	1.6	2.2	0.2	0.5	NP	NP	2.1	2.7
	DB	0.0	0.0	0.2	0.0	1.5	1.8	0.3	0.3	0.1	0.3	2.1	2.4
uPA	DSE	0.2	0.1	1.8	2.0	4.4	3.9	1.8	1.5	NP	NP	8.2	7.5
	DP	0.3	0.3	1.8	1.7	4.4	4.1	2.4	2.6	NP	NP	8.9	8.7
	DB	0.4	0.5	2.1	2.0	5.4	5.3	4.7	5.5	1.3	1.1	13.9 [§]	14.4
uPAR	DSE	0.3	0.5	0.9	1.8	5.6	3.8	1.8	1.5	NP	NP	8.6	7.6
	DP	0.2	0.0	0.7	1.7	5.5	4.3	1.6	1.7	NP	NP	8.0	7.7
	DB	0.4	0.8	0.7	2.2	5.4	5.0	2.7	2.5	0.9	1.2	10.1	11.7

^a Specific antigens of pericellular acting proteases in DSE, DP and DB were determined by immunohistochemistry, scored per cell type and compared within subjects. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis within groups and the general linear model was used to analyse differences between the two groups.

^{b,c} The staining was expressed as mean staining indices per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), perivascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue.

NP = not present. [§] $p < 0.05$ in DSE versus DP and $p < 0.05$ in DB versus DP and DSE in the early first-trimester group. * $p < 0.05$ in late vs early first-trimester decidua.

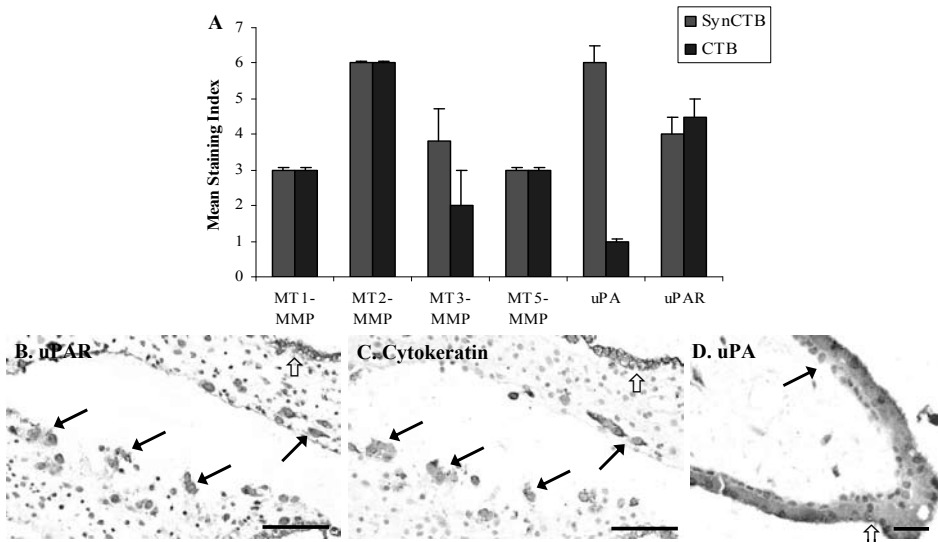


Figure 3. Protein levels of proteases in villous and extra-villous trophoblasts (EVT) of early first-trimester decidua.

The presence of protease antigens in villous and extravillous trophoblasts in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Immunostaining in syncytio- and cytotrophoblasts (synCTB and CTB) was expressed as the mean staining index $\pm$ SEM. **B.** and **C.** uPAR and cytokeratin antigens in serial expression in EVT (closed arrows) and epithelium (open arrows). **D.** uPA antigens in syncytiotrophoblast (open arrow) and not in cytotrophoblast (closed arrow). **B-D.** Bar = 50 μ m.

(see colour figures supplement)

endothelial protein expression. However, it should be noted that MT3-MMP was present in the endothelium of DSE, but largely reduced in DP and undetectable in those cells of DB. The same pattern was also displayed by endothelial MT2-MMP and by the staining of MT2- and MT3-MMP in smooth muscle cells (Table 3, Figure 1 and 2).

All proteases studied were present in extra-villous trophoblasts, although MT5-MMP stained only dimly. Furthermore, all proteases were detectable in villous trophoblasts; uPAR, MT1-, MT2-, MT3- and MT5-MMP were moderately expressed in both syncytiotrophoblasts as cytotrophoblasts. The expression of uPA was weak in cytotrophoblasts, while it was abundantly present in syncytiotrophoblasts (Table 3, Figure 3).

Proteases in early versus late first-trimester decidua

The mRNA levels of proteases in late first-trimester decidua were compared to those in early first-trimester decidua (100%, Table 4). The levels of MT1-MMP (206%, $p < 0.02$) and MT2-MMP (1047%, $p < 0.02$) mRNAs were elevated, whereas MT3-MMP mRNA content was reduced (62%, $p < 0.05$) in DSE of late compared to early first-trimester pregnancies. Comparison of DP between both groups showed elevated mRNA levels of MT1-MMP

Table 4. mRNA expression in late first-trimester pregnancies compared to early first-trimester pregnancies (100%).^a

	Late 1st DSE ^b	Late 1st DP ^b	Late 1st DB ^b
MT1-MMP	206 %*	215 %*	150 %
MT2-MMP	1047 %*	80 %	110 %
MT3-MMP	38 % [#]	23 % [#]	92 %
MT5-MMP	111 %	98 %	107 %
uPA	131 %	205 %	168 %
uPAR	160 %	207 %*	137 %

^a mRNA levels was determined by RT-PCR in DSE, DP and DB of early and late first-trimester (n=25 and n=8 respectively) and compared within subjects. The 'repeated measures ANOVA' was performed to analyse the data.

^b The mRNA levels in DSE, DP and DB of early first- trimester decidua were set at 100% to compare with mRNA levels in DSE, DP and DB of late first-trimester decidua.

*p<0.02, [#] p<0.05

(215%, p<0.02) and uPAR (207%, p<0.02) and reduced mRNA expression of MT3-MMP (77%, p<0.05) in late first-trimester tissues. The mRNA levels of these proteases in DB showed no differences between the two groups (Table 4).

At the protein level, total and mean staining indices of MT1- and MT2- MMP were reduced in DSE and DP of late first-trimester compared to early first-trimester (p<0.05). The total and mean SI of MT3-MMP were reduced in all cell types of all tissues in late first-trimester decidua (Table 3, p<0.05).

The mean SI's of MT1-, MT2- and MT3-MMP in endothelium were reduced in late versus early first-trimester DSE, DP and DB. Strikingly, endothelial MT1 and MT3-MMP expression was not even detectable in late first-trimester decidua. uPA and uPAR displayed comparable staining indices in endothelial cells at the two time points, while MT5-MMP was absent in these cells in both conditions (Table 3).

Analysis of uNK cells (CD56 +) and macrophages (CD68 +)

The presence of CD56 and CD68 antigens was determined in DSE, DP and DB of both the early and late first-trimester group and expressed as the percentage positive cells per total number of stromal cells.

The CD56 staining showed inhomogeneous staining, mainly localised around vessels and glands, which was corrected for by analysing various samples per patient. The number of CD56+ (uNK) cells in DP and DB was comparable, $18 \pm 6\%$ and $21 \pm 4\%$ respectively, of the total number of stromal cells. Both DP and DB showed a significantly larger number of CD56+ cells than DSE ($9 \pm 2\%$, p<0.05). No significant differences were detected between early and late first-trimester decidua (Figure 4 A and C).

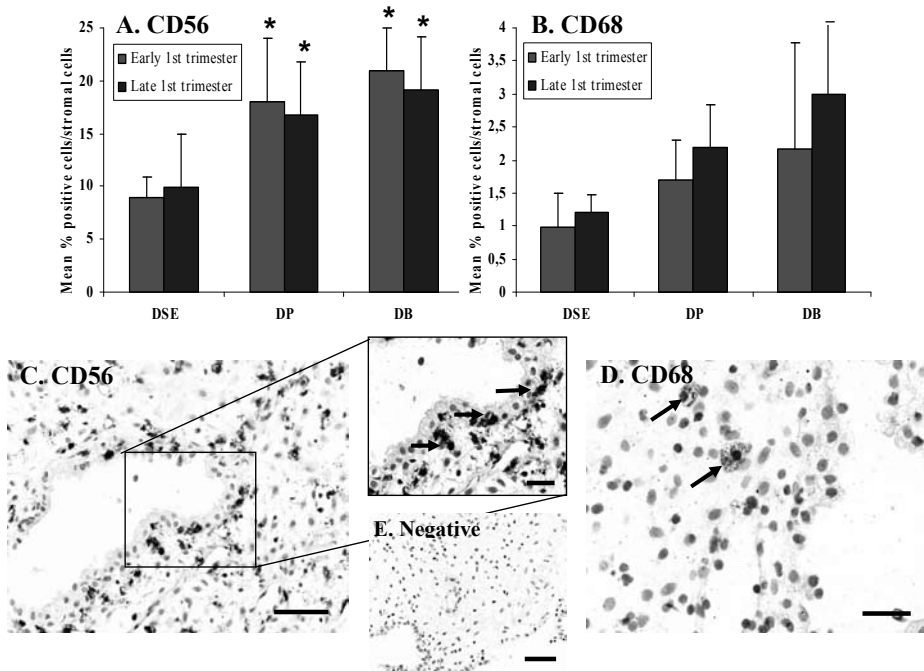


Figure 4. CD56- and CD68- positive cells in early and late first-trimester decidua.

A. CD56-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean $\pm$ SD). **B.** CD68-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean $\pm$ SD). **C.** CD56 expression in decidua basalis, bar = 100 μ m. Blow up shows CD56+ cells (arrows) surrounding a vessel and gland, bar = 50 μ m. **D.** CD68-positive cell (arrow) in decidua parietalis, bar = 50 μ m. **E.** Negative control, bar = 100 μ m. * $p < 0.05$ versus DSE (see colour figures supplement)

The CD68 staining showed scattered positive cells throughout the tissues (range 1.0–2.2%). Although the number of macrophages appeared to increase from DSE to DP to DB, these differences were not significant. The two time points also showed no significant differences (Figure 4 B and D).

DISCUSSION

The present study demonstrates that the mRNA and protein content of the pericellular-acting MT-MMPs and uPA/uPAR varied between decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP). Furthermore, the percentage of CD56-positive cells was two-fold lower in DSE than in DB and DP. A comparable increase in macrophages did not reach statistical significance. It is likely that the differential expression of the proteases is mainly due to the different effects of pregnancy-

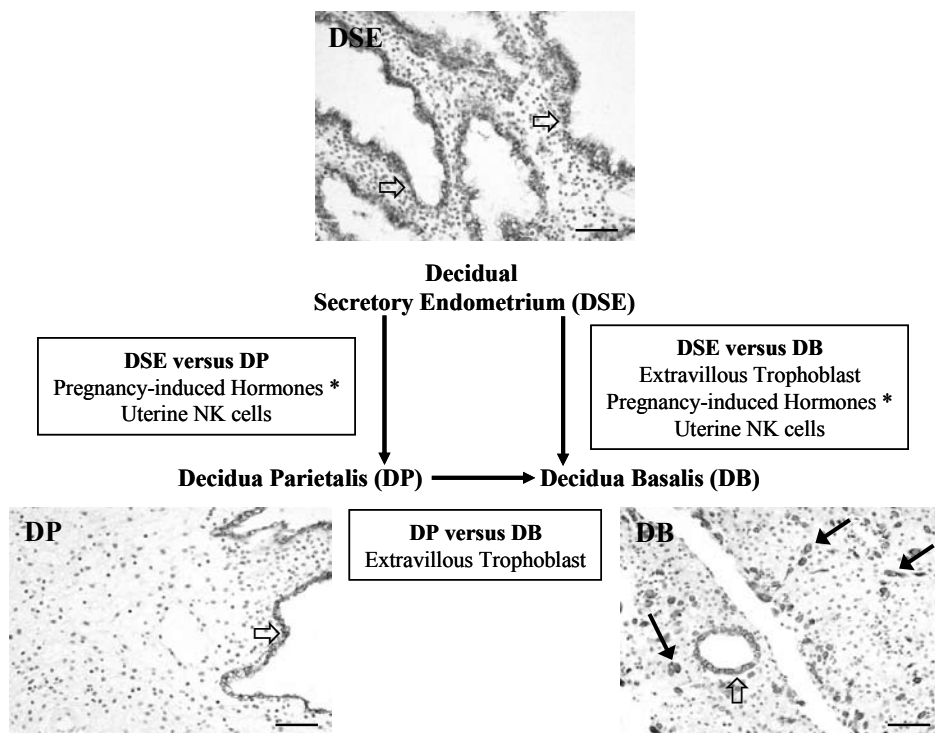


Figure 5. Human first-trimester decidual tissues.

Representation of the influences of immune cells, extra-villous trophoblast and pregnancy-induced hormones (*hCG, oestradiol and progesterone) on first-trimester decidual tissues. Differentiation between decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB), was obtained by HPS and cytokeratin staining. DSE and DP express cytokeratin in glandular epithelium (open arrows), whereas cytokeratin is also expressed in EVT (closed arrows) of DB. Bar = 100 μ m. (see colour figures supplement)

induced hormones (hCG, oestradiol and progesterone), the extra-villous trophoblast and/or immune cells on the three decidual tissues. Our study suggests that uPAR and MT1-MMP are enhanced by pregnancy-induced hormones, and possibly uNK cells, and uPA, MT2-, MT3-MMPs by EVT. In addition, MT5-MMP appears reduced by the EVT. Finally, we show differences in protease expression and presence of immune cells as gestation progresses.

Decidual tissues and their contributors

Endometrial adaptation to pregnancy is induced by pregnancy-induced hormones, i.e. hCG, oestradiol and progesterone, immune cells, and the extra-villous trophoblasts (EVT). Pregnancy-induced hormones are involved in the development of decidua parietalis (DP) from the decidual secretory endometrium (DSE), while the additional presence of the EVT induces the generation of the decidua basalis (DB). This may imply that

differences in protease expression between DB and DP are due to interactions of the decidual tissue with the trophoblast. However, NK cells have also been suggested to vary between term DB and DP [Sindram-Truijillo *et al.*, 2003]. As these uNK cells are known to produce many chemokines and angiogenic growth factors [Hanna *et al.*, 2006; Li *et al.*, 2001a; Lash *et al.*, 2006], the uNK cells may also be able to influence the production of proteases in DB and DP. Other investigators were unable to detect differences in uNK numbers in DP and DB [Khong 1987; Haller *et al.*, 1995; Bulmer, Lash 2005]. Indeed, in our study we also found similar amounts of uNK cells in DB and DP being 18% and 21%, respectively. This excludes an involvement of the number of uNK on the difference between DB and DP, but possible differences in the functional properties of the uNK cells in DB and DP cannot yet be excluded.

The percentual contribution of uNK cells and macrophages to the immune cell and overall stromal cell populations were comparable with those reported by other investigators [Tuckerman *et al.*, 2007; Quenby *et al.*, 1999; Bulmer *et al.*, 1991]. Interestingly, the amounts of uNK cells were twofold higher in DB and DP than in DSE (9%). A comparable increase in macrophages did not reach statistical significance (range 1.0–2.2% of total stromal cells). To our knowledge the difference between DSE and DB/DP has not been systematically investigated before.

The foregoing information is summarised in Figure 5, which presents a model that describes the influences of uNK cells, extra-villous trophoblast and pregnancy-induced hormones on DSE, DP and DB. This model enables us to discuss changes that occur independently of trophoblast invasion, and those that are mainly induced by pregnancy-induced hormones and/or uNK cells, from changes influenced by the invasive extra-villous trophoblast [Plaisier *et al.*, 2007]. The effects of uNK cells require further functional studies.

Pericellular proteases in early first-trimester decidua

MT1-MMP mRNA and protein expression were significantly induced in decidua basalis and parietalis (DB and DP) as compared to decidual secretory endometrium (DSE), but no differences were found between DB and DP. This suggests that decidual MT1-MMP expression is induced rather by pregnancy-induced hormones and/or uNK cells than by invasive trophoblasts (EVT). This confirms an earlier study which showed that invasive trophoblasts are not required for the induction of MT1-MMP [Nakano *et al.*, 2001]. Furthermore, the apparent induction of MT1-MMP in DP and DB might be explained by the influx of NK cells in those tissues, as NK cells are known to contain MT1-MMP [Albertson *et al.*, 2000]. MT1-MMP may also play a role in uNK cell migration.

Although the regulation of MT1-MMP expression seems unaffected by the EVT, MT1-MMP is expressed by the EVT. This suggests a role in trophoblast invasion, which indeed has been reported previously [Hurskainen *et al.*, 1998; Nawrocki *et al.*, 1996; Tanaka *et*

al., 1998]. Finally, the fact that MT1-MMP is induced in various cell types of decidua parietalis and basalis also points to a role in decidual remodelling and/or vascularisation. MT2-MMP and MT3-MMP mRNA levels were increased and MT5-MMP mRNA expression was reduced in DB compared to both DSE and DP. This suggests that these mRNA levels are mainly influenced by the presence of the EVT. Together with their antigens being present in invasive trophoblasts, this suggests a role in trophoblast invasion. However, no significant differences were detected in the overall presence of MT2-, MT3- and MT5-MMP proteins between the three types of decidua. MT2-MMP and MT3-MMP protein levels were reduced in endothelium and perivascular smooth muscle cells of DP and DB as compared to DSE. Endothelial protein expression of the other proteases showed no differences between the tissues. Interestingly, we recently determined the vascularisation pattern in serial sections of the same specimens, which showed highly enhanced vascularisation at the implantation site. Comparison of these data with the endothelial expression of MT2- and MT3-MMPs shows that the amounts of these endothelial MT-MMPs correlated well with the number of vascular structures/mm² and correlate inversely with the luminal surface of these vascular structures [Plaisier *et al.*, 2007]. Furthermore, experiments with endometrial endothelial cells in vitro indicated that MT2- and MT3-MMP are potential candidates for regulation of endometrial angiogenesis [Plaisier *et al.*, 2006; Plaisier *et al.*, 2007]. These data leads us to hypothesise that MT2-MMP and MT3-MMP play a role in vascular adaptation to pregnancy at the implantation site.

UPA and uPAR were detected in the various decidual cell types. In contrast to cycling secretory endometrium, in which endothelial and stromal cells contained uPA antigen and glandular epithelium did not [Koolwijk *et al.*, 2001], glandular epithelial cells are positive for uPA antigen in first-trimester tissues together with the endothelium and part of the stromal cells. The expression of uPAR largely follows the pattern of uPA and is clearly present in endothelial cells. The presence of uPAR in EVT is confirmed by previous studies and suggests a role in trophoblast invasion [Floridon *et al.*, 1999; Pierleoni *et al.*, 1998].

Interestingly, the expression of uPA mRNA and antigen showed different patterns. The mRNA levels suggest a stimulatory influence of pregnancy-induced hormones and/or uNK cells, as well as an inhibiting influence of the EVT. This latter observation may be explained by the downregulation of uPA mRNA by hCG, an important product of trophoblasts [Salamonsen 1999; Yagel *et al.*, 1993]. However, uPA was increased at the protein level in all cell types in DB compared to DP and DSE. The presence of uPA is not only determined by the ability of the cells to produce uPA, but also by their content of uPAR, which binds and internalises uPA in complex with its inhibitor PAI-1 [Blasi, Carmeliet 2002; Kroon *et al.*, 1999]. Furthermore, uPA and uPAR are present in a part of

the stromal cells that probably include the uNK and other leukocytes. This is in concert with the notion that the uPA/uPAR system is used, probably together with MT-MMPs, by leukocytes for their invasion into tissues [Albertsson *et al.*, 2000; Al Atrash *et al.*, 2001]. Moreover, the increase in uNK cells may thus contribute to the increased uPA antigens observed in DB as compared to DSE.

Early versus late first-trimester pregnancies

The expression of uPA and uPAR mRNA and proteins showed no differences between early and late first-trimester decidua, indicating that the uPA/uPAR system remains under a constant regulation as gestation progresses. No data are available in literature regarding uPA/uPAR during these time points. In contrast, the differential mRNA expression of the matrix metalloproteinases suggests that these proteases are regulated over time. The overall amounts of MT₁-, MT₂- and MT₃-MMP were reduced in all decidual tissues in late compared to early first-trimester pregnancy.

Furthermore, the MT₁- MT₂- and MT₃-MMP antigen expression in endothelium and perivascular smooth muscle cells was also reduced in all types of decidua of late first-trimester pregnancy. We recently determined the vascularisation pattern in serial sections of the same early and late first-trimester decidua samples and this showed that vascularisation is regulated as gestation progresses. The endothelial expression of MT₁-, MT₂- and MT₃-MMPs correlated well with the differences in vascularisation between early and late first-trimester decidua [Plaisier *et al.*, 2007]. These data again point to a role for these MT-MMPs in determining the degree and pattern of neovascularisation as gestation progresses.

Conclusion

Decidual adaptation is important in the development of a healthy pregnancy. We showed that the expression of various pericellular-acting proteases varied between decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP). Furthermore, uNK cells (CD56+) and macrophages (CD68+) were present in all decidual tissues and only the percentage of uNK cells differed between the tissues. The differential presence of several proteases enabled hypothesising about their regulation, i.e. by pregnancy-induced hormones, the EVT and/or immune cells, as well as their functions, e.g. in immune cell infiltration, trophoblast invasion and/or vascularisation (Figure 5). UPAR and MT₁-MMP expression appeared regulated by pregnancy-induced hormones and/or uNK cells, whereas the presence of uPA, MT₂-, MT₃-, and MT₅-MMP appeared regulated by the extra-villous trophoblast. All proteases were expressed by the EVT and might be involved in trophoblast invasion. MT₂- and MT₃-MMP are known candidates in regulating angiogenesis and together with their differential expression this suggests that they may not only support decidual remodelling and trophoblast invasion during

implantation but also, partially, account for the vascular changes at the implantation site. Finally, we show differences in protease expression as gestation progresses. A better understanding of decidualisation may contribute to new insights in currently non-explicable pathological events associated with pregnancy, such as miscarriages and pre-eclampsia.

Chapter 7

**Decidual vascularisation and the expression of
angiogenic growth factors and proteases in
first-trimester spontaneous abortions.**

M. Plaisier
I. Dennert
E. Rost

P. Koolwijk
V.W.M. van Hinsbergh
F.M. Helmerhorst

ABSTRACT

Objective: Decidual vascular development is important for implantation. This study analysed decidual vascular adaptation to implantation in correlation with miscarriage in decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB) of miscarriage patients and matched controls.

Methods: Decidua was obtained during first-trimester termination of pregnancy (controls) and vacuum-aspiration in case of missed abortion (cases). Vascularisation and the expression of VEGF-A, PlGF, flt-1, KDR, Angiopoietin-1, Angiopoietin-2, TIE-2, and membrane-type matrix metalloproteinases MT1-, MT2-, MT3- and MT5-MMP were determined at mRNA and protein level. uNK cells (CD56), macrophages (CD68), proliferation (Ki67) and apoptosis (activated-caspase-3) were evaluated in consecutive sections.

Results: Decidual vascularisation showed differences between cases and controls, i.e. fewer vessels with larger circumference in cases. This correlated with the differential expressions of various factors at mRNA/antigen level and with increased endothelial flt1, KDR, MT2- and MT5-MMP expression miscarriage patients. The differences between cases and controls were probably not based on altered proliferation and/or apoptosis, since Ki67 and active caspase-3 showed comparable expression levels in both groups. Although DB of cases and controls showed similar amounts of CD56- and CD68-positive cells, the missed abortion group did show elevated levels of CD56 in DSE and of CD68 in DSE and DP compared to the control group.

Conclusion: The differences in vascularisation and in the expression of angiogenic factors and proteases between both groups suggest a correlation between decidual vascularisation and the occurrence of miscarriages.

INTRODUCTION

Decidual vascular adaptation to implantation is important for the success of pregnancy and already starts during the receptive secretory phase of the menstrual cycle. Decidualisation, vascular remodelling and invasion of immune cells are prominent processes in the first weeks after fertilisation [Bulmer *et al.* 1991; Salamonsen *et al.* 2003; Smith 2000]. Immune cells increase from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70% of these leucocytes are uterine-NK (uNK) cells and 10% are macrophages [Bulmer *et al.* 1991].

Vascular adaptation includes (pseudo-)vasculogenesis, arterial remodelling, and angiogenesis, the formation of new vessels out of existing ones [Burton *et al.* 1999; Pijnenborg *et al.* 1983]. Angiogenesis is characterised by increased vascular permeability, endothelial cell proliferation and migration, and is regulated by various growth factors, e.g. vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and angiopoietins, and proteases like the membrane-type matrix metalloproteinases [Carmeliet 2003; Pepper 2001]. Disturbances in vascular development may play a role in the pathogenesis of miscarriages [Vailhe *et al.* 1999; Vuorela *et al.* 2000; Zygmunt *et al.* 2003].

The angiogenic growth factors VEGF-A and PlGF are extensively studied in the vascular development of the placenta [Plaisier *et al.* 2007; Smith 2000; Vuorela *et al.* 1997]. VEGF is one of the earliest genes activated during pre-implantation embryo development and is produced by maternal decidual cells and by the invading blastocyst and trophoblast [Kapiteijn *et al.* 2006; Jackson *et al.* 1994; Plaisier *et al.* 2007]. VEGF is a potent inducer of angiogenesis and interacts with the Flt-1 (VEGFR-1) and KDR (VEGFR-2) to promote endothelial cell proliferation, migration and vascular permeability. KDR is generally recognized as the central VEGF receptor in angiogenesis, while Flt-1 plays a supporting role. During pregnancy, also a soluble variant of Flt-1 (sFlt-1) is formed, which quenches VEGF and thereby limits its activity [Maynard *et al.* 2003].

PlGF shares biochemical and functional features with VEGF, but only interacts with Flt-1. PlGF and VEGF have synergistic effects regarding angiogenesis, however PlGF induced vessels are suggested to be more mature and stable than vessels induced by VEGF alone [Carmeliet *et al.* 2001]. PlGF is abundantly expressed in human placenta and may be an important paracrine regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Sherer, Abulafia 2001; Plaisier *et al.* 2007].

The angiopoietins and their receptor TIE-2 are also known for their regulating capacities regarding angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Angiopoietin-1 (Ang-1) maintains vessel integrity and probably plays a role in the later stages of vascular remodelling [Geva *et al.* 2000]. Angiopoietin-2 (Ang-2) is a

functional antagonist of Ang-1 and leads to loosening of cell-cell interactions and allows access to angiogenic inducers like VEGF [Geva *et al.* 2000].

Ang-1, Ang-2 and TIE-2 are detected in various maternal cells, endovascular trophoblasts, and (syn-) cytotrophoblasts during human first-trimester pregnancy. These findings suggest an additional role for angiopoietins in regulating trophoblast behaviour in the development of uteroplacental circulation [Plaisier *et al.* 2007; Dunk *et al.* 2000; Zhang *et al.* 2001]. An association of the angiopoietins with miscarriage has not been described, but reduced endothelial TIE-2 expression has been linked to the occurrence of miscarriage [Vuorela *et al.* 2000].

The membrane-associated localisation of MT-MMPs makes them suited for pericellular proteolysis [Hotary *et al.* 2000; van Hinsbergh *et al.* 2006]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24), since these MT-MMPs have the proteolytic potential to induce capillary tube formation. The GPI-anchored MT4- and MT6-MMP were unable to do so [Hotary *et al.* 2000].

MT1-MMP is the best known MT-MMP and promotes cell migration, angiogenesis and tumour metastasis [Visse, Nagase 2003]. MT2- and MT3-MMP are less well studied but are also involved in cell migration and invasion. MT1-, MT2- and MT3-MMP induce angiogenesis *in vitro* and MT2- and MT3-MMP may even be potential regulators of endometrial angiogenesis *in vivo* [Hotary *et al.*, 2000; Plaisier *et al.* 2004/2006]]. MT5-MMP is known for the induction of embryonic brain development and axonal growth [Llano *et al.*, 1999].

MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, stromal cells, and the extra-villous trophoblast [Bjorn *et al.* 2000; Nawrocki *et al.* 1996; Curry, Osteen 2003]. These MT-MMPs are assumed to regulate trophoblast invasion during implantation [Salamonsen *et al.* 2003]. To which extent migration of other cell types, e.g. immune and endothelial cells, is also regulated by MT-MMPs remains to be seen.

Vascular adaptation to implantation has mainly been studied with regard to physiology [Craven *et al.* 1999; Kam *et al.* 1999]. Recently, the potential regulation of decidual vascularisation by both the extra-villous trophoblast (EVT) and pregnancy-induced hormones has been described. Moreover, the differential expression of VEGF-A, the angiopoietins, PlGF, Flt-1, MT2- and MT3-MMP suggested their role in regulating the vascular adaptive process in uncomplicated first-trimester pregnancies [Plaisier *et al.* 2007/2008]. Few studies have addressed the question whether vascularisation and its regulatory factors are involved in the pathogenesis of first-trimester complications, like miscarriage. A higher decidual vessel density was correlated with missed abortions [Vailhe *et al.* 1999]. Also, differential antigen expression of VEGF and its receptors has been correlated to re-

current miscarriage [Vuorela *et al.* 2000]. Moreover, elevated uterine MMP and reduced TIMP expression has been linked to failed implantation [Jokimaa *et al.* 2002].

The present study determined decidual vascular adaptation to implantation in correlation with failure of implantation, i.e. missed abortion. Vascularisation pattern and the expressions of the above described angiogenic factors and proteases were determined in three first-trimester decidual tissues, decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB) of miscarriage patients and matched controls. The comparison of these parameters between the two groups enabled hypothesising about their correlation with the occurrence of miscarriages.

MATERIALS AND METHODS

Study group

The study was approved by the ethics committee of the Leiden University Medical Centre and informed consent was provided by all study subjects.

Decidua samples in the case group were obtained during vacuum-aspiration from missed abortion patients. Missed abortion was diagnosed based on ultrasound examination and absence of clinical miscarriage symptoms. Aspirated tissue was analysed by an independent pathologist and samples with pathologic abnormalities were excluded. Patients with pathological conditions known to interfere with implantation, like haemostatic abnormalities, auto-antibodies, etc, were also excluded. Gestational age at foetal death was determined by ultrasound.

Decidua samples in the control group were obtained from women with a healthy, viable intrauterine gravidity, undergoing vacuum aspiration in case of a legal voluntarily abortion. Foetal cardiac activity and gestational age were confirmed by ultrasound. Women with symptoms of miscarriage, with underlying illnesses, and cases with discrepancy between the ultrasound-determined gestational age and last day of menstruation were excluded in both control and case group.

Cases and controls were matched based on maternal and gestational age. These parameters and number of previous pregnancies and miscarriages did not differ significantly between the cases and the controls. Patient characteristics of the study groups are given in Table 1.

Tissue Samples

Decidua samples were obtained from the aspirated tissue (vacuum aspiration), fixed in formaldehyde overnight and embedded in paraffin. Hematoxylin Phloxin Safrane (HPS) and anti-cytokeratin staining were performed. Hematoxylin (50 g potassium aluminium sulfate, 1 g hematox, 500 mg citric acid, 25 g chloralhydrate, 200 mg NaJO₃ in 1000 ml

Table 1. Characteristics of the study subjects.

	Controls (n=16)	Missed Abortions (n=11)
Maternal Age (years) ^a	31.0 ± 1.3	33.3 ± 1.6
Gestational age (days) ^a	50.4 ± 2.7	53.8 ± 3.3
Gestational age (weeks) ^a	7.2 ± 0.4	7.7 ± 0.5
Number of previous pregnancies ^a	1.5 ± 0.4	2.6 ± 0.5
Number of previous miscarriages ^a	0.3 ± 0.1	0.8 ± 0.4

^a All $p > 0.05$.

aqua dest) stains nuclei and calcium blue. Phloxin (0.25 g phloxin in 100 ml aquadest) stains erythrocytes, cytoplasm, fibrin and muscle red. Safrane (3 g safrane in 1000 ml 100% alcohol) stains calcium free bone, cartilage and collagen yellow.

The HPS staining was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (see section immunohistochemistry). DSE, DP and DB were obtained from the same curettement and therefore originate from the same depth and area of the uterine wall. Only subjects with at least two complete sets of DSE, DB and DP were included (n=11 in missed abortion group and n=16 in control group). Serial sections of the paraffin embedded tissue samples were used for all experiments and all parameters were compared between tissues within subjects.

RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples [Plaisier *et al.* 2007]. Several samples per tissue per patient were used and these samples contained a proportionate amount of cells and cell types. In short, 5 µm sections were mounted on RNase-free glass slides. The first and last sections were used to verify the presence of the tissues of interest. The other sections were deparaffinised and the tissues of interest, DSE, DP or DB (without villous tissue), were dissected and dissolved in 190 µl lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% SDS) and 10 µl proteinase K (20 mg/ml proteinase K, Life-Technologies Gibco BRL, Gaithersburg) for 18 hours at 60°C. Subsequently, 400 µl Solution D (4 M guanidium isothiocyanate, 0.75 M sodium citrate, 10% sarkosyl and 2-mercapto-ethanol) was added and RNA was isolated [Plaisier *et al.* 2007].

RNA quantity and quality were analysed in a spectrophotometer (Nanodrop ND-1000). The OD_{260/280} of the RNA samples ranged between 1.85 and 2.00. Reverse transcription was performed with 1 µg total RNA, random primers and cDNA synthesis kit according to the manufacturer's protocol and the obtained 32 µl cDNA was diluted 1:3 (Ready-to-go You-Prime first strand beads, Amersham Biosciences, Buckinghamshire, UK).

Real-time RT-PCR

The mRNA levels were quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R1 (Flt-1), VEGF-R2 (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2, MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP. glycerlyaldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene. Other genes, β -actin, β_2 -microglobulin and cyclophilin, were also used as reference genes and showed comparable results (data not shown, all primer/probe sets purchased from Applied Biosystems, Foster City, CA USA).

RT-PCR reactions for target gene/GAPDH pairs were performed in 12.5 μ l reactions, containing 2.5 μ l cDNA solution, using mastermix (RT-QP2X-03, Eurogentec, Maastricht, The Netherlands), DNase free water and the above described primer/probe sets. Reactions were performed in duplicate, expressed in cycle times (Ct) and quantified into ng/ μ l using a standard curve of total RNA. The amount of RNA expression of DSE was set at 100% to compare DSE with DP and DB and the gene expression of DP was set at 100% to compare DP with DB. Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry

Serial sections were deparaffinised, endogenous peroxidase was quenched with 3% H_2O_2 /methanol and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in trypsin was used for the detection of CD34, TIE-2, MT2- and MT5-MMP. Heat retrieval in 0.1 M citrate buffer was used for the detection of cytokeratin, Ang-2, Ki67, CD56 and CD68.

The first antibodies are described in Table 2. Polyclonal rabbit anti-MT1-MMP was produced and characterised as previously described [Plaisier *et al.* 2004]. The following second antibodies were used: biotinylated horse anti-mouse antibody (1:300, BA-2000, Vector), biotinylated donkey anti-rabbit antibody (1:300, RPN1004, Amersham Biosciences), biotinylated rabbit anti-goat (1:300, E-0466, DakoCytomation).

Primary antibodies were applied overnight at 4°C followed by one hour incubation with biotinylated secondary antibody. Antibody binding was visualised using StreptABComplex/HRP, a streptavidin complexed with biotinylated peroxidase (Ko377, DakoCytomation, Glostrup, Denmark) and NovaRED™ substrate (SK-4800, Vector, Burlingame, USA) according to the manufacturer's protocol. Only CD56 immunohistochemistry was stained with DAB (diaminobenzidine). All incubations were performed in 1% bovine serum albumin in PBS.

Sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reaction was verified by the omission of the first antibody as well as using non-immune mouse IgG₁, concentration adjusted to primary IgG₁ concentration, and

Table 2. Specifications of the primary antibodies.

Antigen	Source	Clone-number	Species	Pre-treatment	Dilution
Cytokeratin	DakoCytomatation	Z0622	Rabbit	Citrate HR	1:2000
CD34	Novocastra	QBEnd/10	Mouse (IgG ₁)	0.1% Trypsin	1:1000
CD56	Monosan	MONX 10844	Mouse (IgG ₁)	Citrate HR	1:50
CD68	DakoCytomatation	M0814	Mouse (IgG ₁)	Citrate HR	1:200
Ki67	Novocastra	Ncl-L-Ki67-MM1	Mouse (IgG ₁)	Citrate HR	1:50
Act Casp 3	Cell Signaling	9661	Rabbit	None	1:300
VEGF-A	Santa Cruz Tech	Sc-152 (A-20)	Rabbit	None	1:100
PIGF	Santa Cruz Tech	Sc-1880 (C-20)	Goat	None	1:50
Flt-1	Santa Cruz Tech	Sc-316 (C-17)	Rabbit	None	1:50
KDR	Santa Cruz Tech	Sc-6251 (A-3)	Mouse (IgG ₁)	None	1:50
Ang-1	Santa Cruz Tech	Sc-6319 (N-18)	Goat	None	1:100
Ang-2	Santa Cruz Tech	Sc-7016 (N-18)	Goat	Citrate HR	1:150
TIE-2	Santa Cruz Tech	Sc-9026 (H-176)	Rabbit	0.1% Trypsin	1:100
MT1-MMP	TNO QoL		Rabbit	None	1:1000
MT2-MMP	Oncogene	IM48L	Mouse (IgG ₁)	0.1% Trypsin	1:250
MT3-MMP	Oncogene	IM50L	Mouse (IgG ₁)	None	1:100
MT5-MMP	Sigma	M6684	Rabbit	0.1% Trypsin	1:200

goat and rabbit serum instead of first antibody. To evaluate the expression in extra-villous trophoblast, cytokeratin and target protein staining were performed on serial sections of 3 μm .

Evaluation of the vascularisation pattern

CD34-stained decidual sections were used to scan sequential fields [Vailhe *et al.* 1999]. Two to four samples of each type of tissue of each study subject were analysed and six fields per sample were scanned at 100x magnification. The area within the stained vessels and the number of vessels per field were analysed using an image analysis software (Qwin, Leica Microsystems) and these data were used to calculate the number of vessels per mm^2 , total vascular surface per mm^2 (mm^2/mm^2) and the luminal surface ($\mu\text{m}^2/\text{vessel}$).

Evaluation of Immunohistochemical staining

In order to avoid intensity differences in the staining we analysed control samples (16 donors) simultaneously with the samples of 11 cases. Immunostaining of Ki67, active Caspase-3 (aCasp-3), CD56 and CD68 was evaluated by counting the number of positive cells and total stromal cells in ten 16 μm^2 fields per tissue per patient. In this way, the mean percentages of positive cells per stromal cells and per μm^2 were determined. In

case of Ki67 and aCasp-3, the expression in endothelial and epithelial cells was also determined similarly.

Immunostaining of the other antigens was evaluated by calculating a staining index (SI): proportion of stained cells x staining intensity. The proportion of stained cells was expressed as 0, 1, 2 or 3, which marks positive staining signal in 0%, <10%, 10-50% or >50% of the cells of a particular cell population. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score 9 [Plaisier *et al.* 2007]. The average score of two independent observers was used to calculate the mean and total staining index (Mean and Total SI). The inter-observer variability was 6% for VEGF-A, 7% for PlGF, 11% for Flt-1, 10% for KDR, 5% for Ang-1, 6% for Ang-2, 9% for TIE-2, 4% for MT1-MMP, 14 % for MT2-MMP, 8 % for MT3-MMP, 7 % for MT5-MMP (mean variability 8%).

The Mean SI represents the protein expression per studied cell type. The Total SI represents the total protein expression per tissue and was calculated as the sum of the Mean SI of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (SC) in DSE and DP samples. The Mean SI of EVT was also included in the Total SI of DB samples.

Statistical analysis

Data are expressed as mean $\pm$ SEM. All parameters were compared between DSE and DP, between DSE and DB and between DP and DB within subjects. A general linear model for repeated measurements, repeated measures ANOVA, was performed to analyze the double paired data within the case and control group as well as to compare data between the case and control group. Where appropriate, the Friedman's test for non-parametric investigations of correlated observations and the wilcoxon test for paired samples were used to study differences within the two groups (SPSS 11.5). The used statistical analyses are described in legends and table footnotes. P-values of < 0.05 were considered significant.

RESULTS

The vascularisation pattern in cases and controls

Intense CD34 staining of vascular structures was observed in all decidual tissues of controls and missed abortion cases. Their vessel density as well as their total and luminal surfaces were quantified in all controls (n=16) and cases (n=11).

Controls

Vessel density was significantly elevated in DSE (116.1 ± 8.5 vessels/mm²) compared to DP (39.1 ± 3.3) and DB (30.9 ± 3.8); vessel density in DP and DB also differed significantly (all $p < 0.01$, Figure 1A). The total vascular surface was significantly smaller in DSE (0.021 ± 0.002 mm²/mm²) compared to DP (0.040 ± 0.004) and DB (0.055 ± 0.007). The total surface in DP differed significantly from that in DB (all $p < 0.01$, Figure 1B). The luminal surface was significantly smaller in DSE compared to DP (203.8 versus 1576.0 μm^2 /vessel) and DB (2928.1 μm^2 /vessel); this parameter was also significantly smaller in DP compared to DB (all $p < 0.01$, Figure 1C). Thus, larger but fewer vessels were present from DSE to DP to DB.

Cases versus Controls

The vessel density was significantly lower in DP and DB of cases compared to the controls; 29.6 vessels/mm² versus 39.1 in DP and 19.8 versus 30.9 in DB respectively (both $p < 0.05$, Figure 1A). The luminal surface of the cases was significantly higher in DP and DB of cases; 3604 μm^2 /vessel versus 1576 in DP and 5661 versus 2928 in DB respectively

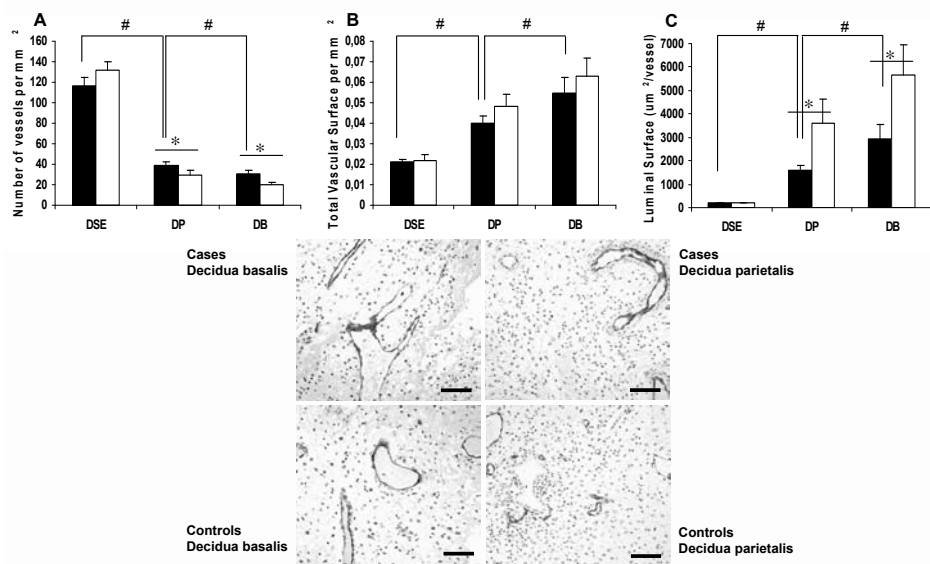


Figure 1. Vascularisation pattern in cases and controls.

The vascularisation pattern in decidual tissues of controls ($n=16$, black bars) and cases ($n=11$, white bars) was determined by image analysis of anti-CD34-stained sections. **A.** Vessel density (number of vessels per mm²), **B.** the total vascular surface (mm²/mm²) and **C.** the luminal surface (μm^2 /vessel) were calculated and expressed as mean $\pm$ SEM. * $p < 0.05$ within controls, * $p < 0.05$ in cases versus controls. The bottom panels show examples of vascularisation in decidual parietalis and basalis of cases and controls (control; left panels) and cases (missed abortions; MA; right panels). (see colour figures supplement)

(both $p < 0.04$, Figure 1C). The total vascular surface and all the parameters in DSE did not differ between cases and controls.

Angiogenic factors and proteases in cases and controls: mRNA levels

The expression of various angiogenic growth factors and MT-MMPs were evaluated at the mRNA level in the three decidual tissues in cases and controls. The data of cases and controls are separately described in Table 3a and 3b. Their relative content in cases versus controls is described in Table 4.

The data of the 16 controls on mRNA levels of angiogenic factors and proteases are given in Table 3a. They were highly comparable to previous data on control samples of 25 different women [Plaisier *et al.*, 2007; Plaisier *et al.*, 2008].

Table 3a Differential mRNA content of angiogenic factors and proteases in first-trimester decidua of controls.

	mRNA content (ng/μl) ^a			mRNA (fold Induction)				
	DSE	DP	DB	DSE ^b	DP	DB	DP ^c	DB
PIGF	1.09 ± 0.22	0.69 ± 0.23	10.1 ± 2.1	1.0	0.7 ± 0.3	12.7 ± 2.7*	1.0	35.8 ± 2.8 [#]
VEGF-A	0.18 ± 0.04	0.20 ± 0.04	0.21 ± 0.05	1.0	1.1 ± 0.2	1.2 ± 0.2	1.0	1.5 ± 0.3
KDR	0.85 ± 0.17	1.68 ± 0.56	1.23 ± 0.50	1.0	1.1 ± 0.1	1.2 ± 0.1	1.0	0.9 ± 0.2
Flt-1	0.81 ± 0.12	0.76 ± 0.09	0.94 ± 0.15	1.0	1.1 ± 0.1	1.2 ± 0.1	1.0	1.3 ± 0.2
Ang-1	0.10 ± 0.02	0.09 ± 0.02	0.03 ± 0.01	1.0	0.9 ± 0.1	0.5 ± 0.1*	1.0	0.6 ± 0.1 [#]
Ang-2	0.51 ± 0.13	0.33 ± 0.04	0.16 ± 0.05	1.0	1.1 ± 0.2	0.4 ± 0.1*	1.0	0.6 ± 0.2 [#]
TIE-2	3.58 ± 0.52	1.87 ± 0.39	0.76 ± 0.20	1.0	0.5 ± 0.1 [§]	0.3 ± 0.1*	1.0	0.6 ± 0.1 [#]
MT1-MMP	3.35 ± 0.43	6.05 ± 0.91	5.69 ± 1.11	1.0	2.1 ± 0.4 [§]	1.7 ± 0.5*	1.0	1.4 ± 0.4
MT2-MMP	0.09 ± 0.03	0.06 ± 0.02	1.45 ± 0.39	1.0	3.1 ± 1.1	23.3 ± 4.2*	1.0	19.3 ± 3.2 [#]
MT3-MMP	7.39 ± 1.89	6.20 ± 1.77	16.3 ± 2.5	1.0	1.4 ± 0.3	7.7 ± 1.0*	1.0	11.4 ± 2.4 [#]
MT5-MMP	39.88 ± 4.89	28.75 ± 7.35	5.94 ± 0.80	1.0	0.7 ± 0.2	0.1 ± 0.0*	1.0	0.3 ± 0.1 [#]

mRNA content was determined by RT-PCR in DSE, DP and DB of early first-trimester pregnancies and compared within subjects of the control group. The 'repeated measures ANOVA' was performed to analyse the data.

a Mean cycle threshold (Ct) per gene: GAPDH 25, VEGF-A 26.0, PIGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, TIE-2 32.1, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/μl using a standard curve of total RNA. Then values were corrected for RNA input by calculating the ratio (ng/μl (target gene))/ (ng/μl (GAPDH)) and mean ± SD ratios are showed in the columns DSE, DP and DB.

b Fold induction was calculated as the ratio (DP)/ (DSE) and (DB)/ (DSE) to compare mRNA levels in DB and DP with those in DSE (1.0) within subjects. Discrepancies between absolute and percental data might originate from inter-individual differences.

c Fold induction was calculated as the ratio DB)/ (DP) to compare DB with DP (1.0) within subjects.

[§] $P < 0.05$ DP vs DSE, * $p < 0.05$ DB vs DSE, [#] $p < 0.05$ DB vs DP

Cases versus Controls

The mRNA levels in cases are given in Table 3b and the comparison of these levels to the mRNA contents of controls is described in Table 4. The mRNA expression in DSE showed an upregulation of KDR (3.5), Flt-1 (3.5), TIE-2 (2.7), and MT1-MMP (2.4) and a reduced MT3-MMP expression (0.7) in DSE of cases compared to controls (1.0) ($p < 0.01$, Table 4). Regarding the mRNA levels in DP, VEGF-A (2.1), KDR (4.4), Flt-1 (3.8), Ang-2 (2.1), and TIE-2 (3.6) expression was significantly elevated and MT3-MMP expression was reduced in the case group (Table 4).

Finally, the mRNA levels in DB showed upregulation of all angiogenic factors, except PlGF, in decidua of the cases. On the other hand, none of the protease were differentially expressed (Table 4).

Angiogenic factors and proteases in cases and controls: immunohistochemistry

The presence and cellular localisation of the various antigens were determined in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues

Table 3b Differential mRNA content of angiogenic factors and proteases in first-trimester missed abortion decidua.

	mRNA content (ng/μl) ^a			mRNA (fold induction)				
	DSE	DP	DB	DSE ^b	DP	DB	DP ^c	DB
PlGF	1.81 ± 0.67	0.44 ± 0.06	10.67 ± 3.11	1.0	0.6 ± 0.1	13.4 ± 3.9*	1.0	21.2 ± 4.2 [#]
VEGF-A	0.29 ± 0.04	0.40 ± 0.05	0.84 ± 0.17	1.0	1.7 ± 0.3	3.7 ± 0.9*	1.0	2.3 ± 0.4 [#]
KDR	2.97 ± 0.57	5.74 ± 1.79	3.90 ± 0.99	1.0	2.6 ± 0.7	2.1 ± 0.8	1.0	1.4 ± 0.6
Flt-1	2.68 ± 0.14	2.88 ± 0.52	2.44 ± 0.47	1.0	1.0 ± 0.2	0.9 ± 0.2	1.0	0.9 ± 0.1
Ang-1	0.12 ± 0.02	0.10 ± 0.01	0.09 ± 0.02	1.0	1.3 ± 0.4	1.0 ± 0.3	1.0	1.0 ± 0.1
Ang-2	0.80 ± 0.14	0.68 ± 0.10	1.46 ± 0.41	1.0	1.3 ± 0.3	3.1 ± 1.0	1.0	2.6 ± 0.6
TIE-2	7.97 ± 0.72	6.04 ± 0.94	4.58 ± 1.08	1.0	0.8 ± 0.1	0.6 ± 0.2	1.0	0.9 ± 0.3
MT1-MMP	8.09 ± 1.49	6.73 ± 0.65	8.40 ± 1.48	1.0	1.1 ± 0.2	1.3 ± 0.3	1.0	1.2 ± 0.1
MT2-MMP	0.12 ± 0.03	0.05 ± 0.03	1.21 ± 0.22	1.0	0.6 ± 0.3	18.0 ± 4.4*	1.0	73.5 ± 12.4 [#]
MT3-MMP	1.93 ± 0.35	0.93 ± 0.23	13.3 ± 2.30	1.0	0.8 ± 0.3	10.2 ± 2.1*	1.0	24.3 ± 5.4 [#]
MT5-MMP	26.97 ± 4.32	28.44 ± 5.82	9.54 ± 1.85	1.0	1.2 ± 0.3	0.4 ± 0.1*	1.0	0.6 ± 0.2 [#]

mRNA content was determined by RT-PCR in DSE, DP and DB of early first-trimester pregnancies and compared within subjects of the Missed Abortion group. The 'repeated measures ANOVA' was performed to analyse the data.

a Mean cycle threshold (Ct) per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, TIE-2 32.1, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/μl using a standard curve of total RNA. Then values were corrected for RNA input by calculating the ratio (ng/μl (target gene)) / (ng/μl (GAPDH)) and mean ± SD ratios are showed in the columns DSE, DP and DB.

b Fold induction was calculated as the ratio (DP) / (DSE) and (DB) / (DSE) to compare mRNA levels in DB and DP with those in DSE (1.0) within subjects. Discrepancies between absolute and fold induction data might originate from inter-individual differences.

c Fold induction was calculated as the ratio DB / (DP) to compare DB with DP (1.0) within subjects.

[#] $p < 0.05$ DP vs DSE, * $p < 0.05$ DB vs DSE, # $p < 0.05$ DB vs DP

and their presence was graded as median staining indices (median SI) per cell type and total staining indices (total SI) based on the sum of the cell types-dependent indices (Table 5a and 5b).

Controls

First, the total expression of the studied antigens in DP and DSE were compared. This showed a more abundant total expression of Flt-1, TIE-2 and MT1-MMP in DP (Table 5a and 5b, $p < 0.05$). Then, the staining patterns in DB and DSE were compared; the total SI's of PlGF, Flt-1, TIE-2, and MT1-MMP were increased whereas the total SI of Ang-1 was decreased in DB. Finally, the comparison between DP and DB showed an increased PlGF total SI and a decreased Ang-1 total SI in DB.

The antigen expression was most abundant in glandular epithelial cells, followed by perivascular smooth muscle and stromal cells. Endothelium displayed the expression of all angiogenic factors, except Ang-1 and MT5-MMP. TIE-2 showed the highest expression in endothelium. The endothelial protein expression of PlGF and Flt-1 showed significant differences between the three decidual tissues and MT2-MMP appeared less abundantly expressed in DB compared to DP and DSE (Table 5a and 5b, Figure 3). All angiogenic factors, except Ang-1 and MT5-MMP very dimly, were also detected in extra-villous tro-

Table 4. Fold induction mRNA expression in first-trimester decidua of cases versus controls (set at 1.0).

	DSE	DP	DB
PlGF	1.7	1.5	1.1
VEGF-A	1.6	2.1*	4.9*
KDR	3.5*	4.4 [#]	5.3*
Flt-1	3.5*	3.8*	2.7*
Ang-1	1.2	1.3	2.8 [#]
Ang-2	1.6	2.1*	8.3*
TIE-2	2.7*	3.6*	5.3*
MT1-MMP	2.4*	1.7	1.8
MT2-MMP	1.3	1.7	0.8
MT3-MMP	2.6 [#]	0.3 [#]	0.8
MT5-MMP	0.6	0.8	1.8

mRNA levels of angiogenic factors and proteases in DSE, DP and DB were compared between cases and matched controls (1.0). The 'repeated measures ANOVA' was performed to analyse the data. Mean cycle threshold (Ct) per gene: GAPDH 25-26, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/μl using a standard curve of total RNA and values were corrected for RNA input by calculating the ratio (ng/μl (target gene)) / (ng/μl (GAPDH)). Fold induction was calculated as the ratio ng/μl (case) / ng/μl (controls).

* $p < 0.01$, [#] $p < 0.05$

Table 3a. Antigen levels of angiogenic factors in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

	EC		PSMC		GE		SC		EVT		Total SI	
	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
VEGF	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (2.3-3.9)	3.0 (3.0-6.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	2.0 (0.0-2.0)	0.0 (0.0-1.5) ^b	NP	10.0 (7-16)	9.0 (8-20)
	DP	0.0 (0.0-0.0)	0.2 (0.0-1.6)	2.0 (0.0-5.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	6.0 (4.5-6.0)	2.0 (2.0-4.0)	3.8 (3.0-6.0)	NP	12.0 (10-16)	15.1 (8-15)
	DB	0.0 (0.0-2.0)	0.0 (0.0-0.0)	3.0 (2.5-3.0)	3.0 (3.0-3.8)	6.0 (3.4-6.0)	4.3 (3.0-6.0)	2.0 (1.0-3.8)	3.4 (2.3-5.6)	3.3 (3.3-4.0)	10.0 (7-16)	14.0 (8-32)
PIGF	DSE	0.0 (0.0-0.1)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (2.5-3.0)	6.0 (3.0-6.0)	3.0 (3.0-4.5)	2.0 (0.0-4.0)	0.0 (0.0-0.0) ^b	NP	13.0 (10-13)	7.8 (6-9) ^a
	DP	0.0 (0.0-0.0)	0.0 (0.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	2.0 (2.0-3.5)	3.0 (2.0-4.5)	NP	11.0 (9-12)	12.0 (11-17)
	DB	1.0 (0.0-2.0)*	0.0 (0.0-2.0)	3.0 (2.7-3.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	3.0 (3.0-4.5)	4.0 (2.3-4.0)	2.5 (2.0-3.0)	2.4 (1.3-3.7)	16.0 (9-18)*	10.5 (10-13)
KDR	DSE	0.3 (0.0-1.0)	0.3 (0.0-0.3)	4.0 (3.0-9.0)	3.0 (1.0-3.0)	6.0 (3.8-9.0)	3.0 (3.0-5.2)	0.0 (0.0-1.5)	0.0 (0.0-0.0)	NP	12.0 (8-20)	8.0 (7-9)
	DP	1.0 (0.0-2.0)	1.0 (0.3-2.0)	3.0 (3.0-5.0)	1.3 (0.0-3.0)	6.0 (3.5-6.0)	3.0 (3.0-6.0)	2.0 (0.5-4.0)	1.5 (1.0-2.4)	NP	14.0 (9-17)	12.0 (10-14)
	DB	1.0 (0.0-2.0)	2.1 (0.0-2.5)*	3.1 (1.5-6.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	3.0 (3.0-3.0)	1.0 (1.0-3.0)	1.3 (1.0-2.0)	1.0 (0.0-1.0)	10.0 (6-17)	9.0 (6-10)
Flt-1	DSE	0.0 (0.0-0.3)	0.0 (0.0-1.0)*	2.5 (1.8-3.0)	3.0 (3.0-3.0)	6.0 (5.3-6.0)	6.0 (4.5-6.0)	1.0 (1.0-2.0)	2.0 (0.0-2.0) ^b	NP	9.0 (8-11)*	11.0 (9-15) ^a
	DP	0.0 (0.0-0.1)	1.0 (0.0-2.0)	2.0 (1.0-3.8)	4.0 (3.0-4.0)	3.0 (2.0-4.0)	6.0 (6.0-6.0)	4.0 (2.0-4.5)	6.0 (3.5-6.0)	NP	12.0 (10-13)	20.0 (16-22)
	DB	0.0 (0.0-1.0)*	1.0 (1.0-1.5)	3.0 (3.0-3.4)	4.5 (3.0-6.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	3.0 (1.0-3.8)	6.0 (4.5-6.0)	2.0 (2.0-2.2)	11.5 (11-14)	19.5 (12-22)

Table 3a. Antigen levels of angiogenic factors in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)													
	EC		PSMC		GE		SC		EVT		Total SI		Case
	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	
Ang-1	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.6 (0.0-3.0)	3.0 (0.8-3.0)	6.0 (3.0-6.0)	3.0 (3.0-5.0)	0.0 (0.0-1.0)	1.0 (0.0-1.0)	NP	NP	6.0 (4-10)	8.5 (5-10)
	DP	0.0 (0.0-0.0)	0.2 (0.0-0.25)	0.0 (0.0-3.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	1.0 (0.0-1.0)	4.5 (2.3-5.0)	NP	NP	6.0 (5-10)	16.5 (12-22)
	DB	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-6.0)	0.0 (0.0-0.0)	5.0 (2.0-6.0)	0.0 (0.0-0.0)	1.0 (0.7-1.0)	3.0 (3-5)*	12.1 (5-19)
Ang-2	DSE	0.0 (0.0-1.0)	0.0 (0.0-1.0)	3.0 (0.0-3.0)	0.0 (0.0-1.5)	6.0 (4.5-6.0)	3.0 (3.0-4.5)	0.0 (0.0-0.0)	0.0 (0.0-1.0)	NP	NP	9.0 (6-10)	3.0 (3-12)
	DP	1.0 (0.0-1.0)	0.0 (0.0-0.5)	0.0 (0.0-3.0)	0.8 (0.0-2.1)	6.0 (6.0-6.0)	3.75 (0.0-4.5)	2.0 (0.5-3.0)	0.7 (0.0-3.0)	NP	NP	10 (8-12)	7.0 (0.0-12.0)
	DB	1.0 (0.0-2.0)	0.0 (0.0-0.5)	1.5 (0.0-3.0)	0.0 (0.0-1.3)	6.0 (3.0-6.0)	2.5 (0.0-3.0)	1.0 (0.5-2.0)	1.5 (0.0-2.5)	0.9 (0.5-1.0)	1.3 (0.0-1.9)	9.0 (7-13)	6.0 (0.0-7.0)
TIE-2	DSE	1.0 (0.0-2.0)	1.0 (0.5-2.0)*	3.0 (3.0-3.0)	3.0 (3.0-3.0)	6.0 (5.3-6.0)	3.0 (2.0-3.0)	1.1 (0.0-2.0)	1.0 (0.5-1.0)*	NP	NP	10.0 (9-13)*	9.0 (7.0-13.0)*
	DP	2.0 (0.0-4.0)	1.5 (1.0-2.0)	3.0 (3.0-3.4)	3.0 (3.0-3.0)	6.0 (3.8-6.0)	3.0 (3.0-3.8)	2.5 (1.3-4.0)	3.0 (2.5-3.0)	NP	NP	13.0 (11-17)	12.5 (11-19)
	DB	1.0 (1.0-2.0)	2.0 (1.0-3.0)	2.1 (1.1-3.0)	3.0 (3.0-3.0)	3.0 (3.0-6.0)	3.0 (3.0-6.0)	3.0 (1.5-5.0)	3.0 (2.0-3.8)	2.8 (2.0-4.0)	3.0 (3.0-3.0)	14.0 (9-16)	15.0 (12-20)

The antigen levels of angiogenic factors in DSE, DP and DB were compared between cases (case) and controls (co). Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis within groups and the general linear model was used to analyse differences between the two groups. The staining was expressed as median staining indices (Median SI) per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue. NP = not present.

Within groups: * $p < 0.05$ in DB vs DP and DSE, # $p < 0.05$ in DSE vs DP and DB.

Between groups: bold $p < 0.05$ in MA versus AP.

Table 5b. Antigen levels of proteases in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)													
	EC		PSMC		GE		SC		EVT		Total SI		
	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	
MT1	DSE	0.0 (0.0-1.0)	0.0 (0.0-0.0)	2.0 (1.0-3.0)	3.0 (0.0-3.0)	3.0 (3.0-3.0)	1.5 (1.0-2.0)	2.0 (2.0-2.0)	NP	NP	6.5 (6.0-9.0)*	8.0 (7.0-8.0)	
	DP	0.0 (0.0-1.0)	0.0 (0.0-1.0)	2.0 (0.0-3.0)	2.0 (0.0-3.0)	2.0 (1.0-3.0)	2.0 (1.0-2.0)	2.0 (2.0-2.0)	NP	NP	9.0 (6.5-10.0)	7.0 (4.5-9.0)	
	DB	1.0 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.0-2.0)	0.0 (0.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-2.0)	2.0 (1.5-2.0)	1.0 (0.5-1.0)	1.0 (0.0-1.0)	9.0 (8.0-10.0)	5.0 (3.5-10.0)	
MT2	DSE	2.0 (0.3-2.0)	1.0 (1.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.5 (3.0-6.0)	1.5 (1.0-2.0)	2.0 (2.0-4.5)	NP	NP	11.0 (8.0-13.8)	10.5 (8.8-12.5)	
	DP	1.0 (1.0-1.0)	1.0 (1.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.0 (3.0-6.0)	2.5 (1.5-3.5)	2.0 (2.0-3.8)	NP	NP	13.0 (8.5-14.5)	10.0 (9.0-13.8)	
	DB	1.0 (0.5-1.0)	1.7 (1.0-2.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	2.5 (2.0-4.0)	2.5 (2.0-3.5)	2.0 (2.0-4.0)	1.5 (1.0-2.0)	2.0 (1.0-2.0)	9.5 (8.3-11.8)	12.0 (10.0-15.0)*	
MT3	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.5 (2.0-4.0)	0.0 (0.0-0.5)	0.0 (0.0-0.0)	NP	NP	9.0 (6.0-11.0)	6.0 (6.0-6.0)	

Table 5b. Antigen levels of proteases in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

	EC		PSMC		GE		SC		EVT		Total SI	
	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
MT5	DP	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.0 (2.0-4.0)	3.0 (3.0-3.0)	1.0 (0.0-1.0)	0.0 (0.0-0.8)	NP	10.0 (8.0-11.0)	6.0 (6.0-6.0)
	DB	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	1.5 (1.0-2.5)	2.0 (1.0-2.0)	0.0 (0.0-1.0)	8.0 (5.5-13.0)	4.0 (4.0-8.0)
	DSE	0.0 (0.0-0.0)	1.0 (0.0-1.0)	2.0 (0.0-2.0)	3.0 (0.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-2.0)	1.0 (0.8-2.0)	NP	2.5 (0.0-3.0)	8.0 (6.5-8.5)
	DP	0.0 (0.0-0.0)	1.0 (0.0-1.0)	0.0 (0.0-1.0)	0.0 (0.0-2.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-1.5)	0.5 (0.0-1.0)	NP	2.5 (0.0-3.5)	4.5 (4.0-6.5)
DB		0.0 (0.0-0.0)	1.0 (0.0-1.0)	0.0 (0.0-1.5)	0.0 (0.0-2.3)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-1.0)	0.5 (0.0-1.3)	0.5 (0.0-1.0)	2.5 (0.0-3.8)	5.0 (4.0-6.5)

The antigen levels of proteases in DSE, DP and DB were compared between cases (case) and controls (co). Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis within groups and the general linear model was used to analyse differences between the two groups. The staining was expressed as median staining indices (Median SI) per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue. NP = not present.

Within groups: * p<0.05 in DB vs DP and DSE, # p<0.05 in DSE vs DP and DB.

Between groups: bold p<0.05 in MA versus AP.

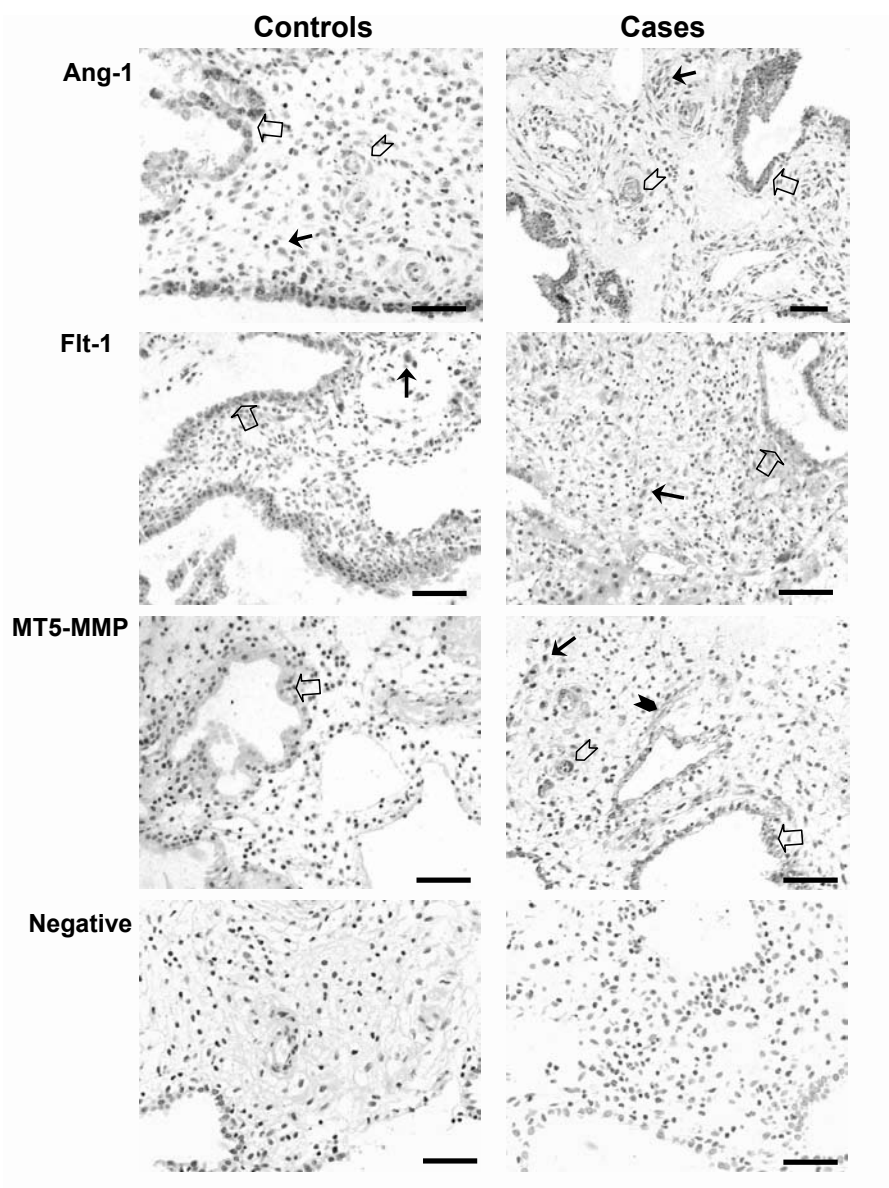


Figure 2. Antigen presence of angiogenic factors and proteases in cases and controls.

Examples of the antigen expression of Ang-1, Flt-1 and MT5-MMP in controls (left site of panel) and cases (right site of panel). **Ang-1** antigen presence in epithelial cells (open arrow) in DB of controls, compared to their presence in stromal (closed arrow), epithelial cells (open arrow) and PSMC (open arrowhead) in DB of cases. **Flt-1** antigen presence in epithelial cells (open arrow) and stromal cells (closed arrow) in DB of controls, compared to their elevated presence in DB of cases. (compare figure 3 for endothelial expression). **M5-MMP** antigens in epithelial cells (open arrow) in DB of controls, compared to their presence in PSMC (open arrowhead), endothelial (closed arrowhead), stromal (closed arrow) in DB of cases. **Negative** controls using non-immune mouse IgG₁ (left) and omission of the first antibody (right). All panels bar = 100 μm. (see colour figures supplement)

phoblasts; PlGF, VEGF, Flt-1 and Tie-2 were moderately expressed, whereas KDR and Angiopoietin-2 expressions were weak (Table 5a and 5b).

Cases versus Controls

The comparison of the antigen expression between cases and controls is described in Table 5a and 5b and Figure 2 and 3. Firstly, comparing protein expressions in DSE of cases and controls showed a reduced total staining index for KDR and increased total SI for MT5-MMP in DSE of cases (Table 5a and 5b, $p < 0.05$).

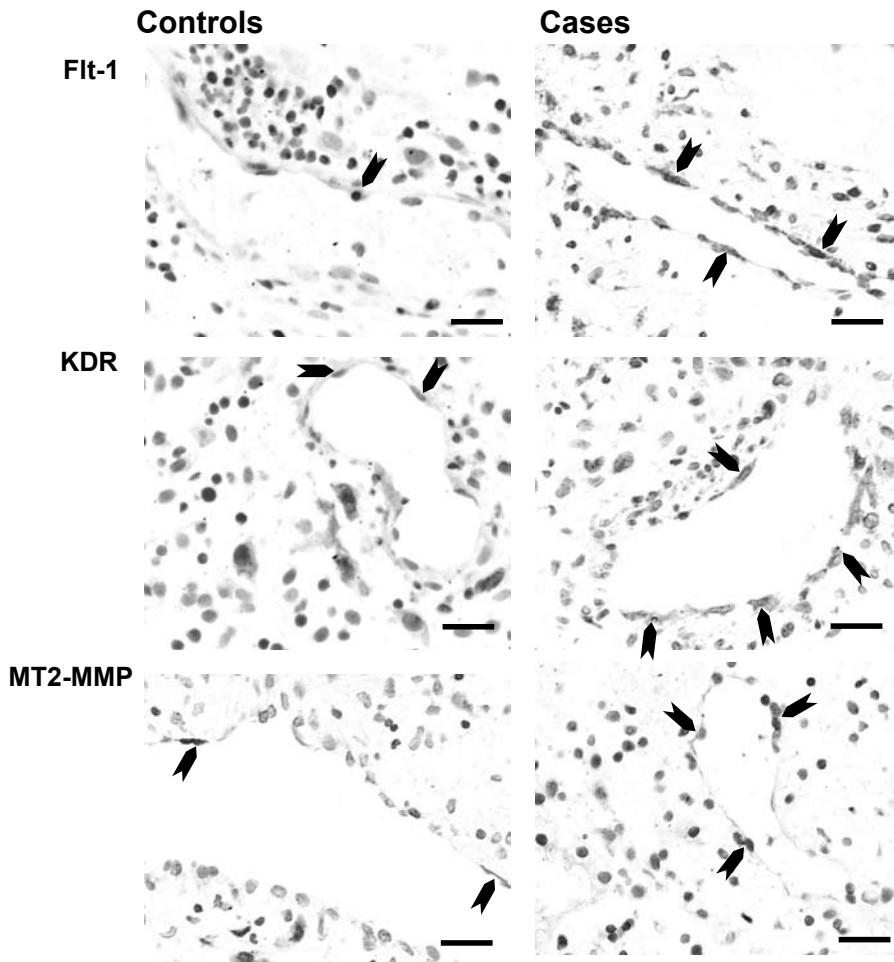


Figure 3. Antigen presence of angiogenic factors and proteases in endothelium in cases and controls. Examples of the differential endothelial expression (arrowheads) of flt-1, KDR and MT2-MMP between DB specimens of cases (right site of panel) and controls (left site of panel) are given. All panels: bar = 50 μm . (see colour figures supplement)

With regard to DP, the total expression of flt-1 was elevated in the case group (Table 5a and 5b, $p < 0.05$). The proteases showed differential expression as well, reflected by a reduced total MT3-MMP and MT5-MMP expression in missed abortion decidua. The angiogenic factors in DB were differentially expressed; the total SI of flt-1 and Ang-1 expression were elevated in missed abortion DB ($p < 0.05$), in particular in perivascular smooth muscle cells and stromal cells. In addition, reduced total MT3-MMP and elevated total MT2- and MT5-MMP were detected in DB of cases ($p < 0.05$, Figure 2). The endothelial protein expression of KDR, Flt-1, MT2- and MT5-MMP showed differential expression between cases and controls (Table 5a and 5b, Figure 3). Finally, the Ang-1 expression was significantly elevated and the Ang-2 expression appeared decreased in DP and DB of cases compared to controls (Figure 2). This trend was also detected in DSE tissues. Subsequently, the Ang-2/Ang-1 ratio was decreased in all tissues of cases compared to controls ($p < 0.02$ for DB and DP, $p < 0.05$ for DSE).

The expression of proliferation (Ki67) and apoptosis (active Caspase 3) markers

To evaluate whether cell proliferation and apoptosis contributed to the observed changes, we evaluated the presence of active caspase-3 and the proliferation marker Ki67. The expression of active Caspase-3 (aCasp-3) in epithelial (range 17-25%), endothelial (range

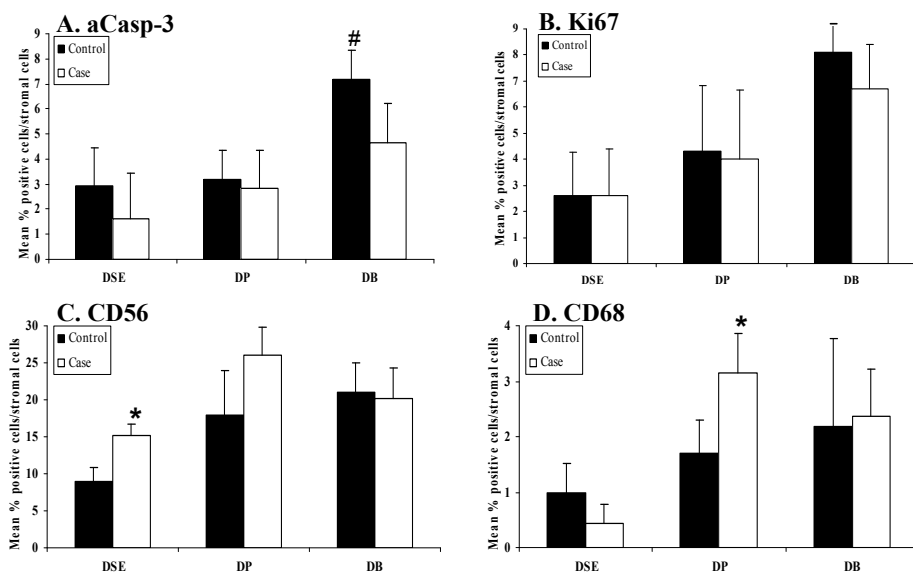


Figure 4. Proliferation, apoptosis, and immune cells in cases and controls.

The number of positive cells per total stromal cells for **A.** active Caspase-3 (apoptosis marker), **B.** Ki67 (proliferation marker), **C.** CD56 (uNK cell marker), and **D.** CD68 (macrophage marker) were determined via immunohistochemistry and expressed in graphs as mean $\pm$ SEM. [#] $P < 0.05$ in DB versus DSE and DP of controls. ^{*} $P < 0.05$ in cases versus controls.

0.0-0.1%) and stromal (range 1.6-7.2%) cells was comparable in both groups. The differences between the decidual tissues within each group were similar, except for the expression in stromal cells. The controls showed a significantly decreased percentage of stromal aCasp-3 expression in DSE and DP compared to DB and the cases displayed the same trend (Figure 4A).

The expression of proliferation marker Ki67 in epithelial (range 0.3-1.9%), endothelial (range 0.3-2.3%) and stromal (range 2.6-8.1%) cells was comparable in cases and controls. Strikingly, the endothelial and stromal expression appeared to be decreased in DSE and DP compared to DB in both cases and controls (Figure 4B).

The expression of uNK cell (CD56) and macrophage (CD68), markers

Because decidualisation is accompanied by a large influx of immune cells, which can influence angiogenesis, we determined the number of uNK cells and macrophages in the tissues of controls and cases. The CD56 staining showed inhomogeneous staining, mainly situated surrounding vessels and glands, which was corrected for by analysing various samples per patient. DSE showed a significantly increased number of uNK cells in the case group of $15 \pm 2\%$ positive cells per total number of stromal cells versus $9 \pm 2\%$ in the control group. No significant differences were detected in between the two groups DP ($18 \pm 6\%$ vs $26 \pm 4\%$) and DB ($21 \pm 4\%$ vs $20 \pm 4\%$, Figure 4C).

The CD68 staining showed scattered positive cells throughout the tissues (range 0.4-3.2%). No significant differences were detected in DSE and DB between the two groups. The number of macrophages was significantly increased in DP of cases compared to controls (Figure 4D).

DISCUSSION

The present study showed that the vascularisation pattern varied in decidual secretory endometrium (DSE), decidua parietalis (DP) and decidua basalis (DB) of missed abortion cases and matched controls, i.e. fewer vessels with larger circumference, were present in DP and DB of cases. Strikingly, these differences correlated with the differential expression of several angiogenic molecules at mRNA and antigen level, including the endothelial protein expression of Flt1, KDR and MT2-, MT3- and MT5-MMP. These findings might indicate a correlation between decidual vascularisation and the occurrence of miscarriages.

Maternal vascular adaptation to implantation and its regulation has been subject to discussion, but has mainly been studied with regard to arterial remodelling and villous vascularisation [Craven *et al.*, 1999; Kam *et al.*, 1999]. Recently the potential regulation

of decidual vascularisation by the extra-villous trophoblast (EVT), immune cells and pregnancy-induced hormones has been described [Plaisier *et al.* 2007/2008]. The present study compared the vascularisation pattern and the expression of angiogenic factors and proteases in miscarriage cases and matched controls, which enabled hypothesising about their correlation with the occurrence of miscarriages. All methods were performed on serial paraffin sections, which allowed studying all parameters in the same tissue. Karyotyping was not allowed. However, no differences in the number of leucocytes in general, in uNK cells and macrophages, and in decidual architecture were previously detected between chromosomally normal and abnormal pregnancies [Quack *et al.* 2001; Greenwold *et al.* 2003; Shimada *et al.* 2006]. Thus, the lack of karyotyping will probably not significantly bias our observations.

The vascularisation pattern showed increasing vascular and luminal surfaces and decreasing vessel density from DSE to DP to DB in both uncomplicated and complicated first-trimester pregnancies. Vailhe *et al.* reported a decreased vessel density in DB compared to DP, but did not study DSE [Vailhe *et al.* 1999]. The authors described a vessel density ranging between 20 and 30 vessels/mm², which is comparable to our findings. The changes in vascular pattern from DSE to DP to DB may be related to mechanical factors and/or proteins regulating angiogenesis. For instance, the lower vessel density and the enlargement of the vascular surface in DB may be associated with the increased blood flow in the proximity of the implantation site [Gibbons *et al.* 1994; Kam *et al.* 1999; Plaisier *et al.* 2007]. Increased luminal size might also be induced by a relatively low oxygen level [Charnock-Jones *et al.* 2002].

The decreased vessel density and increased vascular surface in DB and DP might also be due to an altered balance between angiogenesis regulating factors. Changes in the relative content of VEGF, angiopoietins and their receptors have been associated with the formation of dilated or enlarged microvessels [Adams, Alitalo, 2007; Nagy *et al.* 2003]. Whether the altered contents of angiopoietin-1 (higher) and MT-MMPs (lower) in smooth muscle cells of cases reflect an altered interaction between pericytes and endothelial cells contributing to changes in vasculature is yet unknown and requires further studies.

Comparison of cases and controls showed that the total vascular surface did not differ between the two groups. However, the arrangement of the vessels did differ in DB and DP, i.e. a significant decreased vessel density and increased luminal surface in DB and DP of cases. This is not in concordance with the previously described increased vessel density in DP of missed abortions tissue specimens (Vailhe *et al.* 1999).

The differences in vascularisation patterns between controls and cases resemble the differences between early and late first-trimester vascularisation, respectively, suggesting that a too fast maturation of the vasculature is associated with the pathogenesis of mis-

carriages [Plaisier *et al.* 2007]. The pre-maturely ripening may allow access of maternal blood in the intervillous space too early in the development of pregnancy, which was also demonstrated in vivo by Doppler ultrasound imaging [Greenwold *et al.* 2003; Jauniaux, Burton 2005]. This might result in increased oxygen levels with subsequent excessive oxidative stress, which is able to modulate the architecture of vasculature and the expression of peri-cellular proteases and angiogenic factors [Burton *et al.* 1999, Sharkey *et al.* 2000, Kingdom, Kaufman 1997, Zhang *et al.* 2001].

The role of smooth muscle cells has been described regarding vascular adaptation to pregnancy. The quantification of smooth muscle cells in the studied samples may provide interesting information in the future.

Steroidal hormones, the EVT, and immune cells are able to modulate vascularisation directly or indirectly via angiogenic factors [Hanna *et al.* 2006; Hyder, Stancel 1999; Simoncini *et al.* 2003; Zygmunt *et al.* 2002]. We confirm the previously described potential role for VEGF-A (via KDR), the angiopoietins (via TIE-2) and PlGF (via Flt-1) in the regulation of decidual vascularisation [Plaisier *et al.* 2007]. Additionally, their possible association with the differential vascularisation pattern in the miscarriage group is evaluated. The closer the tissue is situated near the implanting embryo, the more angiogenic factors show increased expressions in cases compared to controls. PlGF, the most abundantly regulated factor in uncomplicated first-trimester decidua [Plaisier *et al.* 2007], is not differentially expressed and appears not to be involved in the pathogenesis of missed abortions. In which way the increased expression of angiogenic factors are related to the fewer but larger vessels remains to be elucidated in future research.

In contrast with the mRNA results, the antigen expression of the angiogenic factors showed remarkable little differences between cases and controls. Apparently, the regulation at transcriptional level could not be detected at the translational level. Reasons for this might be an increased cytoplasmic storage of the antigens, reduced bio-availability or the higher sensitivity of RT-PCR. Only the endothelial and total expression of Flt-1 and KDR antigens and the total expression of Flt-1 and Ang-1 were differentially expressed. Thus, the receptors of VEGF and PlGF, and not the growth factors themselves, seem to be regulated at the protein level. It should be noted that our assays did not differentiate between the expression of Flt-1 and the soluble form of Flt-1, which is known to have a counter regulatory role in VEGF signal transduction during in early pregnancy [Maynard *et al.* 2003].

Decidual tissues of controls showed an Ang-2/Ang-1 ratio in favour of Ang-2, whereas decidual tissues of cases showed a ratio in favour of Ang-1. This altered ratio might be based on different oxygen levels in both groups, since hypoxia enhances Ang-2 transcription and destabilises Ang-1 [Zhang *et al.* 2001]. The favour of Ang-1 in cases might in part explain the observed decreased vessel density with increased circumference, since Ang-1

promotes the generation of a more complex vascular network and vessels of increased luminal size [Geva *et al.* 2000; Asahara *et al.* 1998]. In addition, less complex and fewer large arteries are reported in Ang-1 $-/-$ mice [Suri *et al.* 1996].

The expression of pericellular acting proteases was assessed in decidua of both groups. MT1-MMP did not show differential expression between cases and controls. The differential expression at protein but not at RNA level suggests that MT2- and MT5-MMP are regulated at translation and not at transcription level. The differential endothelial antigen levels of MT2, MT3- and MT5-MMP may be partially responsible for the differences in vascular pattern in DB and DP. Especially, since we know that MT2- and MT3-MMP are likely to be regulators of decidual angiogenesis [Hotary *et al.* 2000; Plaisier *et al.* 2004/2006]. In which way MT5-MMP is involved in decidual vascularisation and possibly other decidual remodelling processes remains a question.

Causal conclusions from histo-pathological studies of abortion tissue are often questioned since foetal death may occur days before evacuation, allowing post-mortem inflammatory or apoptotic reactions during this “retention time”. No effect has been described of retention time on the outcome of vascular parameters [Meegdes *et al.* 1988; Nelen *et al.* 2000; Lisan *et al.* 2004]. This most likely excludes a contribution of “retention time” to the observed vascularisation differences between cases and controls. Nevertheless, inflammatory, proliferation, and apoptotic processes were analysed.

The expression of proliferation and apoptosis markers suggests an increased cellular turnover from DSE to DP to DB in both cases and controls. The level of proliferation and apoptosis was comparable in cases and controls, suggesting that our data were not seriously biased by differences in proliferation and apoptosis in response to foetal loss. The amount and distribution of apoptosis is comparable with earlier reports and the elevated apoptosis level in DB compared to DP has been described in uncomplicated pregnancies [Joswig *et al.* 2003; Marx *et al.* 1999; Von Rango *et al.* 2003]. No references data were available regarding proliferation in the various decidual tissues, although moderate Ki67 expression has been described in DB [Korgun *et al.* 2006].

Similar numbers of uNK cells were demonstrated in DP and DB of cases and controls, but their number was significantly increased in DSE of cases. Reports on uNK cells and miscarriages describe increased uNK cells in receptive endometrium of patients with recurrent miscarriages (RM) [Quenby *et al.* 1999; Tuckerman *et al.* 2007]. The abundant presence of uNK cells in DSE samples may reflect these observations. The difference between cases and controls were levelled in DP and DB, possibly by hormone regulated uNK cell migration to normal values [Van den Heuvel *et al.*, 2005b].

The number of macrophages has been reported not to be related to miscarriages [Hill *et al.* 1995; Quack *et al.* 2001]. We detected increased amounts of macrophages in DP of

cases and not in DSE and DB. Strikingly, the number of uNK cells and macrophages at the actual implantation site did not differ between the two groups

Conclusion

Vascular adaptation to implantation is important in the development of a healthy pregnancy. Here, the involvement of decidual vascularisation was studied in relation to miscarriages. Differences were observed in vascularisation pattern between decidual tissues of cases and controls. Strikingly, the vascular differences correlated with the differential expressions of several angiogenic molecules at mRNA and antigen level. Furthermore, the endothelial antigen levels of MT₃-MMP were decreased and of Flt₁, KDR and MT₂- and MT₅-MMP were enhanced in DP and DB of cases. These findings might indicate a correlation between decidual vascularisation and the occurrence of miscarriages.

Chapter 8

The background of the page features a large, stylized, light gray number '8' centered vertically and horizontally. The '8' is composed of two overlapping circles. Within the lower-right circle of the '8', there is a complex, wavy, white line pattern that resembles a topographical map or a microscopic view of tissue. The rest of the background is a solid dark gray.

The expression of angiogenic factors in first- trimester human decidua of pregnancies further complicated by pre-eclampsia or foetal growth restriction.

M. Plaisier
E. Streefland
P. Koolwijk
F.M. Helmerhorst
J.J. Erwich

ABSTRACT

Objective: Disturbances in decidual and placental vascular development may play a role in the pathogenesis of pregnancy complications. This study focused on the role of angiogenic factors in the first-trimester in the pathogenesis of pre-eclampsia (PE) and/or foetal growth restriction (FGR).

Materials and Methods: First-trimester decidua was obtained during chorionic villous sampling. The expression of VEGF-A, PlGF, Flt-1, KDR, Angiopoietin-1, Angiopoietin-2 and TIE-2 mRNA was determined by RT-PCR and related to the pregnancy outcome.

Results: First-trimester decidua expressed all angiogenic factors. VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared increased in FGR compared to matched controls, whereas PlGF, Ang-1 and TIE-2 appeared increased in PE compared to controls. Flt-1 mRNA levels were comparable in both PE and FGR compared to the controls. The differential expression of angiogenic factors appeared to be more prominent in FGR than in PE.

Conclusions: These first-trimester samples provided a unique opportunity to obtain information regarding the onset of PE and FGR. First-trimester changes in angiogenic factor expression may well occur as a compensatory mechanism. In turn, this may unintentionally set the stage for increased angiogenesis and altered decidual/placental vascular adaptation, which may be part of the pathogenesis of PE and/or FGR.

INTRODUCTION

Disturbances in decidual and placental vascular development may play a role in complications of pregnancy, like pre-eclampsia (PE) and foetal growth restriction (FGR) [Ahmed, Perkins 2000; Ong *et al.*, 2000; Torry *et al.*, 2004]. PE affects 5-10% of pregnancies and is responsible for substantial maternal and neonatal morbidity and mortality. Predominant features are maternal systemic vascular endothelial dysfunction and microangiopathy. Target organs are the brain (seizures or eclampsia), liver (Hemolysis, Elevated Liver enzymes, Low Platelets (HELLP) Syndrome), kidney (proteinuria) and the utero-placental unit (fetal distress and growth restriction). Failure of placental vascular development has been linked to the pathogenesis of PE and FGR [Luttun, Carmeliet 2003; Maynard *et al.*, 2003].

Decidual vascular adaptation to pregnancy is characterised by high levels of both angiogenesis, the formation of new vessels from existing vasculature, and vasculogenesis, blood vessel generation from angioblast precursor cells [Burton *et al.*, 1999; Pijnenborg *et al.*, 1983]. Additionally, vascular mimicry or pseudo-vasculogenesis is also taking place where cytotrophoblasts convert to an endothelial phenotype. Failure of angiogenesis and pseudo-vasculogenesis has been proposed as processes in the inception of PE and FGR [Zhou *et al.*, 2002].

Numerous factors are thought to play a role in normal vascular adaptation to implantation [Geva *et al.*, 2002]. Vascular Endothelial Growth Factor-A (VEGF-A) and Placental Growth Factor (PlGF), are probably the best studied factors [Das *et al.*, 1997a; Plaisier *et al.*, 2007; Chung *et al.*, 2004]. VEGF interacts with VEGFR-1 (Flt-1) and VEGFR-2 (KDR) to promote endothelial cell proliferation, cell migration and vascular permeability. In rabbits, VEGF, together with its receptor Flt-1, plays an active role in the angiogenic process during implantation [Das *et al.*, 1997a]. PlGF shares biochemical and functional features with VEGF and interacts with VEGFR-1 (Flt-1). PlGF and VEGF-A have synergistic effects regarding angiogenesis, but vessels induced by PlGF are more mature and stable than vessels induced by VEGF-A [Carmeliet *et al.*, 2001; Luttun *et al.*, 2002]. PlGF is abundantly expressed in human placenta. Both VEGF-A and PlGF may be important paracrine regulators of decidual angiogenesis and autocrine mediators of trophoblast function [Plaisier *et al.*, 2007; Sherer, Abulafia 2001]. The soluble variant of Flt-1 has been associated with the pathogenesis of PE [Luttun, Carmeliet 2003; Maynard *et al.*, 2003]. A second family of growth factors, the angiopoietins, is also known for their regulating capacities regarding angiogenesis [Geva, Jaffe 2000]. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) bind with equal affinity to their receptor TIE-2, but have different functions. Ang-1 maintains vessel integrity and plays a role in the later stages of vascular remodelling [Geva, Jaffe 2000]. Ang-2 is a functional antagonist of Ang-1 and leads to

loosening of cell-cell interactions and allows access to angiogenic inducers like VEGF [Maisonpierre *et al.*, 1997]. Co-expression of VEGF and Ang-2 induces angiogenesis, but Ang-2 results in vascular regression in the absence of angiogenic signals [Asahara *et al.*, 1998]. Ang-1 and Ang-2 have both been detected in decidual and placental tissues [Maisonpierre *et al.*, 1997; Plaisier *et al.*, 2007].

Various decidual celltypes are capable of producing angiogenic factors. We recently showed the production of PlGF, KDR, Flt-1, Ang-2 and TIE-2 by endothelial cells and extra-villous trophoblasts. Decidual stromal cells, glandular epithelium and perivascular smooth muscle cells were found to produce all studied angiogenic factors [Plaisier *et al.*, 2007]. Uterine NK cells are also abundantly present in first-trimester decidua and are known to produce PlGF, VEGF, Ang-1 and Ang-2 [Li *et al.*, 2001a].

The possible correlation of PE and FGR with impaired vascular development has been studied in placental tissues mainly after the onset of disease [Geva *et al.*, 2002; Roberts, Cooper 2001; Taylor *et al.*, 2003]. However, whether decidual vascularisation is also related to the pathogenesis of both diseases is not known. Information regarding the expression of angiogenic factors in first-trimester tissues would be helpful in understanding PE and FGR. The present study focused on the role of first-trimester angiogenic growth factors in the pathogenesis of PE and/or FGR. The expression of VEGF-A, PlGF, Flt-1, KDR, Ang-1, Ang-2 and TIE-2 was determined in first-trimester decidua obtained during routine chorion villous sampling. The included patients were followed throughout their pregnancy to be able to relate the angiogenic factor expression to the pregnancy outcome, i.e. uncomplicated, PE or FGR.

MATERIALS & METHODS

Study group and tissue collection

Decidual samples were obtained from viable first-trimester pregnancies during routine chorionic villous sampling (CVS). CVS was performed vaginally between 10 and 12 weeks of gestation. Main indications were maternal age and serum screening related risk for aneuploidy. Gestational age was calculated according to the last menstrual period and confirmed by ultrasound (crown lump length). After CVS, surplus decidual tissue was microscopically separated from the chorion villi and immediately stored at -20 °C until RNA isolation. One sample per person was collected with an average size of 5-15 mg. Follow-up of the pregnancies was available through a postpartum questionnaire. Patients experiencing FGR (FGR, n=10) and PE/HELLP (n=9) were selected. PE was defined as diastolic blood pressure (BP) of minimal 90 mmHg, or a raise of 15 mmHg compared to first-trimester BP, combined with proteinuria of at least 300 mg/day. For FGR, criteria

according to Kloosterman were used [Kloosterman 1969]. Birth weight was corrected for gestational age and FGR was defined as a corrected birth weight below the 5th percentile. All cases were matched to controls based on gestational age, maternal age and parity at time of CVS (n=19). Patients with concurrent morbidity (e.g. diabetes) or medication were excluded. Foetal karyotyping showed no chromosomal abnormalities. The weight and the number of smokers were comparable between cases and controls. Patients were informed that surplus material was used for research, according to the "Guideline for Good Use" by the Federation of Medical Scientific Associations and informed consent was provided by all study subjects. Patient characteristics are described in Table 1.

RNA isolation and cDNA synthesis

Tissue samples were incubated in 1.5 ml RNA Later Ice (Ambion, Austin, TX, USA) for 48 hours at -20°C. After centrifugation, the tissue samples were lysed in lysisbuffer (RLT buffer (Qiagen) and β -mercapto-ethanol) using plastic mortars and RNA isolation was performed using RNeasy columns (Qiagen Benelux BV, Venlo, Netherlands) following recommended protocol. mRNA was dissolved in RNase free water. mRNA quantity and quality was determined by measuring its absorbance in a spectrophotometer (Nano-drop ND-1000) and reverse transcription was performed with 1 μ g total RNA, random primers and a cDNA synthesis kit (Ready-to-go You-Prime first strand beads, Amersham Biosciences, Buckinghamshire, UK). The obtained 32 μ l cDNA was diluted 1:3.

Real-time RT-PCR

mRNA expression was quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, Flt-1, KDR, PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene (all purchased from Applied Biosystems, Foster City, CA, USA).

Table 1. Characteristics of the FGR and PE study groups and matched control groups.

		FGR (n=10) mean $\pm$ SD	Control (n=10) mean $\pm$ SD	PE/HELLP (n=9) mean $\pm$ SD	Control (n=9) mean $\pm$ SD
At CVS	Age (years)	39.5 $\pm$ 2.1	39.6 $\pm$ 2.1	39.2 $\pm$ 1.9	39.2 $\pm$ 1.9
	GA (days)	75.6 $\pm$ 3.2	76.5 $\pm$ 2.5	78.6 $\pm$ 3.9	77.3 $\pm$ 4.4
	Parity	1.5 $\pm$ 1.4	1.1 $\pm$ 0.6	0.7 $\pm$ 0.9	0.7 $\pm$ 0.9
At delivery	GA (days)	278 $\pm$ 11	282 $\pm$ 12	261 $\pm$ 22	271 $\pm$ 10
	Birth weight (g)	2477 $\pm$ 286*	3648 $\pm$ 475	2648 $\pm$ 798	3420 $\pm$ 382

* p<0.05 versus control group

β -actin, β_2 -microglobulin, and cyclophilin were also used as reference genes and showed similar results (data not shown).

RT-PCR for target gene/GAPDH pairs was performed in 12.5 μ l reactions, containing 2.5 μ l cDNA solution, using mastermix (RT-QP2X-03, Eurogentec, Maastricht, the Netherlands), DNase free water and the above described primer/probe sets. Reactions were performed in duplicate and mRNA levels were expressed in cycle threshold (Ct). To correct for the RNA input, the difference in Ct values of target and reference genes ($dCt = Ct(\text{target gene}) - Ct(\text{GAPDH})$) was calculated. The difference between cases and controls ($ddCt$) was calculated as $ddCt = dCt(\text{case}) - dCt(\text{control})$ and the fold induction was calculated as 2^{-ddCt} . In addition, Ct values were quantified into ng/ μ l using a standard curve of total RNA. Cases and controls were compared by the ratio ng/ μ l(case)/ ng/ μ l(control). Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Statistics

The mRNA expression levels were compared between cases and matched controls. A general linear model for repeated measurements, ANOVA, was performed to analyse the paired data between matched cases and controls (SPSS 11.5). P-values of < 0.05 were considered significant.

RESULTS

The expression of the angiopoietins and their receptor TIE-2 and of VEGF, PlGF and their receptors KDR and Flt-1 were evaluated. The mRNA levels in all cases were calculated and expressed as $ddCt$, ng/ μ l and as fold induction compared to the control group (Table 2 and 3). All factors were present at mRNA level in decidua of cases as well as controls.

Control group

The control patients were matched to the cases; ten controls to FGR patients and nine controls to PE patients. First, mRNA expression was analysed in all the control patients. In these controls, PlGF showed the most abundant expression with a mean of 4.6 ng/ μ l (± 1.8). KDR, Flt-1 and Ang-2 showed a moderate expression with means of 0.4 ng/ μ l (± 0.1), 0.5 ng/ μ l (± 0.1) and 0.8 ng/ μ l (± 0.3) respectively. VEGF-A, Ang-1 and TIE-2 were only dimly expressed with means of 0.04 ng/ μ l (± 0.01), 0.01 ng/ μ l (± 0.00) and 0.15 ng/ μ l (± 0.06) (data not shown).

Table 2. First-trimester decidual mRNA expression of angiogenic factors (in cycle threshold (Ct)).

Angiogenic Factor	mRNA content (dCt) mean $\pm$ SEM ^a		Fold induction mean $\pm$ SEM ^b	
	FGR (n=10)	PE (n=9)	FGR (n=10)	PE (n=9)
VEGF-A	-1.5 $\pm$ 1.2	-0.5 $\pm$ 0.8	83 $\pm$ 70	3 $\pm$ 1
PlGF	0.5 $\pm$ 1.6	-2.0 $\pm$ 1.4	31 $\pm$ 21	51 $\pm$ 34
KDR	-0.5 $\pm$ 1.8	-1.8 $\pm$ 1.1	32 $\pm$ 25	17 $\pm$ 9
Flt-1	0.4 $\pm$ 0.6	-1.1 $\pm$ 0.7	2 $\pm$ 0.9	8 $\pm$ 6
Ang-1	-1.5 $\pm$ 1.9	-1.7 $\pm$ 1.6	84 $\pm$ 50	68 $\pm$ 40
Ang-2	-0.4 $\pm$ 1.3	0.1 $\pm$ 1.3	11 $\pm$ 5	6 $\pm$ 5
TIE-2	-1.2 $\pm$ 1.5	-3.1 $\pm$ 1.3	34 $\pm$ 25	110 $\pm$ 83

First-trimester decidual mRNA expression was determined by RT-PCR and compared between pregnancies resulting in FGR or PE and their matched controls. The 'repeated measures ANOVA' was performed to analyse the data.

^a mRNA content was expressed in cycle threshold (Ct). Mean Ct per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, and TIE-2 32.1. The Ct values were corrected for the mRNA input, by calculating the difference in Ct of target and reference genes: dCt = Ct(target gene) – Ct(GAPDH).

^b The difference between cases and controls (ddCt) was calculated as ddCt = dCt(case) – dCt(control). Fold induction in cases compared to controls was calculated as 2^{-ddCt} .

Fetal growth restriction group

The FGR group showed no significant differential expression compared to the control group (100%). Strikingly, the expression of VEGF-A (53-fold), PlGF (33-fold), KDR (26-fold), Ang-1 (59-fold), Ang-2 (8-fold) and TIE-2 (37-fold) appeared unregulated in cases compared to controls. However, significance was not reached due to the large inter-individual variation. Flt-1 showed comparable expression levels in cases and controls. The same pattern was found with regard to the data described as dCt (Table 2 and 3).

Pre-eclampsia group

The PE group showed no significant differential expression compared to the control group (100%). PlGF (35-fold), Ang-1 (29-fold) and TIE-2 (68-fold) appeared unregulated in cases compared to controls. However, these differences were not significant due to the large inter-individual variation. VEGF-A, KDR, Flt-1 and Ang-2 showed comparable expression levels in cases and controls. With regard to the data described as dCt, the same pattern was found (Table 2 and 3).

Table 3. First-trimester decidual mRNA expression of angiogenic factors (in ng/ μ l).

Angiogenic Factor	mRNA content mean $\pm$ SEM ^a		Fold induction mean $\pm$ SEM ^b	
	FGR (n=10)	PE (n=9)	FGR (n=10)	PE (n=9)
VEGF-A	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	53 $\pm$ 46	2 $\pm$ 1
PlGF	4.1 $\pm$ 1.8	8.2 $\pm$ 3.1	33 $\pm$ 21	35 $\pm$ 24
KDR	0.7 $\pm$ 0.3	1.7 $\pm$ 0.8	26 $\pm$ 17	13 $\pm$ 8
Flt-1	0.6 $\pm$ 0.2	2.0 $\pm$ 1.3	2 $\pm$ 1	3 $\pm$ 2
Ang-1	0.02 $\pm$ 0.0	0.4 $\pm$ 0.4	59 $\pm$ 34	29 $\pm$ 21
Ang-2	0.4 $\pm$ 0.2	3.6 $\pm$ 2.0	8 $\pm$ 4	5 $\pm$ 3
TIE-2	0.9 $\pm$ 0.8	19.6 $\pm$ 19.5	37 $\pm$ 30	68 $\pm$ 47

First-trimester decidual mRNA expression was determined by RT-PCR and compared between pregnancies resulting in FGR or PE and their matched controls. The 'repeated measures ANOVA' was performed to analyse the data.

^a mRNA content was expressed in cycle threshold (Ct). Mean Ct per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, and TIE-2 32.1. Cycle threshold were converted into ng/ μ l using a standard curve of total RNA. Values were corrected for mRNA input by calculating the ratio (ng/ μ l (target gene))/ (ng/ μ l (GAPDH)) and these values are expressed as "mRNA content" in the table.

^b Fold induction in cases compared to controls was calculated as the ratio ng/ μ l (case)/ ng/ μ l (controls).

DISCUSSION

The results showed that first-trimester decidual tissues obtained from chorionic villous sampling expressed all examined angiogenic factors. mRNA levels of VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared unregulated in FGR cases compared to matched controls. In addition, PlGF, Ang-1 and TIE-2 mRNA appeared unregulated in PE cases compared to matched controls. However, the large inter-individual variation disallowed an unequivocal conclusion. The differential expression of angiogenic factors appeared more prominent in FGR than in PE.

The origin of both PE and FGR probably starts in the first-trimester, before the onset of overt disease during the last trimester. Our first-trimester decidual samples provide a unique opportunity to obtain information regarding the onset of PE and FGR. The fact that the decidual samples were obtained together with villous tissue offers valid information on the original localization *in utero*, namely underneath the implantation site. Shortcomings are also present. No histological investigation could be performed on the number of trophoblasts and other cell types, because of the small amounts of material in each sample. Also, the study groups are small because of the rarity of the samples. This results in rather high standard deviations, which makes interpretation of the data

difficult. However, the 10- to 100-fold induction of genes in these small groups promises significant differences when groups could be expanded.

Current knowledge regarding PE suggests that defective vascular remodelling of maternal spiral arteries leads to impaired invasion of trophoblasts, placental insufficiency and ischemia [Luttun, Carmeliet 2003]. The affected placenta releases soluble anti-angiogenic factors, like soluble Flt-1 (sFlt-1) and endoglin (sEng) [Maynard *et al.*, 2003; Clark *et al.*, 1998b; Tjoa *et al.*, 2007]. These factors alter the angiogenic balance, mainly by neutralising VEGF and PlGF. Furthermore, these factors are able to induce systemic endothelial dysfunction and finally to induce clinical PE [Maynard *et al.*, 2003; Ahmad, Ahmed 2004; Hayman *et al.*, 1999; Sugimoto *et al.*, 2003]. The serum and placental levels of these factors have been studied extensively in term pregnancies, without reaching complete consensus with regard to VEGF. Several reports described increased, comparable and even decreased VEGF levels in placenta of term PE pregnancies [Geva *et al.*, 2002; Chung *et al.*, 2004; Ranheim *et al.*, 2001; Sgambati *et al.* 2004]. PlGF was decreased in serum of PE in the third-trimester [Lam *et al.*, 2005; Roberts, Cooper 2001]. Term placental tissue showed reduced Ang-2 mRNA and equal Ang-1 mRNA in PE [Zhang *et al.*, 2001]. No reports on these angiogenic factors are available regarding first-trimester tissues.

In the present study, mRNA levels of PlGF, Ang-1 and TIE-2 appeared unregulated in future PE patients compared to controls. VEGF-A, KDR, Flt-1 and Ang-2 showed comparable expression levels. No previous reports were found to reference these data in first-trimester tissues. Only first-trimester serum data are available, showing reduced PlGF and increased VEGF and sFlt-1 in first-trimester serum of PE patients [Levine *et al.*, 2004; Bosio *et al.*, 2001].

The studied Flt-1 represents both the soluble and membrane-bound Flt-1 and unfortunately no differentiation can be made between both types. The comparable overall Flt-1 expression in PE cases probably corresponds to the described decline of membrane bound Flt-1 and the known increase of soluble Flt-1 [Tsatsaris *et al.*, 2003].

FGR has been proposed to be induced by relatively high oxygen levels, which cause a disturbed expression of angiogenic factors and altered angiogenesis in placental villi [Burton 1997; Kingdom, Kaufmann 1997; Regnault *et al.*, 2003]. These relative hyperoxic conditions may cause the decreased VEGF and increased PlGF expression in FGR placentas, since these factors are inversely regulated by oxygen [Lyll 1997; Khaliq *et al.*, 1999]. An ovine model for FGR demonstrated increased Ang-1, Ang-2 and TIE-2 levels in early gestation before FGR was clinically detected. Later in gestation decreased TIE2 expression was associated with FGR [Erickson Hagen *et al.*, 2005]. Whether decidual angiogenesis is also affected by the altered oxygen levels in FGR is still not demonstrated but plausible.

Present study showed that the mRNA levels of angiogenic factors in first-trimester decidua were altered in the FGR group compared to the control group, although differences were not significant. The expression of VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared unregulated in FGR cases and Flt-1 showed comparable expression levels in cases and controls.

The relative hyperoxia in FGR and the oxygen effect on angiogenic factors are mainly described in feto-placental tissues and trophoblasts. If the theory is valid for first-trimester decidua, one would expect decreased VEGF and Ang-2 and increased Ang-1 and PlGF mRNA levels in FGR patients [Zhang *et al.*, 2001; Regnault *et al.*, 2003; Khaliq *et al.*, 1999; Sharkey *et al.*, 2000]. However, mRNA levels of all four genes appeared increased in FGR compared to matched controls. This might be explained by a different expression and regulation of angiogenic factors in first-trimester compared to third-trimester tissues. For example, VEGF-A and Ang-1 were shown to increase and Ang-2 to decrease as gestation progresses [Geva *et al.*, 2002]. In contrast, others demonstrated a decline in VEGF-A mRNA as gestation progresses [Cooper *et al.*, 1996]. In addition, oxygen influences in decidua may differ from its influences in villous tissue and/or trophoblasts. No references were found regarding oxygen regulation in decidua. Finally, histological differences or differences in the number of trophoblasts between the studied specimens cannot be ruled out.

The differential expression of angiogenic factors appeared more prominent in FGR than in PE and therefore a more pronounced disbalance between angiogenic factors might be involved in the pathogenesis of FGR. Overall, enhanced angiogenic factors in both patient groups might represent an (ineffective) vascular rescue mechanism in response to unidentified situation(s) and/or event(s). This compensatory mechanism may in turn unintentionally induce increased non-branching angiogenesis, altered decidual and placental vascular adaptation and functional placental insufficiency that may all result in PE and/or FGR during late gestation.

Chapter 9

General Discussion



Pregnancy demands significant physiologic adaptations, including vascular remodelling, of the uterine environment to facilitate adequate implantation. The timing of angiogenic processes is of paramount importance for the development of a receptive endometrium suitable for implantation. Disturbances in uterine blood supply are associated with first-trimester miscarriages and third trimester perinatal morbidity and mortality caused by pre-eclampsia (PE) and foetal growth restriction (FGR). This thesis assessed the role of angiogenesis in cycling endometrium and during human implantation. Furthermore, we attempted to evaluate the involvement of angiogenesis in the pathogenesis of missed abortions, PE and FGR.

ANGIOGENESIS AND PERICELLULAR PROTEASES IN CYCLING ENDOMETRIUM

Angiogenesis occurs on a regular basis as part of the growth and regression of the human endometrium during the menstrual cycle. There is evidence for multiple mechanisms of endometrial angiogenesis; angiogenesis during post-menstruation repair, elongative angiogenesis during the early and late proliferative phase and intussusception during the secretory phase. However, neither the timing of vascular growth during the menstrual cycle nor the mechanisms by which endometrial vessels are formed are currently completely understood, thus placing major limitations on our understanding of how angiogenesis promoters and inhibitors may act in the endometrium [Rogers, Gargett 1998]. With the development of *in vitro* systems mechanistic questions regarding the regulation of endometrial angiogenesis may become answered.

Previously, an *in vitro* model for endometrial angiogenesis was developed using human endometrial microvascular endothelial cells (hEMVEC) on fibrin and/or collagen 3D matrix. These cells have unique characteristics. They express high amounts of urokinase-type plasminogen activator (uPA), express various matrix metalloproteinases (MMPs) and membrane-type-MMPs (MT-MMPs), and display high angiogenic activity in response to vascular endothelial growth factor (VEGF). These characteristics are demonstrated in their *in vivo* counterparts as well [Chung *et al.*, 2002; Freitas *et al.*, 1999; Goffin *et al.*, 2003; Koolwijk *et al.*, 2001; Maatta *et al.*, 2000; Skinner *et al.*, 1999; Zhang *et al.*, 2000]. The combination of enhanced uPA expression and the high angiogenic activity directed us to determine the contribution of pericellular proteolysis to endometrial angiogenesis *in vitro*.

We demonstrated that both uPA/plasmin and MMPs contribute to the invasion and tubular structure formation of hEMVEC. Besides uPA, these cells express various MMPs, amongst which MT3-MMP (MMP-16) and MT4-MMP (MMP-17) and smaller amounts of MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT5-MMP (MMP-24) and MT6-MMP (MMP-25). These data are in agreement with observations reported from immunohis-

tochemical studies in endometrial tissue [Chung *et al.*, 2002; Freitas *et al.*, 1999; Goffin *et al.*, 2003; Maatta *et al.*, 2000; Skinner *et al.*, 1999; Zhang *et al.*, 2000].

MT1-, MT2-MMP and MT3-MMP are known inducers of capillary-tube formation and their mRNA levels increase during tube formation *in vitro* [Hotary *et al.*, 2000; Lafleur *et al.*, 2002; Shofuda *et al.*, 2001]. Experiments with overexpression of MMP inhibitors (tissue inhibitor of matrix metalloproteinases (TIMP) -1 and -3), which are also expressed by endometrial endothelial cells *in vivo* [Freitas *et al.*, 1999; Maatta *et al.*, 2000; Rodgers *et al.*, 1994], indicated that hEMVEC utilise a unique pattern of MT-MMPs during angiogenesis compared to endothelial cells originating from other tissues [Collen *et al.*, 2003; Galvez *et al.*, 2001; Hotary *et al.*, 2002; Lafleur *et al.*, 2002]. Moreover, the relative high expressions of MT3-MMP mRNAs in hEMVEC, the presence of MT3-MMP protein on endometrial endothelial cells and the inhibition of capillary tube formation by inhibiting MT3-MMP are strongly in favour of a contribution of MT3-MMP in capillary-like tube formation by hEMVEC. This was a striking finding, since up to now MT1-MMP was thought to be the most important MT-MMP involved in angiogenesis [Pepper 2001; Seiki, Yana 2003; Sounni *et al.*, 2002; Stetler-Stevenson 1999].

Certainly, *in vitro* models comprise several limitations. For instance, our model uses fibrin/collagen matrices, whereas the endometrial ECM consists of several proteins, among which collagen, and its composition and the presence of fibrin varies during the menstrual cycle [Aplin *et al.*, 1988; Bulletti *et al.*, 1988; Iwahashi *et al.*, 1996; Okada *et al.*, 2001]. We attempted to mimic endometrial ECM by isolating ECM of cultured endometrial stromal cells (hESC). hEMVEC cultured on fibronectin and on hESC ECM for 24-96 hours displayed differential gene expression. For example, ECM cultured hEMVEC showed markedly induced expression of VE-cadherin, fibronectin, and SPARC (inhibits proliferation of endothelial cells). Alternatively, the MT-MMPs, TIMPs, uPA, uPAR, PAI-1 and CD31 showed less than 2-fold (re)inductions. Thus, the gene expressions of the proteases of interest were not considerably changed by culturing on fibronectin.

Only by a combination of mechanistic *in vitro* and observational *in vivo* or *in situ* studies, an overall picture can emerge. In view of the proposed angiogenic involvement of MT3-MMP, we were interested whether MT3-MMP was expressed in human endometrium *in vivo*. All MT-MMPs were expressed in human endometrium. The elevated expression of MT-MMP antigens during the late secretory and menstrual phase indicates a role in the shedding process during menstruation [Lockwood *et al.*, 1998; Salamonsen, Woolley 1996; Zhang *et al.*, 2000]. Several MT-MMPs also showed a reduced expression during the receptive window, probably under the influence of steroidal hormones, which suggest a contribution to the generation of a stable environment in preparation for embryonic implantation.

With regard to the endothelium, MT2-, MT3-, and MT4-MMP, but not MT1-MMP, were expressed by *in vivo* endothelial cells. The endothelial expression of MT3-MMP and the consistent absence of endothelial MT1-MMP are in accordance with previous *in vivo* and *in vitro* work, but no data regarding the cellular expression of MT3- and MT4-MMP have been reported prior [Zhang *et al.*, 2000; Maatta *et al.*, 2000]. Strikingly, endothelial MT2, and MT3-MMPs were expressed during the proliferative and late secretory phase, which both display high angiogenic activity [Ferenczy *et al.*, 1979; Goodger, Rogers 1995; Morgan *et al.*, 1996; Rogers, Gargett 1998; Smith 2001]. This confirms the suggested contribution of MT3-MMP to angiogenesis *in vitro*. Moreover, MT2-MMP might be an additional angiogenic regulator to consider [Hiraoka *et al.*, 1998; Hotary *et al.*, 2000; Lafleur *et al.*, 2002]. So overall, the pericellular proteases uPA, MT2- and MT3-MMP, but not MT1-MMP, were shown to be involved in endometrial angiogenesis.

CONTRIBUTORS TO THE FORMATION OF FIRST-TRIMESTER DECIDUA

The endometrium transforms during the secretory phase into a well vascularised receptive tissue in response to oestradiol and progesterone. Influx of uterine NK (uNK) cells, decidual remodelling and angiogenesis are dominating features in this stage and continue in the presence of fertilisation and implantation. The differential presence of hormones, extra-villous trophoblasts, and uNK cells results in the generation of different types of decidua; pregnancy-induced hormones are involved in the development of decidua parietalis (DP) from the decidual secretory endometrium (DSE), while the additional presence of the extra-villous trophoblast (EVT) induces the generation of the decidua basalis (DB).

The invasion of immune cells to the implantation site is enormous; from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70 % of these leucocytes are uNK cells and 10% are macrophages [Bulmer *et al.*, 1991]. Various growth factors and cytokines are expressed by uNK cells, including the angiogenic factors and proteases Ang-1, Ang-2, TIE-2, VEGF-c, PlGF, uPA, uPAR and MT1-MMP [Al-Atrash *et al.*, 2001; Albertsson *et al.*, 2000; Hanna *et al.*, 2006; Lash *et al.*, 2006; Li *et al.*, 2001]. Consequently, an influx of uNK cells and macrophages may influence angiogenesis, decidualisation and implantation.

For that reason, we evaluated the presence of these cells in the three types of decidua. uNK cells (CD56+) and macrophages (CD68+) were present in DSE, DP and DB. The percentage of uNK cells showed an increase in DP and DB compared to DSE, while a comparable increase in macrophages did not reach statistical significance.

The foregoing information is summarised in a model, which describes the influences of uNK cells, EVT and pregnancy-induced hormones on DSE, DP and DB (Figure 1). This

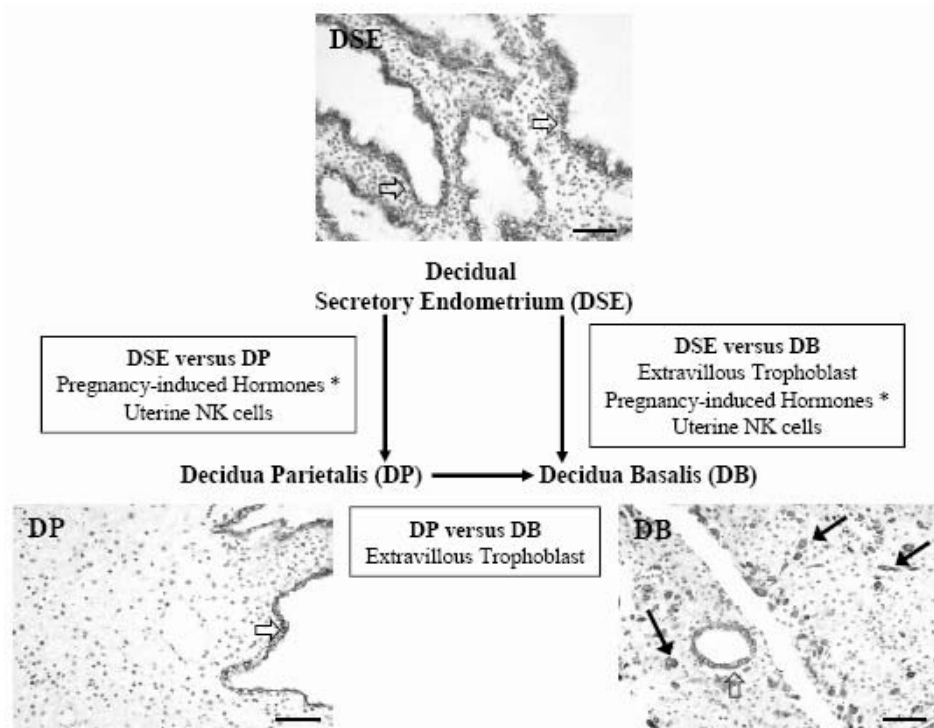


Figure 1. Schematic representation of the contributors to the formation of decidua parietalis and decidua basalis out of decidual secretory endometrium in the first-trimester of pregnancy. *) hCG, oestradiol and progesterone. DSE and DP express cytotokeratin in (open arrows), whereas cytotokeratin is also expressed in EVT (closed arrows) of DB. Bar =100 µm.

(see figure 5 of chapter 6 in colour figures supplement)

model enables hypothesising about vascular changes that occur independently of trophoblast invasion, and those that are mainly induced by pregnancy-induced hormones and/or uNK cells.

VASCULARISATION PATTERNS IN FIRST-TRIMESTER DECIDUA

Vascular adaptation to pregnancy is not well studied in human tissues and as a consequence, the regulation of decidual vascularisation is not clear. In rats, two separate mechanisms appear to control endothelial cell proliferation during implantation; a maternal control mechanism throughout the endometrium during the first days after fertilisation and a second post-implantation mechanism in the vicinity of the embryo [Goodger, Rogers 1993]. However, whether human decidual vascular adaptation to implantation is regulated by hormones, immune cells and/or the EVT and which mediators are used is unknown. In order to evaluate this, we determined the vascularisation

pattern and expression of angiogenic factors in decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP).

Differences in vascularisation pattern, enhanced total vascular surface and luminal diameter and reduced vessel density, from decidual secretory endometrium (DSE) to decidua parietalis (DP) to decidua basalis (DB). This vascular adaptation appeared to occur under influence of pregnancy-induced hormones, uNK cells, and the EVT. The reduced vascular density at the implantation site has been described before in rats and was suggested to result from oedema [Goodger, Rogers 1993]. However, the vascular density still differed after correction for oedema. The increase in luminal diameter may represent an adaptation to increased blood flow or may be due to vasodilatation reported to occur prior to infiltration of endovascular trophoblasts [Charnock-Jones 2002; Huppertz, Peeters 2005]. Furthermore, increased vessel diameter could also be induced by a relatively low oxygen level, which has been shown previously for pregnancies at high altitude [Zhang *et al.*, 2002]. The enlargement of the total vascular surface in DB and DP has been reported previously and likely is functionally related to the increased blood supply required to serve the growing demands of the foetus. [Gibbons, Dzau 1994; Kam *et al.*, 1999].

ANGIOGENIC FACTORS IN FIRST-TRIMESTER DECIDUA

The differential vascular pattern in various decidual tissues enlarged our interest in vascular regulation. Endometrial vasculature already undergoes expansion during the pre-implantation period. Apparently the embryo prepares its implantation site by stimulating angiogenesis before any physical contact between embryo and endometrium has been made. We demonstrate that the human blastocyst is able to stimulate *in vitro* angiogenesis via the production of significant amounts of vascular endothelial growth factor-A (VEGF-A). Das *et al.*, reported comparable events during rabbit implantation [Das *et al.*, 1997a]. In humans, VEGF has been shown to be one of the earliest genes activated during pre-implantation embryo development [Krussel *et al.*, 2003]. Thus, VEGF-A appears to be a crucial contributor to the inception of pregnancy.

We attempted to further reveal whether VEGF-A and other angiogenic factors are mediators in the regulation of the described differential vascularisation. in the three types of decidua, DSE, DP and DB. VEGF-A was abundantly, but not differentially, expressed in all decidual tissues. KDR and flt-1 and VEGF proteins were expressed on endothelium of both DB and DP. Together, these findings confirm both the role of VEGF in decidual angiogenesis throughout implantation and the hypothesis of VEGF being an important factor during implantation.

Other mediators appeared to play their role more locally at the implantation site. The abundant induction of PlGF mRNA and proteins in decidua basalis in general and of PlGF and Flt-1 proteins on endothelial cells in particular was remarkable. PlGF, via Flt-1, is able to potently induce angiogenesis resulting in mature and stable vessels [Arroyo *et al.*, 2004; Carmeliet *et al.*, 2001; Khaliq *et al.*, 1996; Luttun *et al.*, 2002; Torry *et al.*, 1999]. These are important features in facilitating and resisting the dramatic increase in blood supply at the implantation site to serve the growing demands of the foetus. Furthermore, the Ang-2/Ang-1 ratio was elevated in decidua basalis (DB), which suggests that vessel destabilisation is favoured over vessel maturation in DB. Overall, the angiopoietins, via Tie-2, and PlGF, via Flt-1, appear to be key regulators of the vascular adaptive process and may, partially, account for the vascular changes at the site of embryonic implantation in decidua basalis.

Solely locating numerous angiogenic growth factors in human decidua presents a problem in understanding the coordination of local events in new vessel growth, as most of these factors display differing effects not necessarily related to angiogenesis. It must be remembered that the mere presence of a particular growth factor does not indicate functional availability and that further studies have to be performed to elucidate vascular regulation.

PERICELLULAR PROTEASES IN FIRST-TRIMESTER DECIDUA

Proteolysis is imperative during the adaptation to implantation since trophoblasts, uNK cells and endothelial cells require proteolytic activity to migrate. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs), in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs), and to the plasmin/plasminogen system [Alfano *et al.*, 2005; Kindzelskii *et al.*, 2004; Reuning *et al.*, 2003]. A synergistic effect of both systems on decidual vascularisation in mice has been shown by Solberg *et al.* MMP inhibition altered the differentiation of the decidua, whereas loss of both MMP and uPA/plasmin was necessary to alter decidual vascularisation and induce embryonic lethality [Solberg *et al.*, 2003]. In light of these observations, the PA/plasmin system and MT-MMPs were also evaluated in human decidua.

The expression of various pericellular-acting proteases varied between DSE, DB and DP. This differential expression enabled hypothesising about their functions as well as their regulation (Figure 1). Regarding their regulation, uPAR and MT1-MMP appeared regulated by pregnancy-induced hormones and/or uNK cells, whereas the presence of uPA, MT2-, MT3-, and MT5-MMP appeared regulated by the extra-villous trophoblast.

Possible decidual functions of the proteases may involve trophoblast invasion, immune cell migration, and /or vascularisation. All proteases were expressed by the EVT, which

suggests involvement in trophoblast invasion. Whether the proteases are involved in immune cell migration could not be answered from our data and is neither supported nor contradicted by the equal presence of immune cells in DB and DP. However, the expression of proteases by stromal decidual cells (our finding) and uNK cells enables both autocrine and paracrine influences on immune cell infiltration [Albertsson *et al.*, 2000; Kim *et al.*, 2000].

An interesting observation came into view with regard to their possible role in vascularisation. Endothelial and perivascular smooth muscle cell MT2- and MT3-MMP antigens were less abundantly present in DP and DB as compared to DSE. The endothelial amount of these MT-MMPs correlated well with the vessel density and correlated inversely with the luminal surface of vascular structures. This differential MT2- and MT3-MMP expression, together with the fact that MT2- and MT3-MMP are likely candidates for regulation of angiogenesis, suggests a role for MT2- and MT3-MMP in the regulation of vascularisation at the implantation site *in vivo*. Their reduced endothelial expression might indicate a control mechanism which prevents too vigorous endothelial migration and thereby too fast vascular remodelling. This has not been described previously. Whether MT-MMPs act as crucial contributors, or merely as trivial bystanders, to implantation remains to be elucidated.

ANGIOGENESIS, PROTEASES AND ANGIOGENIC FACTORS IN LATE FIRST-TRIMESTER PREGNANCY

As gestation progresses, vascular adaptation continues and is represented by decreased vessel density and a non-significant increased luminal surface in DB and DP. These vascular changes correlated with decreased total and endothelial expression of MT1-, MT2- and MT3-MMP proteins, whereas endothelial expression of PlGF and VEGF was increased in late first-trimester. The increased levels of growth factor and decreased levels of proteases in endothelial cells at the implantation site appear contradictory. Possibly, the regulatory mechanisms alter as gestation proceeds, perhaps under the influence of changing concentrations of hormones. The observed induced endothelial VEGF and PlGF might be correlated to the enhanced vascular remodelling, whereas the reduced endothelial MT-MMP expression might control vascular infiltration. Furthermore, the reduced overall MT-MMP expression might function in controlling a too destructive infiltration of EVT.

ANGIOGENESIS, PROTEASES, ANGIOGENIC FACTORS AND IMMUNE CELLS IN MISCARRIAGE

Vascularised, decidua is important for implantation and disturbances in vascular development may play a role in the pathogenesis of miscarriages. Few studies have addressed this hypothesis; higher decidual vessel density and differential expression of VEGF and its receptors were correlated with miscarriages [Vailhe *et al.*, 1999; Vuorela *et al.*, 2000]. Additionally, differential MMP and TIMP expression has been detected in receptive endometrium of recurrent miscarriage patients [Inagaki *et al.*, 2003b, Jokimaa *et al.*, 2002].

We demonstrated the importance of vascular remodelling for implantation. The occurrence of miscarriage was associated with altered vascularisation: significant decreased vessel density and increased luminal surface in DB and DP in decidua of miscarriages. The vascular differences correlated with the differential expression of angiogenic factors and proteases at the implantation site of miscarriages. Furthermore, the endothelial antigen expression of Flt1, KDR and MT2- and MT5-MMP was enhanced in DP and DB of miscarriages. Surprisingly, PlGF, the most regulated factor in uncomplicated first-trimester decidua, appeared not to be involved in the pathogenesis of missed abortions. Overall, more angiogenic activity generated by angiogenic growth factors as well as proteases, appeared to be generated in decidua of miscarriages.

The altered vascularisation and expression of angiogenic factors in miscarriages must have an embryonic or a maternal origin. The first category remains fairly hypothetical and might include genetical modifications, like polymorphisms and mutations, which possibly result in changed expression of angiogenic factors. These DNA variations are not detectable by conventional karyotyping techniques and therefore performing karyotyping would not have helped clarify this hypothesis. In addition, previously no differences in the number of leucocytes, in uNK cells and macrophages, and in decidual architecture were previously detected between chromosomally normal and abnormal pregnancies [Greenwold *et al.*, 2003; Quack *et al.*, 2001; Shimada *et al.*, 2006]. Thus, the lack of karyotyping will probably not significantly bias our observations.

Maternal-related causes might include a fundamentally disturbed angiogenic reaction to fertilisation and implantation. Strikingly, this “miscarriage vascularisation” resembles the differences between early and late first-trimester vascularisation, suggesting that too fast maturing vasculature is associated with the pathogenesis of miscarriages. The pre-maturely ripening may allow maternal blood in the intervillous space too early in the development of pregnancy, also demonstrated in vivo by doppler ultrasound imaging [Greenwold *et al.*, 2003; Jauniaux, Burton 2005]. The early-onset of maternal circulation could result in increased oxygen levels with subsequent oxidative stress. This could

modulate the architecture of vasculature and the expression of peri-cellular proteases and angiogenic factors [Burton 1997; Kingdom, Kaufmann 1997; Sharkey *et al.*, 2000]. Another maternal cause might be the altered regulation of oxygen levels, resulting in an even more hypoxic situation than during uncomplicated implantation. The relative “super-hypoxic” situation can induce non-branching angiogenesis and affect the expression of various angiogenic factors. These compensatory mechanisms generate the needed increase in blood supply, but may occasionally induce disproportionate angiogenic activity and set the stage for embryonic loss [Burton 1997, Khaliq *et al.*, 1999; Kingdom, Kaufmann 1997; Regnault *et al.*, 2003; Sharkey *et al.*, 2000; Zhang *et al.*, 2002]. This hypothesis is confirmed by the described increased vascular area and altered VEGF expression and Ang-2/Ang-1 ratio, but not by the unchanged PlGF expression in miscarriage decidua. A shortcoming of this hypothesis is that the effects of oxygen on vascularisation and the expression of angiogenic factors are derived from experiments in term placental tissue and whether these effects are true in first-trimester decidua has not been described yet.

Conclusions from histo-pathological studies of abortion tissue are often questioned since foetal death may occur days before evacuation, allowing post-mortem inflammation or apoptosis during this “retention time”. Retention time has been reported to have no effect on vascularisation [Lisman *et al.*, 2004; Meegdes *et al.*, 1988; Nelen *et al.*, 2000], which appears to exclude a contribution of “retention time” to the observed vascularisation differences between miscarriages and controls. To analyse whether our specimens demonstrated differential inflammatory or apoptotic events in response to foetal death, we determined proliferation, apoptosis and the number of immune cells. Ki67 and active Caspase-3 (aCasp-3) showed similar expression patterns suggesting an increased cellular turnover from DSE, to DP to DB in both cases and controls. The cases and controls showed equal amount of proliferation and apoptosis, thus indicating that increased apoptotic events in the miscarriage samples are unlikely. Comparable numbers of uNK cells were demonstrated in DP and DB of cases, but their number was significantly increased in DSE. Most reports on the relation of uNK cells to miscarriages describe increased uNK cells in receptive endometrium of patients with recurrent miscarriages (RM) [Quenby *et al.*, 1999; Tuckerman *et al.*, 2007]. The abundant presence of NK cells in our DSE samples may reflect these observations. The difference between cases and controls were levelled in DP and DB, possibly by hormone regulated NK cell migration to normal values [van den Heuvel *et al.*, 2005b].

ANGIOGENIC FACTORS IN PRE-ECLAMPSIA AND FOETAL GROWTH RESTRICTION

Both pre-eclampsia (PE) and foetal growth restriction (FGR) are extensively studied in serum/plasma, third-trimester tissues and also, to a smaller extent, by *in vitro* models [Chung *et al.*, 2004; Eriksson Hagen *et al.*, 2005; Geva *et al.*, 2002; Maynard *et al.*, 2003; Sugimoto *et al.*, 2003]. Information about first-trimester molecular and morphological events would be very valuable.

Current knowledge suggests that the pathogenesis of PE starts with defective vascular remodelling of maternal spiral arteries leading to non-invasion of trophoblasts, placental insufficiency and ischemia [Luttun, Carmeliet 2003; Zhou *et al.*, 1997]. The diseased placenta releases soluble anti-angiogenic factors, like soluble flt-1 (sFlt-1) and endoglin (sEng). These factors alter the angiogenic balance, mainly by neutralising VEGF-A and PlGF, induce systemic endothelial dysfunction and finally clinical PE [Ahmad, Ahmed 2004; Clark *et al.*, 1998; Fisher 2004; Hayman *et al.*, 1999; Maynard *et al.*, 2003; Sugimoto *et al.*, 2003; Tjoa *et al.*, 2007; Yuan *et al.*, 2005]. FGR, or Foetal growth restriction (FGR), has been proposed to be induced by relatively high oxygen levels, which cause disturbed expression of angiogenic factors and altered angiogenesis in placental villi [Burton 1997; Kingdom, Kaufmann 1997; Regnault *et al.*, 2003]. These relative hyperoxic conditions may explain the decreased VEGF and increased PlGF expression found in FGR placentas, since both factors are inversely regulated by oxygen [Lyll 1997; Khaliq *et al.*, 1999]. Whether the altered oxygen levels also affect decidual angiogenesis is not documented, but is likely.

We showed that pre-eclampsia and foetal growth restriction were both associated with the induction of angiogenic factors in first-trimester decidua. Unfortunately, the study groups are small because of the rarity of the samples. This results in rather high standard deviations, which prevents significance and makes interpretation of the data difficult. However, the 10 to 100-fold induction of genes in these small groups promises significant differences when groups could be expanded.

The early first-trimester changes in angiogenic factor expression may well occur as a compensatory mechanism, but in turn may unintentionally induce increased non-branching angiogenesis, altered decidual and placental vascularisation and insufficiency that result in pre-eclampsia and/or foetal growth restriction during late gestation.

FUTURE PERSPECTIVES

This thesis includes studies focussing on angiogenesis and its regulation in human endometrium and decidua and provides new insights into the regulation of endometrial and decidual angiogenesis.

In human, very little work has been done in relation to decidual angiogenesis apart from few studies focussing on the location of numerous angiogenic growth factors. This approach presents a problem in understanding the coordination of local events in new vessel growth and its controls and limits, as most of these factors display widely differing effects. It must be remembered that the presence of a growth factor does necessarily indicate functional availability. Therefore, observations obtained from these *in situ* studies have to be accompanied by *in vitro* studies in order to confirm and further elucidate the mechanisms controlling decidual vascular development. For example, analysing the involvement of uNK cells and the role of MT2- and MT3-MMP, but also MT5-MMP, in regulating capillary like tube formation *in vitro* will be very interesting. However, it is essential to optimise the *in vitro* tube formation model to the “decidual situation” in order to obtain valuable results.

The interaction between EVT and vascularisation and the regulation of angiogenic factors and proteases by pregnancy-induced hormones, uNK cells and/or the extravillous trophoblast is best studied *in vitro*. The first item can be studied by performing co-cultures of trophoblasts and endothelial cells and for example subsequent gene expression profiling. The latter item is probably best studied by analysing the effect of the three possible regulators on separate cell types. In addition, determining the presence of hormone receptors is imperative as well. Merely the combination of *in vivo* and *in vitro* assays will achieve the understanding of human implantation.

Additionally, the interesting regulatory effects of hypoxia on decidual vascularisation must be further elucidated. The oxygen level in the different types of first-trimester decidua, DSE, DP and DB, has to be established and the regulation of angiogenic factors and proteases in first-trimester tissues by oxygen has to be determined. This information will contribute to a better understanding of oxygen mechanisms in both physiologic and pathologic pregnancies. Moreover, new morphological imaging as well as new ultrasound techniques and measurements hopefully will allow quantification of altered angiogenesis and blood flow in physiologic and complicated first-trimester pregnancies and pregnancies complicated by miscarriage, PE and FGR.

Another promising future research subject is the role of endothelial progenitor cells during pregnancy. Endothelial progenitor cells are mobilised from the bone marrow by oestrogen and are proposed to play an important role in the regulation and maintenance of vasculature during pregnancy. Their increased mobilisation, probably via ischemia, may underlie pre-eclampsia [Robb *et al.*, 2007].

Finally, angiogenesis and its regulation in endometrium and decidua is a fascinating research topic. Angiogenesis and various angiogenic factors and proteases appear crucial contributors to the inception of pregnancy. The obtained knowledge adds to a better understanding of physiologic and pathologic conditions regarding menstruation and pregnancy.

Chapter 10

Conclusions & future perspectives



Angiogenesis and pericellular proteases in cycling endometrium

- Tube formation by endometrial endothelial cells depends on uPA/plasmin and MMPs (Ch. 2).
- MT3-MMP is a potential regulator of endometrial angiogenesis *in vitro* (Ch. 2).
- MT-MMPs are expressed in endometrium in a cycle-dependent pattern (Ch. 3).
- MT2- and MT3-MMP appear associated with endometrial angiogenesis *in vivo*, which is a confirmation of earlier *in vitro* data (Ch. 3).

Contributors to the formation of first-trimester decidua

- Pregnancy induced hormones, i.e. oestradiol, progesterone and hCG, uterine NK cells and the extra-villous trophoblast are the main regulators of decidual development (Ch. 5, 6).

Vascularisation patterns in first-trimester decidua

- The extra-villous trophoblast (EVT), the pregnancy-induced hormones (oestradiol, progesterone and hCG), and uterine NK cells contribute to vascular regulation (Ch. 5).
- Vasculature at the implantation site displays fewer vessels with greater circumference and an enlarged total vascular area (Ch. 5).

Angiogenic factors in first-trimester decidua

- The human embryo is able to stimulate decidual angiogenesis via VEGF-A (Ch. 4).
- VEGF-A is probably involved in vascularisation in all decidual tissues (Ch. 5).
- PlGF, via Flt-1, and the angiopoietins appear to be related to decidual angiogenesis at the implantation site (Ch. 5).

Pericellular proteases in first-trimester decidua

- The pericellular proteases, uPA (and uPAR), MT1-, MT2-, MT3- and MT5-MMP were differentially regulated by the EVT, pregnancy-induced hormones, and uterine NK cells (Ch. 6).
- All MT-MMPs might be involved in trophoblast and uNK cell invasion, whereas MT2- and MT3-MMP also appear to contribute to decidual vascularisation (Ch. 6).

Angiogenesis, proteases and angiogenic factors in late first-trimester pregnancy

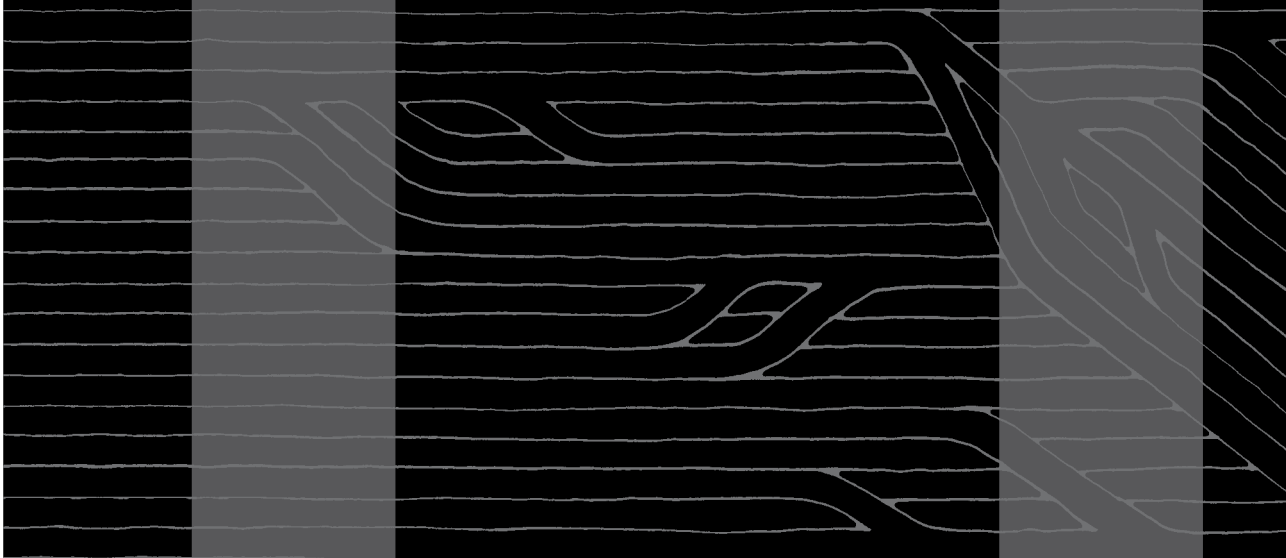
- Decidual vascularisation is altered as gestation progresses, showing less but larger vessels (**Ch. 5**).
- This altered vascularisation correlated with elevated expression of VEGF and PlGF and decreased expression of MT₁, MT₂- and MT₃-MMP in endothelium and in general (**Ch. 5/6**).

Vascularisation and miscarriage, pre-eclampsia and foetal growth restriction

- Decidual vascularisation appears to adapt too rapidly to embryonic implantation in miscarriage (**Ch. 7**).
- The disturbed vascular development correlated with differential expression of angiogenic factors and proteases (**Ch. 7**).
- Altered expression of angiogenic factors in first-trimester decidua appears correlated with the pathogenesis of pre-eclampsia or foetal growth retardation later in pregnancy (**Ch. 8**).

Chapter 11

Summary & Samenvatting



SUMMARY

Endometrial angiogenesis is indispensable for the formation of a richly vascularised receptive endometrium and decidua. The endometrium is a unique tissue which undergoes rapid remodelling during each menstrual cycle. During the secretory phase endometrial receptivity is enhanced by stimulation of oestradiol and progesterone. This process is called pre-decidualisation. By secretion of prostaglandin, cytokines and hCG the blastocyst will further induce decidualisation of the endometrium at the implantation site, and eventually throughout the endometrium. Vascularisation during decidualisation and in the decidua is of importance for the success of the embryo-maternal interaction. Disturbances in vascular development may play an important role in pathologies during pregnancy. For instance, early pregnancy wastage, pre-eclampsia (PE) and foetal growth restriction (FGR) are disorders associated with impaired angiogenesis.

Angiogenesis is a multi-step process involving degradation of the basal membrane, activation, migration and proliferation of endothelial cells and finally the formation of capillaries and the recruitment of pericellular smooth muscle cells. Numerous factors are involved in these processes, e.g. vascular endothelial growth factor (VEGF), placental growth factor (PlGF), angiopoietins and proteases, each of which can regulate the process in different ways. Pericellular proteolysis plays an important role in angiogenesis being required for endothelial cell migration, invasion and tube formation. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs), in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs), and to the plasmin/plasminogen system. However, neither the timing of vascular growth during the menstrual cycle and implantation, nor the mechanisms by which vessels are formed are currently completely understood.

In **Chapter 2** we studied the expression of MMPs and urokinase-type plasminogen activator (uPA) by human endometrial microvascular endothelial cells (hEMVEC) and their involvement in the formation of capillary tubes. We demonstrated that both uPA/plasmin and MMPs, expressed by hEMVEC, contribute to the invasion and tubular structure formation of hEMVEC in 3D fibrin matrices. MT₁-, MT₂- and MT₃-MMP are known inducers of capillary-tube formation and their mRNA levels increase during tube formation *in vitro*. Experiments with overexpression of MMP inhibitors (tissue inhibitor of matrix metalloproteinases (TIMP) -1 and -3), which are also expressed by endometrial endothelial cells *in vivo*, indicated that hEMVEC utilise a unique pattern of MT-MMPs during angiogenesis compared to endothelial cells originating from other tissues. The relative high expression of MT₃-MMP mRNA in hEMVEC, the presence of MT₃-MMP protein on endometrial endothelial cells and the inhibition of capillary tube formation by

inhibiting MT3-MMP are strongly in favour of a contribution of MT3-MMP in capillary-like tube formation by hEMVEC. This was a striking finding, since up to now MT1-MMP was thought to be the most important MT-MMP involved in angiogenesis.

Although membrane-type-3 metalloproteinase (MT3-MMP) appeared associated with endometrial capillary-like tube formation *in vitro*, the involvement of MT-MMPs with endometrial angiogenesis *in vivo* is unknown. **Chapter 3** describes the presence of MT-MMPs (MT1-MMP to MT6-MMP) in human endometrium and their correlation with endometrial neovascularisation. The various MT-MMP antigens were expressed in the various cell types of the endometrium. Their presence was decreased during the early secretory phase, which might suggest a contribution to the generation of a stable environment in preparation for embryonic implantation. Only MT2-, MT3- and MT4-MMP antigens were expressed by endothelial cells. Strikingly, this vascular expression of MT2- MT3- and MT4-MMP correlated with angiogenic episodes of the cycle. These findings support earlier *in vitro* data on the regulatory role of MT3-MMP in endometrial angiogenesis. Additionally, MT2-MMP appears to be associated with endometrial neovascularisation as well.

Whereas the previous chapters concentrate on endometrial angiogenesis, **Chapters 4-8** focus on decidual angiogenesis. We demonstrated in **Chapter 4** that the human blastocyst is able to stimulate *in vitro* angiogenesis via the production of significant amounts of vascular endothelial growth factor A (VEGF-A). This inducing effect was counteracted by adding soluble VEGF-R1 (flt-1). Other mediators, which have been described as produced by the early embryo/trophoblast, had no effect on tube formation by hEMVEC. This confirmed the hypothesis that VEGF-A plays a key role during early pregnancy and supports the finding that VEGF-A expression is reduced in trophoblastic tissue and in maternal endometrium of recurrent miscarriage cases.

Chapter 5 evaluates vascular adaptation to implantation by studying vascularisation and angiogenic factors in decidual secretory endometrium (DSE) decidua parietalis (DP), and basalis (DB) of first-trimester pregnancies. The vascularisation pattern differed among the three tissues, for example an enhanced vascular and luminal surface and reduced vessel density were found at the implantation site. This raised questions regarding the regulation of decidual vascularisation. Comparing the three tissues provided information about the regulation of vascularisation and showed that both pregnancy-induced hormones and extra-villous trophoblast (EVT) influenced vascularisation. The observed vascular differences were also correlated to the differential expression of angiogenic factors. VEGF-A mRNAs and proteins were abundantly, but not differentially, expressed in all decidual tissues and KDR, flt-1 and VEGF-A proteins were expressed on

endothelium of both DB and DP. These findings suggest a role for VEGF-A in supporting decidual angiogenesis throughout implantation and further confirm the hypothesis of VEGF-A being an important factor during implantation.

Other mediators, like PlGF and the angiopoietins, appeared to have a specific role at the implantation site. These factors may, partially, account for the vascular changes at the site of embryonic implantation in decidua basalis.

Proteolysis is essential for decidual development during embryonic implantation. Therefore, the decidual expression of pericellular proteases was studied in **Chapter 6**. The expression of MT-MMPs and urokinase/urokinase receptor (uPA/uPAR) varied between decidua basalis (DB), decidua parietalis (DP) and decidual secretory endometrium (DSE). This enabled hypothesising about their regulation by pregnancy-induced hormones, immune cells and/or the extra-villous trophoblast (EVT) and their relationship with decidual remodelling, trophoblast invasion and vascularisation. uPAR and MT1-MMP appeared to be regulated by pregnancy-induced hormones and uPA, MT2-, MT3-, and MT5-MMP by EVT. All proteases were expressed by the EVT themselves and might be involved in trophoblast invasion. Furthermore, MT2- and MT3-MMP may also regulate vascularisation at the implantation site.

Additionally, decidual vascularisation and its regulation were compared between early and late first-trimester in **Chapter 5 and 6**. As gestation progresses, vascular adaptation continues and is represented by decreased vessel density and a non-significant increased luminal surface in DB and DP. These vascular changes correlated with increased endothelial expression of PlGF and VEGF-A in late first-trimester. Also, decreased total and endothelial expression of MT1-, MT2- and MT3-MMP proteins were observed in later gestation, which might function in controlling a too destructive infiltration of endothelial cells and extra-villous trophoblasts.

Chapter 7 describes vascular adaptation to implantation in missed abortions. A significant decreased vessel density and increased luminal surface was demonstrated in DB and DP in decidua of miscarriages. Strikingly, these differences in vascularisation patterns resemble the differences between early and late first-trimester vascularisation, suggesting that too fast maturing vasculature is associated with the pathogenesis of miscarriages.

The vascular differences between missed abortions and controls correlated with the differential expression of angiogenic factor, showing an induction of VEGF-A, VEGF-receptor-1 and -2 (flt-1 and KDR), the angiopoietins Ang-1 and Ang-2, angiopoietin receptor TIE-2, MT2- and MT5-MMP and reduction of MT3-MMP at the implantation site. Furthermore, the endothelial expression of flt1, KDR, MT2- and MT5-MMP antigens were

enhanced in DP and DB of missed abortions cases. Surprisingly, placental growth factor (PLGF), the most regulated factor in uncomplicated first-trimester decidua, appears not to be involved in the pathogenesis of missed abortions.

Differential inflammatory or apoptotic events, in response to foetal death, were excluded in our specimens, since cases and controls showed equal amounts of proliferation and apoptosis and comparable numbers of uNK cells in DP and DB. However, uterine natural killer (uNK) cells numbers were significantly increased in DSE. Most reports on the relation of uNK cells to miscarriages describe increased numbers of uNK cells in receptive endometrium of patients with recurrent miscarriages (RM). The abundant presence of uNK cells in our DSE samples may reflect these observations.

Current knowledge suggests that the pathogenesis of pre-eclampsia (PE) includes defective vascular remodelling of maternal spiral arteries, non-invasion of trophoblasts, placental insufficiency and ischemia and that foetal growth restriction (FGR) has been proposed to originate from defective angiogenic factor expression and angiogenesis in placental villi in response to relatively high oxygen levels.

Whether decidual angiogenesis is also affected was studied in **Chapter 8**. We showed the differential mRNA expression of various angiogenic factors in first-trimester decidua of third-trimester PE and FGR patients compared to matched controls. The expression of VEGF-A, PLGF, KDR, Ang-1, Ang-2 and TIE-2 appeared unregulated in FGR cases and PLGF, Ang-1 and TIE-2 appeared upregulated in PE cases. Significance was not reached, which is probably due to the large inter-individual variation. This is rather normal for a pilot study but restricts data interpretation. However, the 10- to 100-fold gene induction in these small groups promises significant differences in expanded groups. The early first-trimester changes in angiogenic factor expression may well occur as a compensatory mechanism, but in turn may unintentionally induce increased non-branching angiogenesis, altered decidual and placental vascularisation and insufficiency that result in PE and/or FGR during late gestation.

In **Chapter 9** the results of the studies of this thesis are discussed. The obtained knowledge generated through this thesis adds to a better understanding of physiologic and pathologic conditions regarding menstruation and pregnancy. Additionally, recommendations for future research are given. **Chapter 10** provides a final conclusion.

SAMENVATTING

Angiogenese is essentieel voor het ontstaan van een zwangerschap, omdat het zorgt voor het ontstaan van een rijk gevasculariseerd en receptief endometrium dat toegankelijk is voor implantatie van de blastocyst. Het endometrium is een uniek weefsel, dat gedurende elke menstruele cyclus enorme veranderingen doormaakt. De receptiviteit van het endometrium wordt gedurende de secretoire fase van de cyclus gestimuleerd door progesteron en oestradiol. Dit proces wordt pre-decidualisatie genoemd. De blastocyst zal daarna op de plaats van implantatie verdere decidualisatie induceren door het lokaal uitscheiden van prostaglandinen, cytokinen en hCG. Uiteindelijk zal het gehele endometrium decidualiseren. Dit wordt gekarakteriseerd door oedeem en de extravasatie van macromoleculen ter bevordering van de receptiviteit. Daarnaast is voldoende vascularisatie belangrijk voor het succes van de implantatie. Verstoringen in de vasculaire ontwikkeling lijken een rol te spelen bij het ontstaan van zwangerschapscomplicaties, zoals miskraam, pre-eclampsie (PE) en intrauteriene groeivertraging (IUGR).

Angiogenese is een proces dat uit verschillende stappen bestaat, te weten degradatie van de basaalmembraan; activatie, migratie en proliferatie van endotheelcellen; en uiteindelijk de formatie van capillairen en het aantrekken van peri-vasculaire gladde spiercellen. Verschillende factoren zijn betrokken bij de regulatie van deze processen, zoals vascular endothelial growth factor (VEGF-A), placental growth factor (PlGF), angiopoietinen en proteases. Daarnaast speelt peri-cellulaire proteolyse een belangrijke rol in het angiogenese proces. Het is noodzakelijk voor endotheelcel-migratie, -invasie en capillairvorming. Belangrijke regulatoren van proteolyse zijn het plasminogeen activator/plasmine systeem, met onder meer urokinase (uPA) en zijn receptor (uPAR), en de matrix metalloproteinases (MMPs), in het bijzonder de membraangebonden MMPs (MT-MMP's).

Zowel de manier waarop nieuwe vaten worden gevormd als de timing van vasculaire groei in de menstruele cyclus en de embryonale implantatie, zijn tot op heden grotendeels onbekend.

In **Hoofdstuk 2** wordt de expressie van MMP's en uPA door humane endometriale micro-vasculaire endotheelcellen (hEMVEC) en hun betrokkenheid bij de vorming van capillairen *in vitro* bestudeerd. Zowel uPA als MMP's blijken een rol te spelen bij het tot stand komen van capillairen door hEMVEC. Naast uPA werden verschillende MMP's aangetroffen in deze cellen.

Het is bekend dat MT₁-MMP, MT₂-MMP, en MT₃-MMP angiogenese kunnen induceren, en dat hun mRNA niveau stijgt tijdens capillairvorming *in vitro*. Een experiment dat gebruikmaakt van twee MMP-remmers (tissue inhibitor of matrix metalloproteinases

(TIMP-1 en -3)) toonde aan dat hEMVEC een unieke set MT-MMPs voor angiogenese gebruiken in vergelijking met endotheelcellen uit andere weefsels. De relatief hoge expressie van MT3-MMP mRNA in hEMVEC, de aanwezigheid van MT3-MMP eiwitten op endometrium endotheelcellen en de remming van capillairvorming door anti-MT3-MMP wezen allen op een rol van MT3-MMP in endometrium angiogenese. Dit was een verrassende observatie, aangezien tot op heden MT1-MMP als de belangrijkste MT-MMP in angiogenese werd beschouwd.

MT3-MMP lijkt dus geassocieerd met endometriale capillairvorming *in vitro*, maar het is onduidelijk of dit ook *in vivo* het geval is. **Hoofdstuk 3** beschrijft de expressie van MT-MMP's en hun correlatie met vascularisatie in humaan endometrium. Alle MT-MMP's werden aangetoond in diverse celtypen in het endometrium en waren verminderd aanwezig tijdens de vroege secretoire fase van de cyclus. Dit wijst op een bijdrage van MT-MMP's aan de stabilisatie van het endometrium in voorbereiding op de embryonale implantatie. Alleen MT2-, MT3- en MT4-MMP antigenen kwamen tot expressie in endotheelcellen. Deze vasculaire expressie correleerde met angiogene perioden gedurende de menstruele cyclus. Deze bevindingen ondersteunen eerdere data met betrekking tot de rol van MT3-MMP in endometrium angiogenese. Bovendien lijkt MT2-MMP ook geassocieerd met neovascularisatie in humaan endometrium.

De eerste hoofdstukken beschrijven angiogenese in het endometrium, maar de **Hoofdstukken 4 t/m 8** richten zich op angiogenese in humaan first-trimester decidua. Diermodellen tonen aan dat het embryo zijn implantatie voorbereidt door het stimuleren van angiogenese, voordat sprake is van fysiek contact tussen embryo en endometrium. In **Hoofdstuk 4** wordt aangetoond dat de humane blastocyst in staat is tot het stimuleren van *in vitro* angiogenese door de productie van significante hoeveelheden VEGF-A. Dit effect kon worden geremd door toevoeging van oplosbaar flt-1. Eerder werd al aangetoond dat VEGF-A één van de eerste genen is die geactiveerd wordt tijdens de embryonale ontwikkeling in de pre-implantatie periode. De hypothese dat VEGF-A een sleutelrol vervult bij het tot stand komen van een jonge zwangerschap wordt verder bevestigd door de observatie dat de VEGF-A expressie in trofoblastweefsel verminderd is in endometrium van patiënten met herhaalde miskramen.

Hoofdstuk 5 bestudeert de aan implantatie gerelateerde vasculaire veranderingen. Daartoe werden het vaatpatroon en de expressie van angiogene factoren bepaald in 1^e trimester deciduaal secretoir endometrium (DSE), decidua pariëtalis (DP), en decidua basalis (DB). Deze deciduale weefsels toonden een verschillend vaatpatroon. Zo toonde decidua basalis, de plaats van implantatie, een toegenomen totale vasculaire oppervlakte in vergelijking met DP en DSE.

Het vergelijken van de drie weefsels toonde dat zowel zwangerschapsgeïnduceerde hormonen als de extravillieuze trofoblast (EVT) vascularisatie beïnvloeden. Welke mediators betrokken zijn bij de regulatie van deze vasculaire veranderingen in de verschillende typen decidua werd bestudeerd in **Hoofdstuk 5**. VEGF-A mRNA en proteïnen kwamen uitbundig tot expressie in alle typen decidua. VEGF-A proteïnen zowel als de receptoren voor VEGF-A, KDR en flt-1, werden aangetroffen op endotheelcellen in zowel DB als DP. VEGF lijkt dus angiogenese te ondersteunen in alle onderzochte decidua-typen. Dit bevestigt wederom de hypothese dat VEGF-A een belangrijke rol speelt in het implantatieproces.

Andere mediators, zoals placental growth factor (PlGF) en de angiopoïetinen, kwamen verhoogd tot expressie in decidua basalis (DB). Zij lijken dus specifiek een rol te spelen bij vascularisatie op de plaats van implantatie en zijn dus mogelijk medeverantwoordelijk voor de vasculaire veranderingen in decidua basalis.

Proteolyse is belangrijk voor de ontwikkeling van decidua. De expressie van pericellulaire proteases in decidua werd daarom bestudeerd in **Hoofdstuk 6**. Ook de regulatie door zwangerschapshormonen, immuuncellen en de EVT, en de relatie tot trofoblastinvasie en vascularisatie werden beschreven. De drie typen decidua toonden verschillende hoeveelheden MT-MMPs, urokinase-type plasminogeen activator (uPA) en uPA receptor (uPAR). MT1-MMP en uPAR bleken gereguleerd te worden door zwangerschapshormonen; uPA, MT2-, MT3- en MT5-MMP door EVT. Alle proteases kwamen tot expressie in EVT en spelen mogelijk een rol bij trofoblastinvasie. Daarnaast lijken MT2- en MT3-MMP ook de vascularisatie te reguleren in decidua basalis.

In **Hoofdstuk 5 en 6** wordt vascularisatie vergeleken in vroege en late 1^e trimester zwangerschappen. Naarmate de zwangerschap vordert worden de aan implantatie gerelateerde vasculaire veranderingen steeds duidelijker, zoals een verminderde vaatdichtheid en een verhoging van lumen-oppervlakte in DB en DP. De veranderingen in het vaatbed correleren met een verhoogde expressie van PlGF en VEGF-A in endotheelcellen. Verder bleken de expressies van MT1-, MT2- en MT3-MMP in verschillende celtypen, inclusief endotheelcellen, verminderd in de late 1^e trimester groep. Dit heeft mogelijk een functie in het controleren van de destructieve infiltratie van endotheel- en trofoblastcellen.

Hoofdstuk 7 beschrijft de vasculaire aanpassingen aan implantatie in missed abortions (MA) in vergelijking met ongecompliceerde zwangerschappen. Een significant afgenomen vaatdichtheid en lumen-oppervlakte werden aangetoond in DB en DP van missed abortions. De verschillen in vaatpatronen tussen MA en controles zijn vergelijkbaar met de gevonden verschillen tussen vroege en late first-trimester zwangerschappen. Dit kan duiden op het te vroeg uitrijpen van het vaatbed in MA.

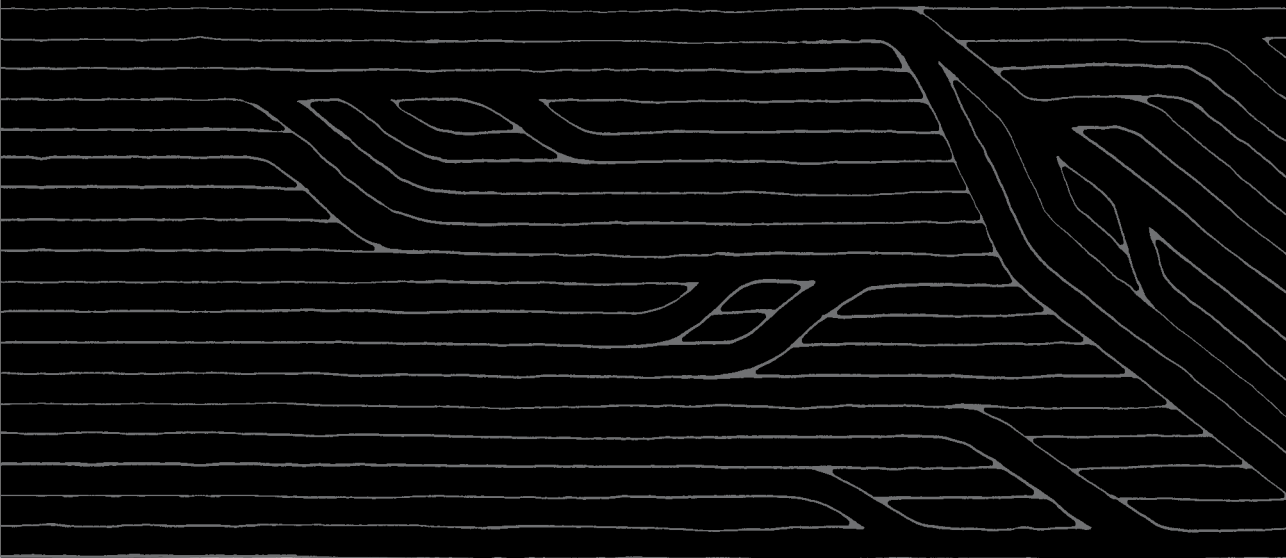
De verschillen op vasculair gebied correleren met verschillende expressie van angiogene factoren. MA decidua toonde een verhoogde expressie van VEGF-A, VEGF receptor-1 en -2 (flt-1, KDR), de angiopoietinen Ang-1 and Ang-2, angiopoietine receptor TIE-2 MT2- en MT3-MMP en verlaagde expressie van MT3-MMP in decidua basalis. Verder bleek de expressie van flt-1, KDR, MT2- en MT3-MMP verhoogd in endotheelcellen in DB en DP van MA. PlGF, de meest gereguleerde factor in ongecompliceerde zwangerschappen, lijkt niet betrokken bij de pathogenese van MA.

De mogelijkheid dat de verschillen tussen beide groepen veroorzaakt worden door ontsteking en apoptose in reactie op foetale dood lijkt verworpen te kunnen worden. De missed abortion en de controle groep tonen namelijk vergelijkbare apoptose-, proliferatie- en ontstekingsparameters. Alleen het aantal uNK cellen in DSE was significant verhoogd in missed abortions. Enkele publicaties over de relatie van uNK cellen met miskramen wijzen op een verhoogd aantal uNK cellen in receptief endometrium van patiënten met herhaalde miskramen. De verhoogde aanwezigheid van NK cellen in DSE zou dit kunnen weerspiegelen.

Recente inzichten suggereren dat pre-eclampsie is geassocieerd met verstoorde remodellering van maternale spiraalarteriën, gevolgd door non-invasie van trofoblasten, placenta-insufficiëntie en ischemie. Intrauteriene groeivertraging (IUGR) lijkt te worden veroorzaakt door relatieve hyperoxie, gevolgd door angiogenese en expressie van angiogene factoren in villi. Of ook deciduale angiogenese een rol speelt, wordt onderzocht in **Hoofdstuk 8**. De expressie van angiogene factoren in 1^e trimester decidua van uiteinde-lijke PE en IUGR patiënten is vergeleken met de expressie in decidua van een gematchte controlegroep. De expressie van VEGF-A, PlGF, KDR, Ang-1, Ang-2 en TIE-2 was verhoogd in IUGR, en de expressie van PlGF, Ang-1 en TIE-2 was verhoogd in PE. Vanwege de grote inter-individuele verschillen in deze pilot-studie zijn de bevindingen niet significant. Dit bemoeilijkt de interpretatie, echter de 10-100-maal inductie doet significante verschillen verwachten bij grotere groepen. De 1^e trimester verschuivingen in de expressie van angiogene factoren lijken te functioneren als een compensatiemechanisme. Dit verstoorde expressiepatroon kan tegelijkertijd non-branching angiogenese veroorzaken, met verstoorde deciduale vascularisatie en PE en IUGR in een later trimester tot gevolg.

In **Hoofdstuk 9** worden de resultaten van deze thesis bediscussieerd, en worden aanbevelingen gedaan voor toekomstig onderzoek. **Hoofdstuk 10** geeft beknopt de conclusies van deze thesis weer.

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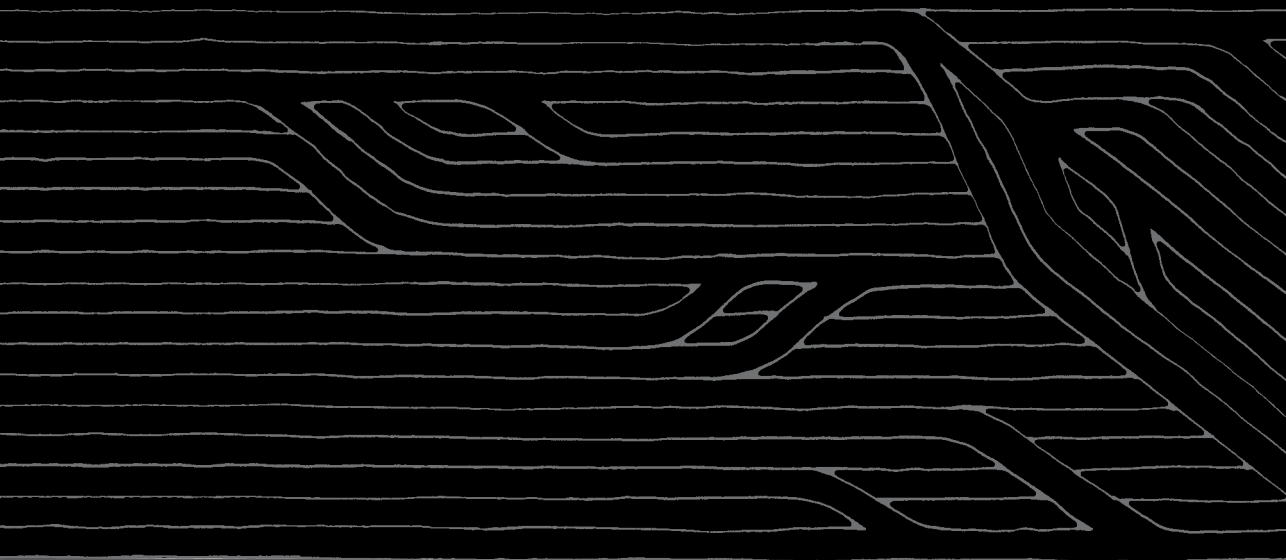
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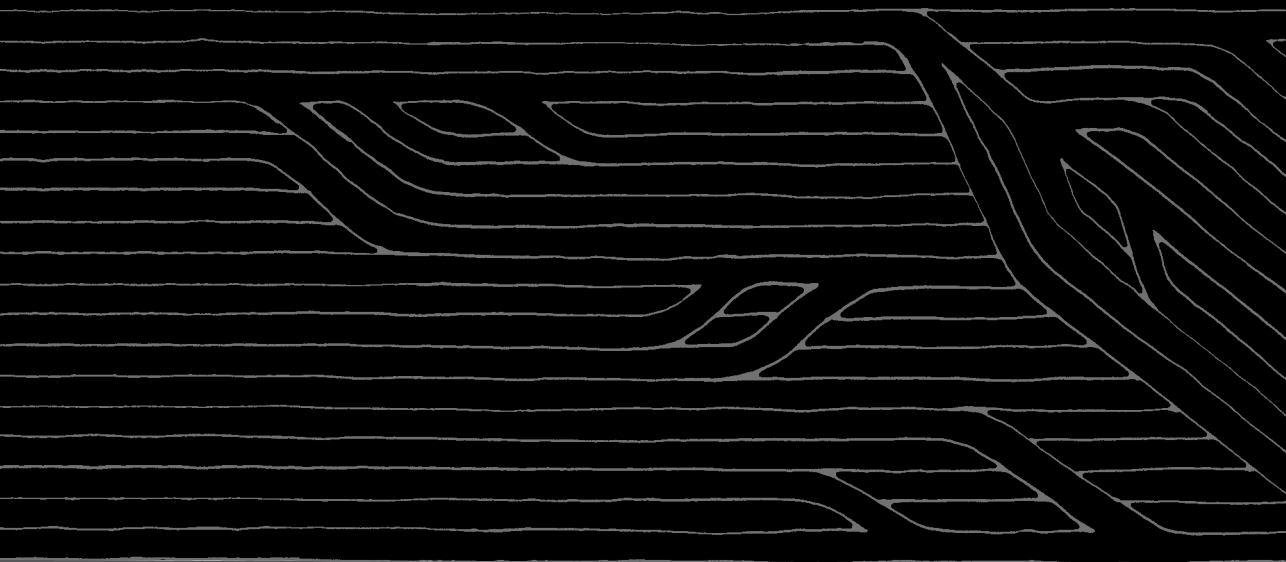
Abbreviations



Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
ANOVA	Analysis of Variance
APLA	Abortus Provocatus Lege Artis
BP	Blood pressure
cDNA	complementary DNA
CSF-1	Colony stimulating factor-1
Ct	Cycle threshold
CVS	Chorion villous sampling
dCt	Difference in Ct values
Δ dCt	Difference in Δ dCt values
DB	Decidua Basalis
DP	Decidua Parietalis
DSE	Decidual Secretory Endometrium
EC	Endothelial cell
ECGF	Endothelial cell growth factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent Assay
ER	Estrogen receptor
EP	Early proliferative
ES	Early secretory
EVT	Extra-villous trophoblast
FAM	6-carboxyfluorescein
FGR	Foetal growth restriction
Flt-1	Fms-related Tyrosine Kinase-1 or VEGF-Receptor 1
GA	Gestational age
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glycosyl-phosphatidylinositol
hCG	Human chorionic gonadotropin
hEEC	Human endometrial epithelial cells
HELLP	Haemolysis, elevated liver enzymes, low platelets
hEMVEC	Human endometrial micro-vascular endothelial cells
hESC	Human endometrial stromal cells
hFMVEC	Human foreskin microvascular endothelial cells
HPS	Haematoxylin phloxin safrane
HRT	Hormonal replacement therapy
HS	Human serum
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin
IL	Interleukin
IVF	In vitro fertilisation
KDR	Kinase insert Domain Receptor or VEGF-Receptor 2
LIF	Leukaemia inhibitory factor
LP	Late proliferative
LS	Late secretory
M	Menstruation
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

MSI	Mean staining index
MT-MMP	Membrane-type matrix metalloproteinase
NBSC	Newborn calf serum
PDGF	Platelet-derived growth factor
PE	Preeclampsia
PI3K	Phosphatidylinositol-3-kinase
PIGF	Placental Growth Factor
PSMC	Perivascular smooth muscle cell
SC	Stromal cell
SD	Standard deviation
SEM	Standard error of mean
S-Flt-1	Soluble Flt-1
SI	Staining Index
RT-PCR	Real time polymerase chain reaction
TAMRA	tetramethyl-6-carboxyrhodamin
TGF	Transforming growth factor
TIE-2	Tyrosine protein kinase receptor for Ang-1 and Ang-2
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF	Tumour Necrosis Factor
tPA	Tissue-type plasminogen activator
TSI	Total staining index
VEGF	Vascular endothelial growth factor
vWF	Van Willebrand Factor
uNK	Uterine Natural Killer cells
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor

Authors and Affiliations



From the department of Obstetrics and Gynaecology, Leiden University Medical Centre, Leiden:

F.M. Helmerhorst, K.Kapiteijn

From the division Biomedical Research, TNO Quality of Life, Leiden:

P.H.A. Quax, A. Mulder-Stapel, R. Hanemaaijer, J.M. Grimbergen, S. Rodrigues, E. Rost, I. Dennert, C. Fijten

From the department of Physiology, Institute for Cardiovascular Research, VU University Medical Centre, Amsterdam:

V.W.M. van Hinsbergh, P. Koolwijk, G.P. van Nieuw Amerongen, C.Jungerius

From the department of Obstetrics and Gynaecology, University Medical Centre Groningen, Groningen:

J.J. Erwich, E. Streefland

From the STIMEZO Plus Clinic, The Hague:

F. Willems

From the department of Obstetrics and Gynaecology, St Fransiscus Gasthuis Rotterdam:

R.M.F. van der Weijden

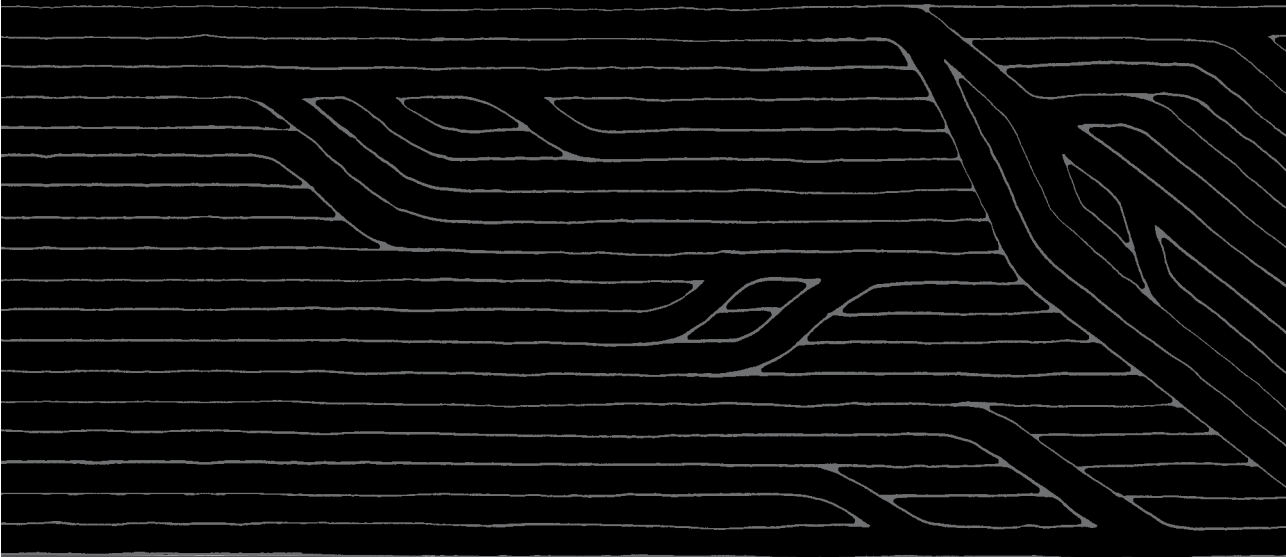
From the department of Obstetrics and Gynaecology, Bronovo Hospital, The Hague:

R.A. Verwey

From the department of Pathology, VU University Medical Centre, Amsterdam:

E.K.J. Risse

Curriculum Vitae

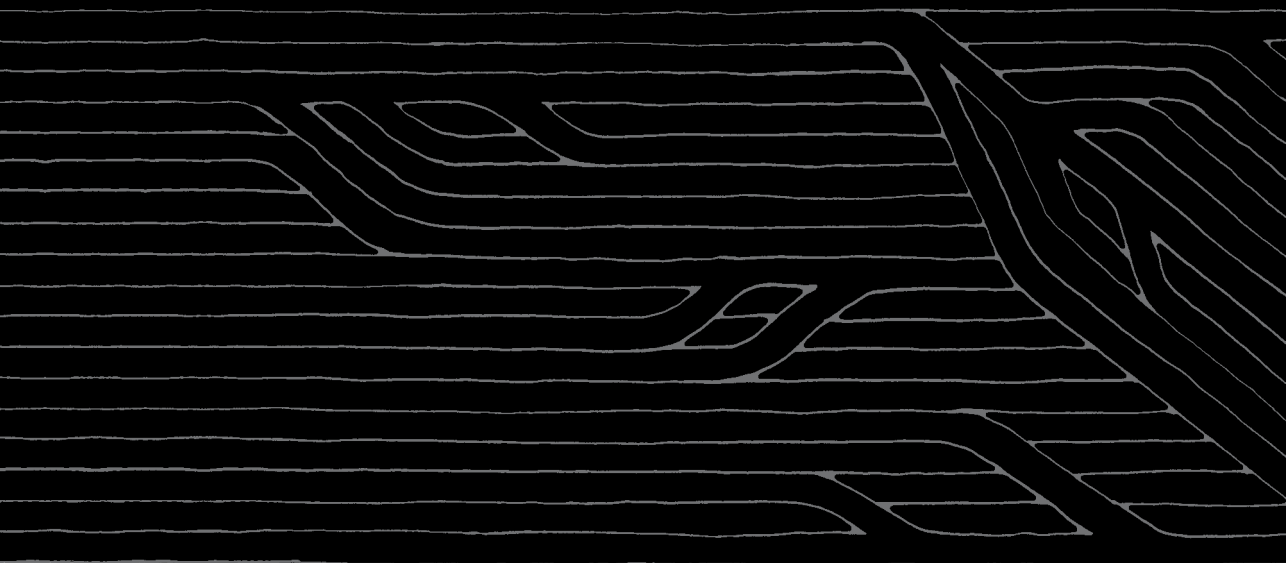


De schrijfster van dit proefschrift werd geboren op 15 december 1975 te Dordrecht. Haar jeugd bracht zij door in Puttershoek, Zuid-Holland. Na het behalen van het eindexamen atheneum aan de Willem van Oranje Scholengemeenschap, te Oud-Beijerland, startte zij in 1994 met de studie Biomedische Wetenschappen aan de Universiteit Leiden. In 1996 werd begonnen met de studie geneeskunde aan dezelfde universiteit. Tijdens de studie geneeskunde werkte zij als student-assistent bij de vakgroep Fysiologie. In 1998 verbleef zij enkele maanden in Bolgatanga, Ghana, en werkte daar aan een onderzoek naar *Oesophagostomum Bifurcum*. Tevens was zij betrokken bij het onderzoek naar de rol van het immuunsysteem bij herhaalde miskramen, onder leiding van Prof. Dr. F.M. Helmerhorst en Prof. Dr. T. Huizinga in het Leids Universitair Medisch Centrum (LUMC). Het doctoraal examen van beide studies behaalde zij in 2000, gevolgd door het artsexamen in 2002.

Na het behalen van haar artsexamen werd in 2002 aangevangen met wetenschappelijk onderzoek in het kader van een AGIKO-aanstelling op de afdeling Gynaecologie (LUMC) en de afdeling Biomedical Research van TNO Kwaliteit van Leven te Leiden. Zij deed onderzoek naar de rol van angiogenese in de jonge zwangerschap, onder begeleiding van Prof. Dr. V.W.M. van Hinsbergh (Vrije Universiteit Medisch Centrum) en Prof. Dr. F.M. Helmerhorst (LUMC). De resultaten van dit onderzoek staan beschreven in dit proefschrift.

In 2005 startte zij met de opleiding tot gynaecoloog in het LUMC (opleiders Prof. Dr. H.H.H. Kanhai en Prof. Dr. G.G. Kenter) en het Bronovo Ziekenhuis (opleider Dr. C.A.G. Hollebom). Margreet woont in Den Haag samen met Roger Froklage en hun zoon Matthijs.

Acknowledgements



Dank...

Mijn voorgangers in onderzoek, voor jullie aanstekelijk enthousiasme

Frédérique en Kitty



De secretaresses, voor jullie onmisbare werk

Wil en Bea



Vriendinnen van vroeger, omdat jullie er nog steeds zijn

Corine, Simone, Miranda en Marjolein



“Mijn” studenten, voor hun vele werk

Sharon, Indra en Ellen



Mijn nieuw gevonden familie

Jaap, Tine en Irving



Ghana girls, voor jullie aandacht en vriendschap

Petra, Marieke, Nicole, Minke en Jutte



Medewerkers van de STIMEZO-plus kliniek Scheveningen, het Bronovo ziekenhuis,
het Spaarne Ziekenhuis, het UMC Groningen en het Leids Universitair Medisch Centrum
voor hun bijdrage aan het verzamelen van weefsel

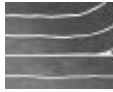


Jutte, omdat je als paranimf naast me staat



“Oud”-kamergenoten, voor de noodzakelijke lach

Bea en Claudia



Medewerkers TNO en de angiogenese club, voor alle gezelligheid en geleende expertise

*Margreet, Erna, Adri, Jos, Erik, Abbey, Marten, Nancy, Martine, Daniël,
Kees, Vincent, Karin, Roeland, Natasja, Monique,
Linda, Lars, Robert en Eric*



Lieve vriendinnen, voor de leuke momenten van afleiding

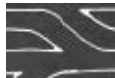
Eiskje, Cindy, Madlenka en Cecile



Mede-auteurs, voor hun waardevolle bijdrage



Medewerkers van de afdeling Gynaecologie en Obstetrie van het
Bronovo Ziekenhuis



Leendert-Jan, grote broer en paranimf, omdat je er altijd voor me bent



Mijn bovenstebeste schoonzus en neefjes

Arianne, Luc en Jesse



Ylang, Ylang, lieve cordial-genootjes, voor jullie gezelligheid en aandeel in zoveel mooie herinneringen

Marjolein, Marion, Susanna, Michele, Myrna, Nicole, Marijke, Karen en Marjolein



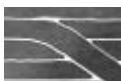
Arts-assistenten Gynaecologie en Obstetrie Cluster Leiden



Mede-AGIKO-ers, voor een luisterend oor en de kroketten
*Marjon, Wendela, Huib, Elles, Esther, Marieke, Ellen, Muriël, Sabrina, Joost,
 Peggy, Heleen, Jessica, Quirine en Henry*



Waar het allemaal begon, dank voor alles!
Pap en Mam



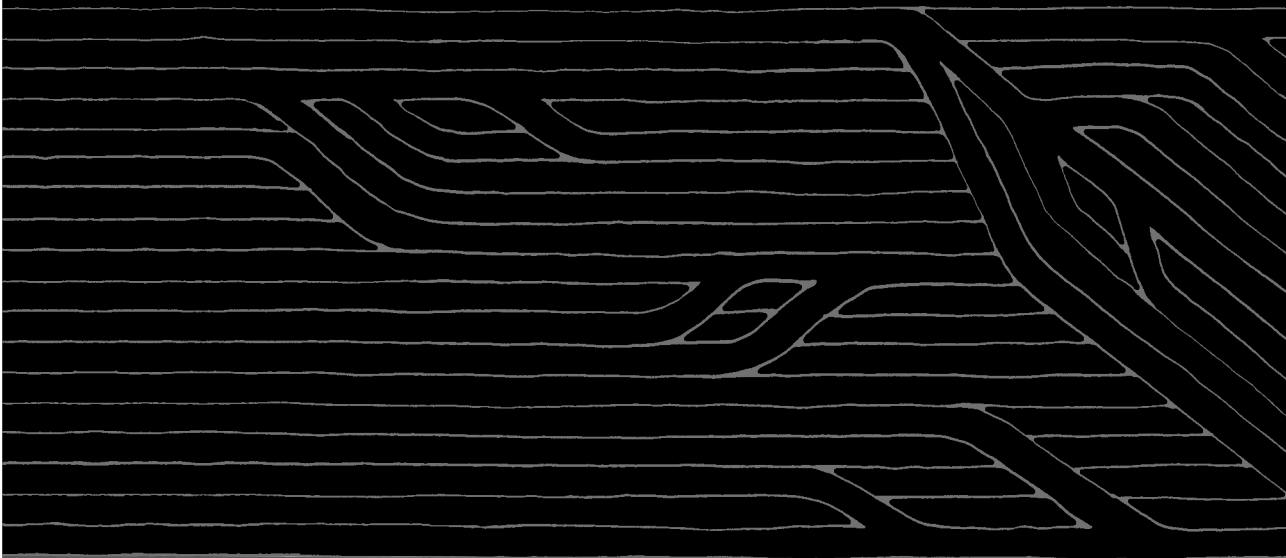
Mijn allerliefste thuishaven
Roger en Matthijs



“We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from a point of view of the direct usefulness of it. It must be done for itself, for the beauty of science. And then, there is always the chance that a scientific discovery may become like the radium.”

By Marie Curie (1867 - 1934)

Colour Figures



CHAPTER 1

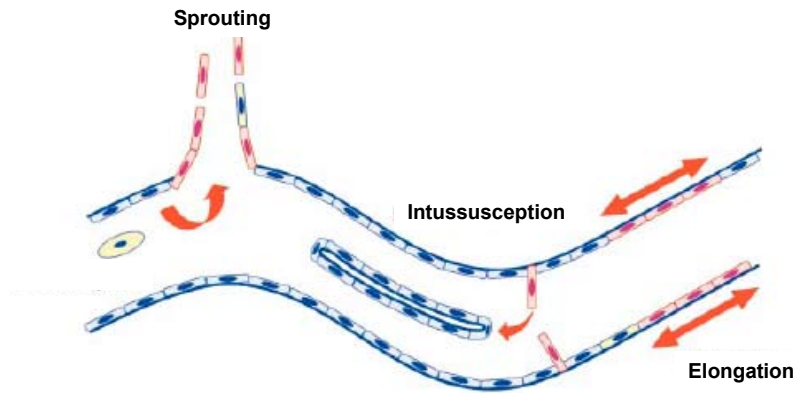


Figure 2. Endometrial angiogenesis proceeds by elongation and intussusception rather than by classical sprout formation. Adapted from Rogers 1998.

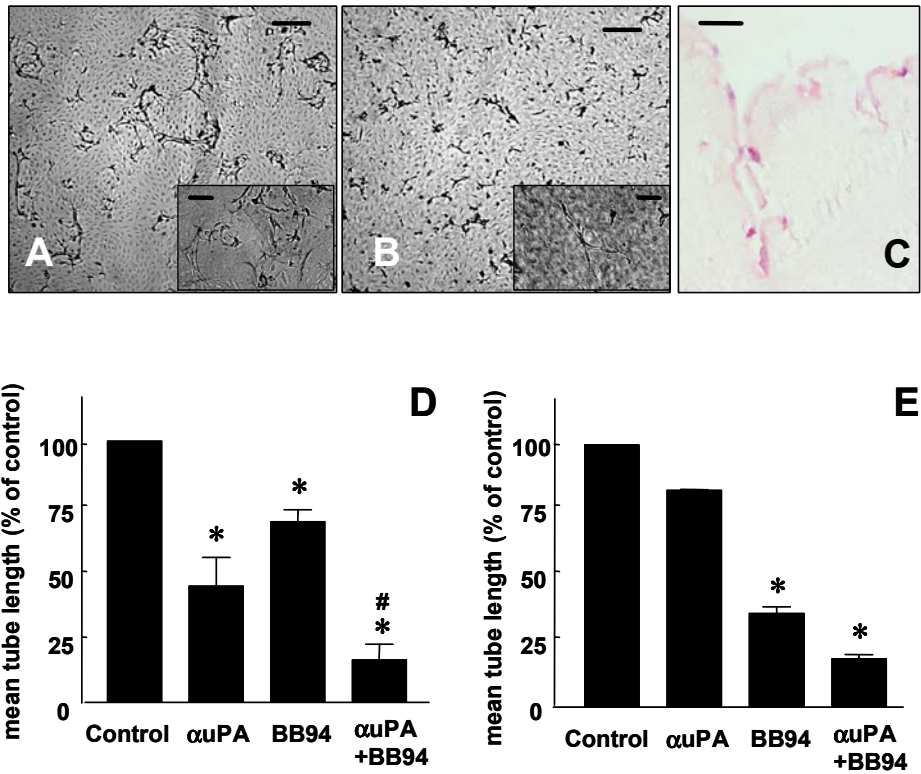


Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.

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 μ m, Bar insets = 100 μ m. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (bar = 50 μ m). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (α uPA, 100 μ g/mL), BB94 (5 μ g/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 $\pm$ 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. α uPA.

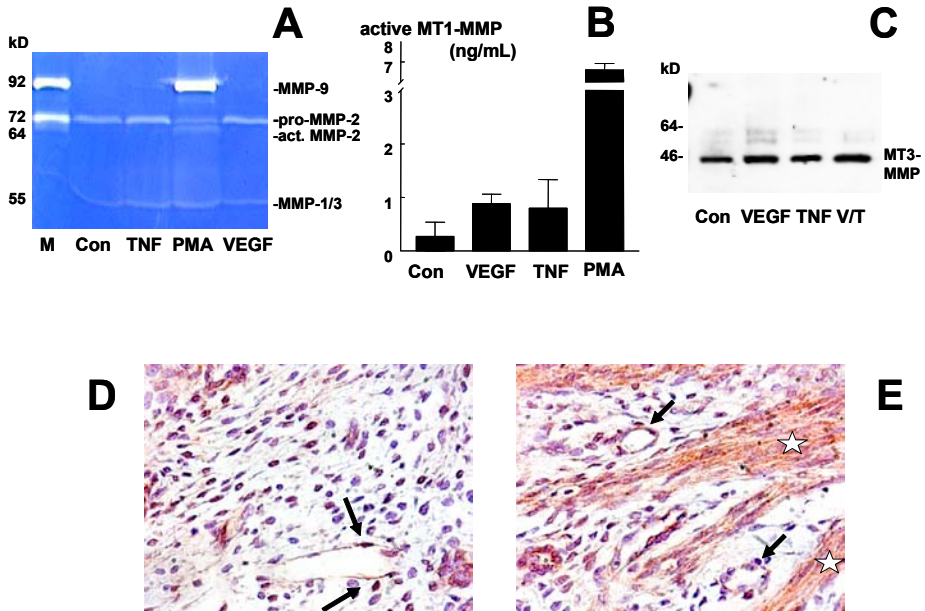


Figure 2. hEMVEC express various MMPs and MT-MMPs.

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 TNF α (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 $\pm$ 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).

CHAPTER 3

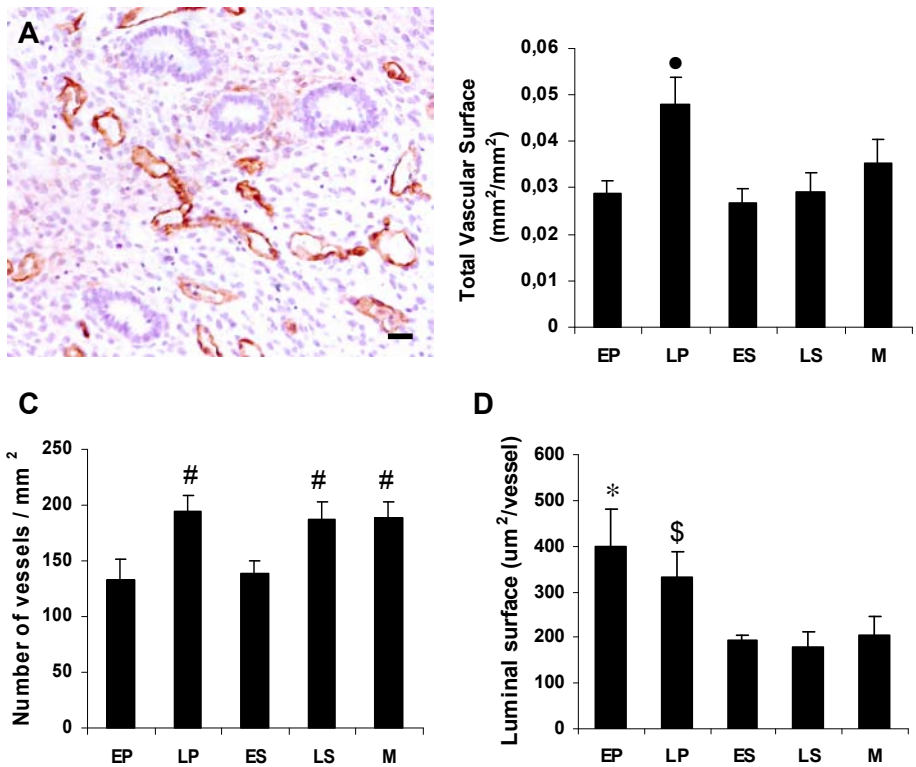


Figure 1. Vascularisation pattern in cycling human endometrium. The vascularisation pattern in human endometrium was determined by image analysis of anti-CD34-stained sections. Six fields per biopsy were scanned at 100x magnification. **A** CD34 antigen expression in endothelial cells in late proliferative endometrium (bar = 50 μm). **B** The total vascular surface (mm²/mm²), **C** the number of vessels per mm² and **D** the luminal surface (μm²/vessel) were calculated for each sample and expressed as mean ± SEM. EP = Early proliferative phase (n=6), LP = Late proliferative phase (n=7), ES = Early secretory phase (n=6), LS = Late secretory phase (n=6), M = menstrual phase (n=2); • p<0.01 versus EP, ES and LS; # p<0.02 versus EP, ES; * p<0.01 versus ES, LS and M; and \$ p<0.05 versus ES, LS and M.

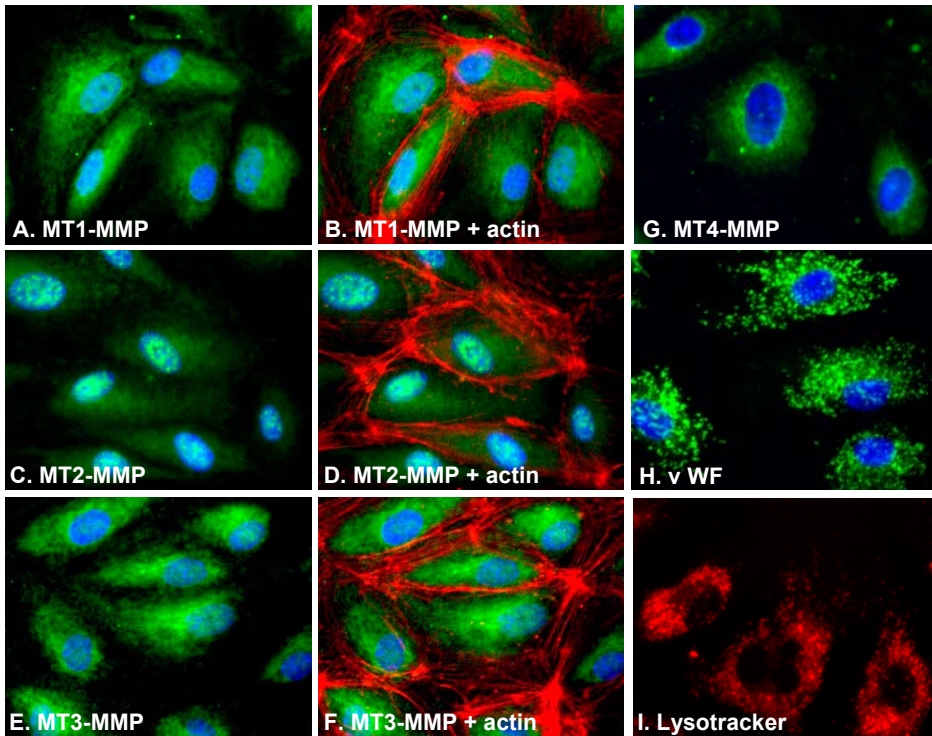


Figure 2. MT-MMP antigen expressions in hEMVEC.

Endothelial MT-MMP expression was determined by fluorescence microscopy (magnification 40x). The expression of MT1-MMP ($\pm$ actin **A, B**), MT2-MMP ($\pm$ actin **C,D**), MT3-MMP ($\pm$ actin **E,F**), MT4-MMP (**G**), von Willebrand Factor in Weibel Palade bodies (**H**) and Lysotracker in lysosomes (**I**) in cultured endometrial endothelial cells (hEMVEC). The negative control only showed blue staining of the nuclei (not shown). Blue = DAPI in nuclei, green = target protein, red = actin or lysotracker.

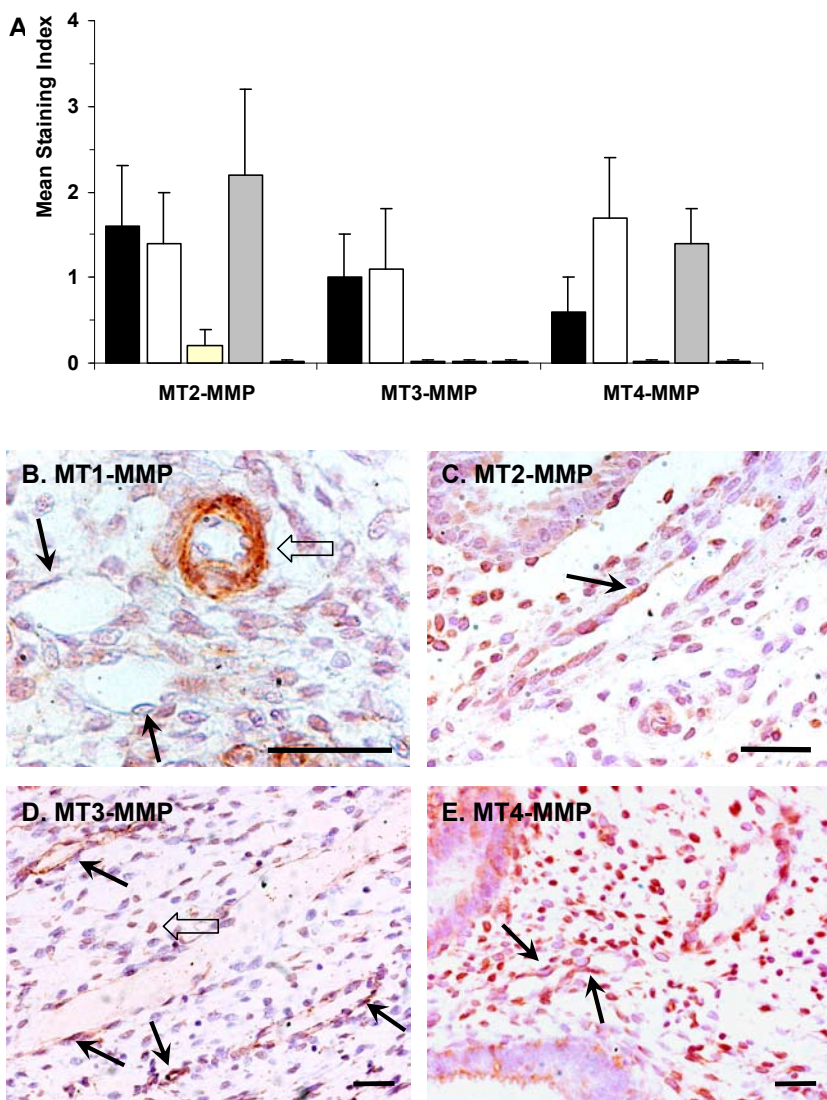


Figure 3. MT-MMP antigen expressions in endometrial endothelium and other cell types.

The expression of MT-MMP antigens by endometrial endothelial cells was determined by immunohistochemistry. **A.** MT-MMP antigen expressions in endothelial cells expressed per cycle phase as mean staining index $\pm$ SEM (all $p > 0.05$). The staining index is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). **B.** MT1-MMP antigen detected in PSMCs (open arrow) but not in endothelial cells (arrows) in late proliferative (LP) endometrium. **C.** MT2-MMP antigen expression in endothelium in LP endometrium (arrow). **D.** MT3-MMP expression in endothelial cells (arrows) and stromal cells (open arrow) in LP endometrium. **E.** MT4-MMP expression in endothelial cells (arrow) in LP endometrium. **F.** Negative control. Bar = 50 μ m.

■ = Early proliferative phase (n=6), □ = Late proliferative phase (n=7), ▤ = Early secretory phase (n=6), ▥ = Late secretory phase (n=6), ▨ = menstrual phase (n=6).

CHAPTER 5

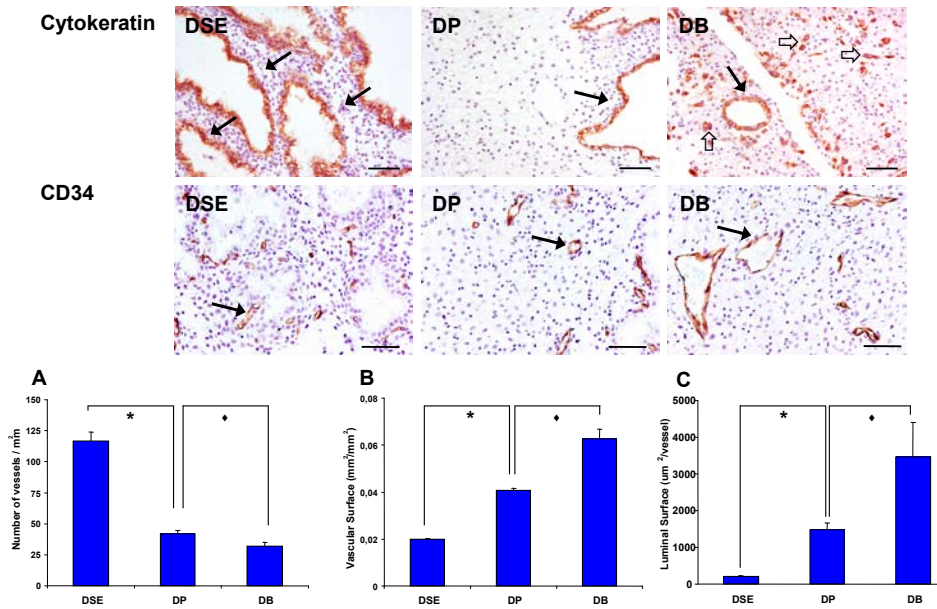


Figure 2. Cytokeratin expression and vascularisation in the three decidual tissues.

Differentiation between three decidual tissues was obtained by HPS and anti-cytokeratin staining (**top panel**). DSE and DP expressed cytokeratin only in glandular epithelial cells (closed arrows), whereas cytokeratin is also expressed in extra-villous trophoblasts of DB (open arrows). The vascularisation pattern in human decidual tissues was determined by image analysis of anti-CD34-stained sections (**middle and bottom panel**). CD34 antigen expression in endothelial cells (arrows) of DSE, DP and DB was compared within subjects. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were calculated and expressed as mean ± SEM. * p < 0.0001; ♦ p < 0.04. Bar = 100 μm.

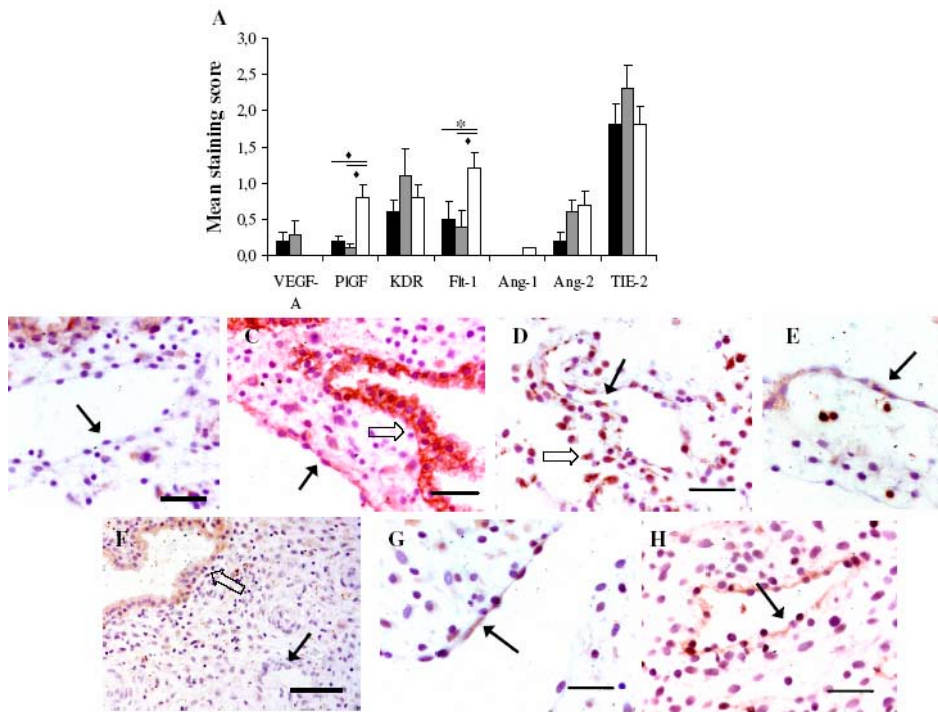


Figure 3. Protein expression of angiogenic factors in endothelium of early first-trimester decidua. The protein expression of angiogenic factors was determined in DSE, DP and DB. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis. **A.** Protein expression of each angiogenic factor in endothelium was expressed as the mean staining index $\pm$ SEM and **B-H** show examples of these expressions. **B.** Endothelial VEGF expression was not detectable in DB. **C.** PlGF was expressed in epithelium (open arrow) and endothelium (closed arrow) of DB. **D.** Endothelial KDR expression (closed arrow) and stromal KDR expression (open arrow) in DB. **E.** Endothelial Flt-1 expression in DB (arrow). **F.** Angiopoietin-1 in DB was detected in epithelium (open arrow), but not in stromal and endothelial cells (closed arrow). **G.** Endothelial angiopoietin-2 expression in DB (arrow). **H.** Endothelial Tie-2 expression in DB (arrow). ■ DSE, ■ DP, □ DB. Bar = 100 μ m (except F = 50 μ m).

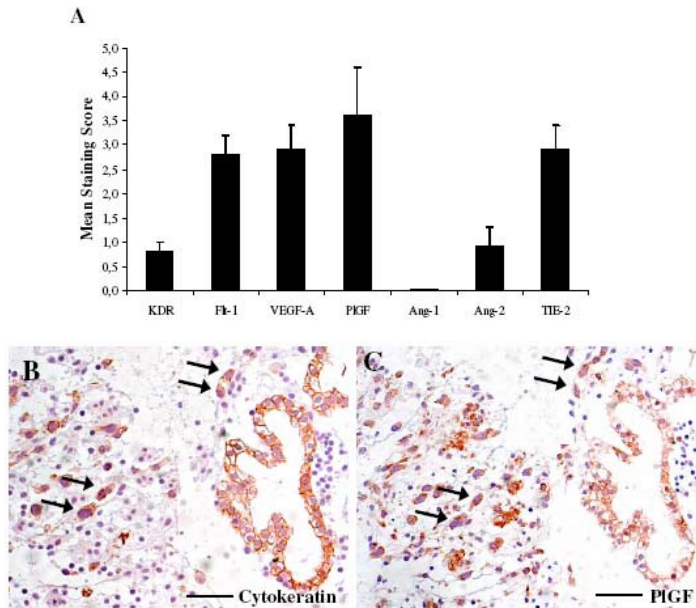


Figure 4. Protein expression of angiogenic factors in extra-villous trophoblasts (EVT) of early first-trimester decidua.

The protein expression of angiogenic factors in EVT in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Protein expression in EVT was expressed as the mean staining index $\pm$ SEM. **B.** Cytokeratin (arrows) and **C.** serial PlGF expression (arrows) in EVT. Bar = 50 μ m.

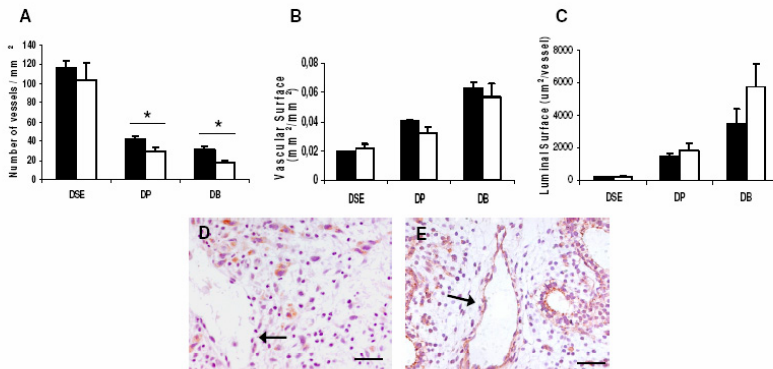


Figure 5. Vascularisation pattern and endothelial protein expression in early versus late first-trimester decidua.

The vascularisation pattern in human early (black bars) and late (white bars) first-trimester decidual tissues was determined by image analysis of anti-CD34-stained sections. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were expressed as mean $\pm$ SEM. VEGF expression was not detectable in early first-trimester endothelium of DB (arrow, **D**), whereas the expression was present in late first-trimester endothelium of DB (arrow, **E**). Early first-trimester and late first-trimester. Bar = 50 μ m, * $p < 0.04$.

CHAPTER 6

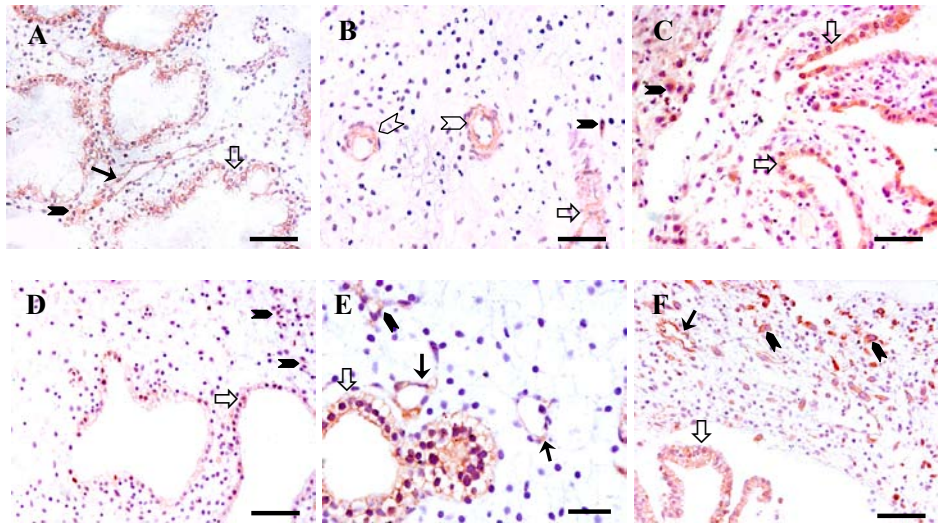


Figure 1. Protein levels of proteases in early first-trimester decidua. The level of proteases was determined in DSE, DP and DB. **A.** MT1-MMP antigen was detectable in epithelial (open arrow), endothelium (closed arrow) and stromal cells (closed arrowhead) of DSE. **B.** MT2-MMP antigen in epithelium (open arrow), stromal cells (closed arrowhead) and pericytes (open arrowhead) of DB. **C.** Epithelial (open arrow) and stromal (closed arrowhead) MT3-MMP antigen in DB. Pericytes not present in this field. **D.** MT5-MMP antigen in epithelium (open arrow), and only dimly in stromal cells (closed arrowheads) of DP. **E.** uPA antigen in epithelium (open arrow), stromal cells (closed arrowhead) and endothelium (closed arrows) in DB. Pericytes not present in this field. **F.** Epithelial (open arrow), endothelial (closed arrows) and stromal cells (closed arrowheads) uPAR protein in DB. **A-D,F** Bar = 100 μ m, **E.** Bar = 50 μ m.

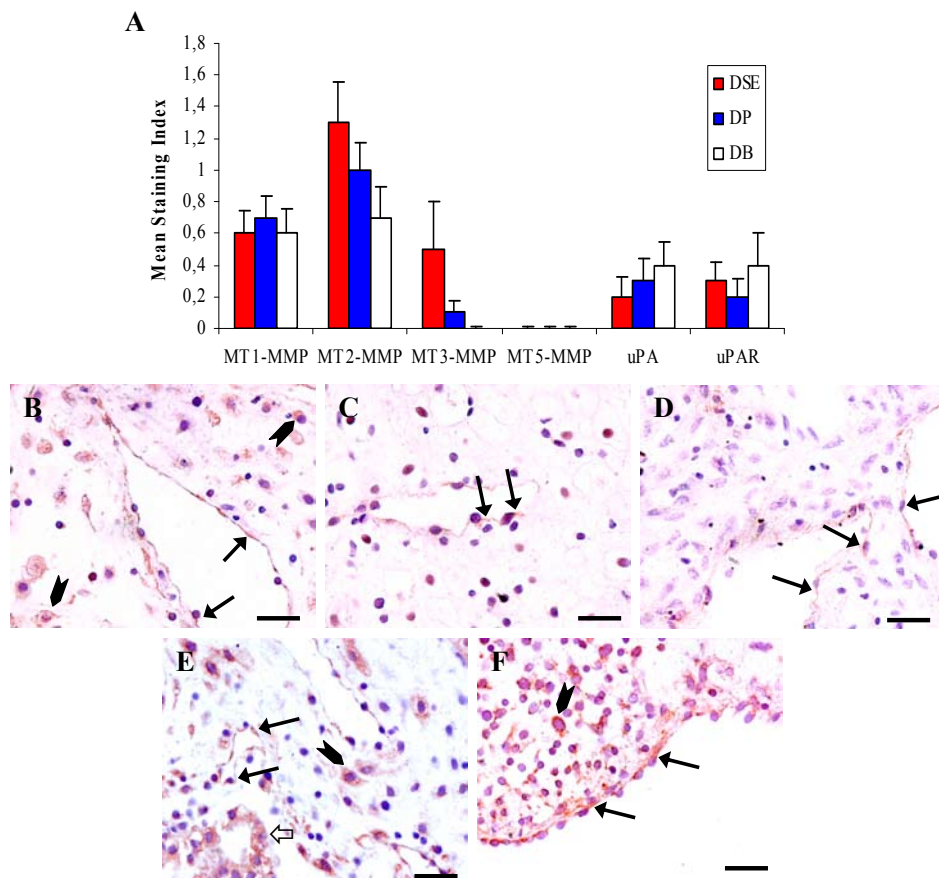


Figure 2. Protein levels of proteases in endothelium of early first-trimester decidua.

A. Presence of protease antigens in endothelium (EC) was expressed as the mean staining index $\pm$ SEM and **B-F** show examples of endothelial protease expression. **B.** Endothelial (arrows) and stromal (arrowheads) MT1-MMP was detectable in DB. **C.** MT2-MMP was detected in endothelium (arrows) of DB. **D.** Endothelial MT3-MMP antigen (arrows) in DSE. **E.** uPA antigen in endothelium (closed arrows), epithelium (open arrow) and stromal cells (arrowhead) in DB. **F.** uPAR in DB was detected in endothelial cells (arrows) and stromal cells (arrowheads). **B-F.** Bar = 50 μ m.

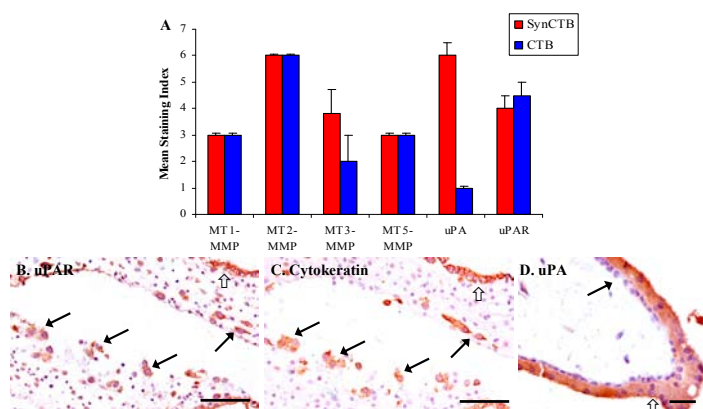


Figure 3. Protein levels of proteases in villous and extra-villous trophoblasts (EVT) of early first-trimester decidua.

The presence of protease antigens in villous and extra-villous trophoblasts in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Immunostaining in syncytio- and cytotrophoblasts (synCTB and CTB) was expressed as the mean staining index $\pm$ SEM. **B.** and **C.** uPAR and cytokeratin antigens in serial expression in EVT (closed arrows) and epithelium (open arrows). **D.** uPA antigens in syncytiotrophoblast (open arrow) and not in cytotrophoblast (closed arrow). **B-D.** Bar = 50 μ m.

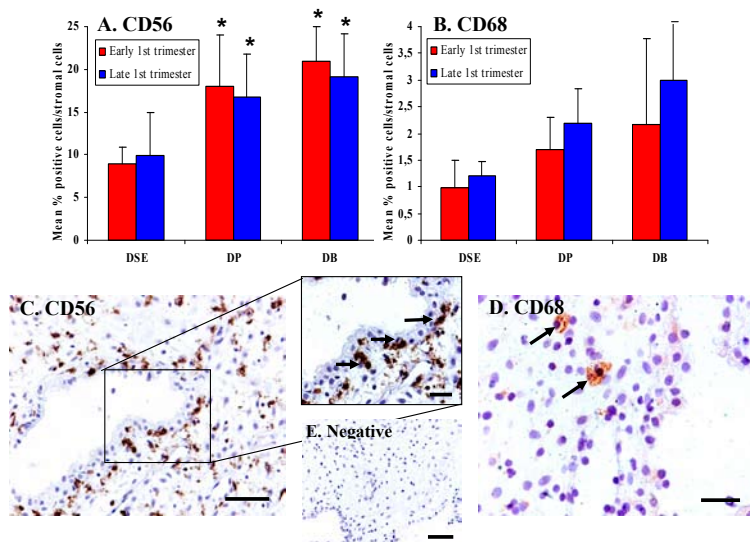


Figure 4. CD56- and CD68- positive cells in early and late first-trimester decidua.

A. CD56-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean $\pm$ SD). **B.** CD68-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean $\pm$ SD). **C.** CD56 expression in decidua basalis, bar = 100 μ m. Blow up shows CD56+ cells (arrows) surrounding a vessel and gland, bar = 50 μ m. **D.** CD68-positive cell (arrow) in decidua parietalis, bar = 50 μ m. **E.** Negative control, bar = 100 μ m. * $p < 0.05$ versus DSE

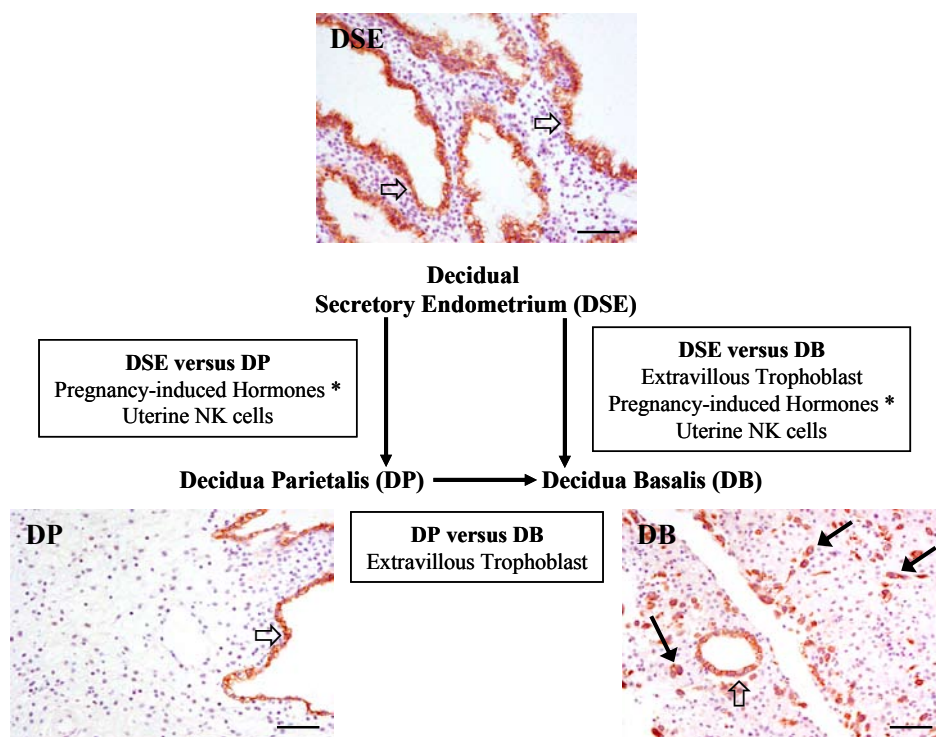


Figure 5. Human first-trimester decidual tissues.

Representation of the influences of immune cells, extra-villous trophoblast and pregnancy-induced hormones (*hCG, oestradiol and progesterone) on first-trimester decidual tissues. Differentiation between decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB), was obtained by HPS and cyokeratin staining. DSE and DP express cyokeratin in glandular epithelium (open arrows), whereas cyokeratin is also expressed in EVT (closed arrows) of DB. Bar =100 μm.

CHAPTER 7

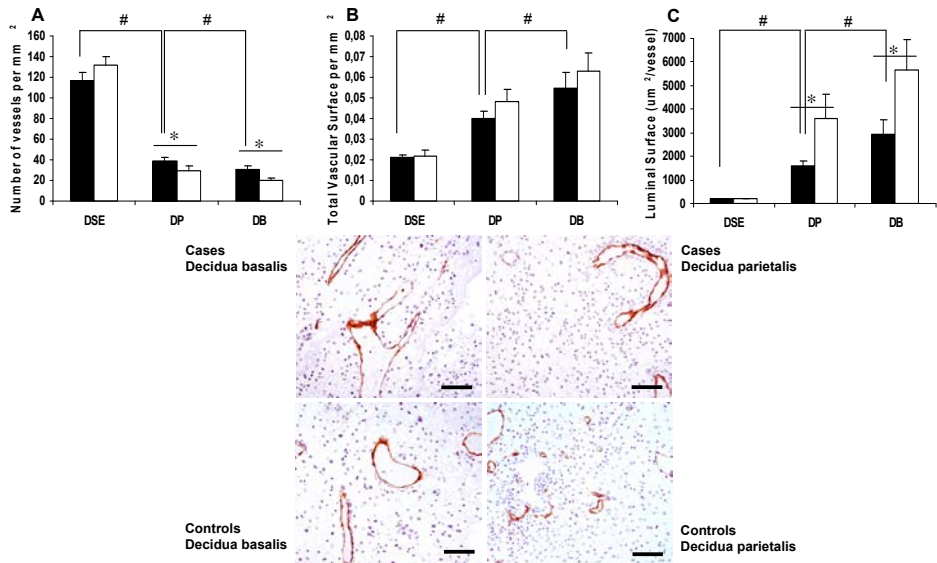


Figure 1. Vascularisation pattern in cases and controls.

The vascularisation pattern in decidual tissues of controls (n=16, black bars) and cases (n=11, white bars) was determined by image analysis of anti-CD34-stained sections. **A.** Vessel density (number of vessels per mm²), **B.** the total vascular surface (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were calculated and expressed as mean±SEM. * p<0.05 within controls, * p<0.05 in cases versus controls. The bottom panels show examples of vascularisation in decidua parietalis and basalis of cases and controls.

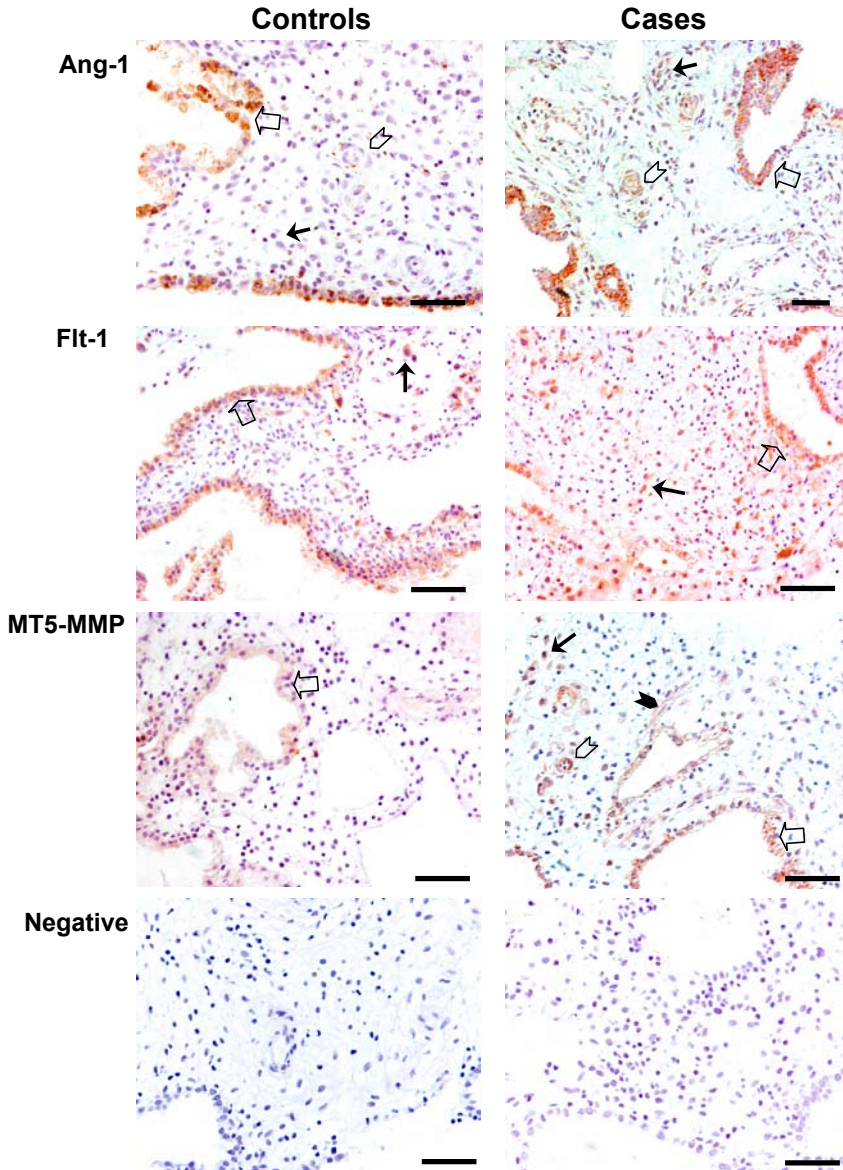


Figure 2. Antigen presence of angiogenic factors and proteases in cases and controls. Examples of the antigen expression of Ang-1, Flt-1 and MT5-MMP in controls (left site of panel) and cases (right site of panel). **Ang-1** antigen presence in epithelial cells (open arrow) in DB of controls, compared to their presence in stromal (closed arrow), epithelial cells (open arrow) and PSMC (open arrowhead) in DB of cases. **Flt-1** antigen presence in epithelial cells (open arrow) and stromal cells (closed arrow) in DB of controls, compared to their elevated presence in DB of cases. No vasculature present in these fields. **MT5-MMP** antigens in epithelial cells (open arrow) in DB of controls, compared to their presence in PSMC (open arrowhead), endothelial (closed arrowhead), stromal (closed arrow) in DB of cases. **Negative** controls using non-immune mouse IgG₁ (left) and omission of the first antibody (right). All panels bar = 100 μ m.

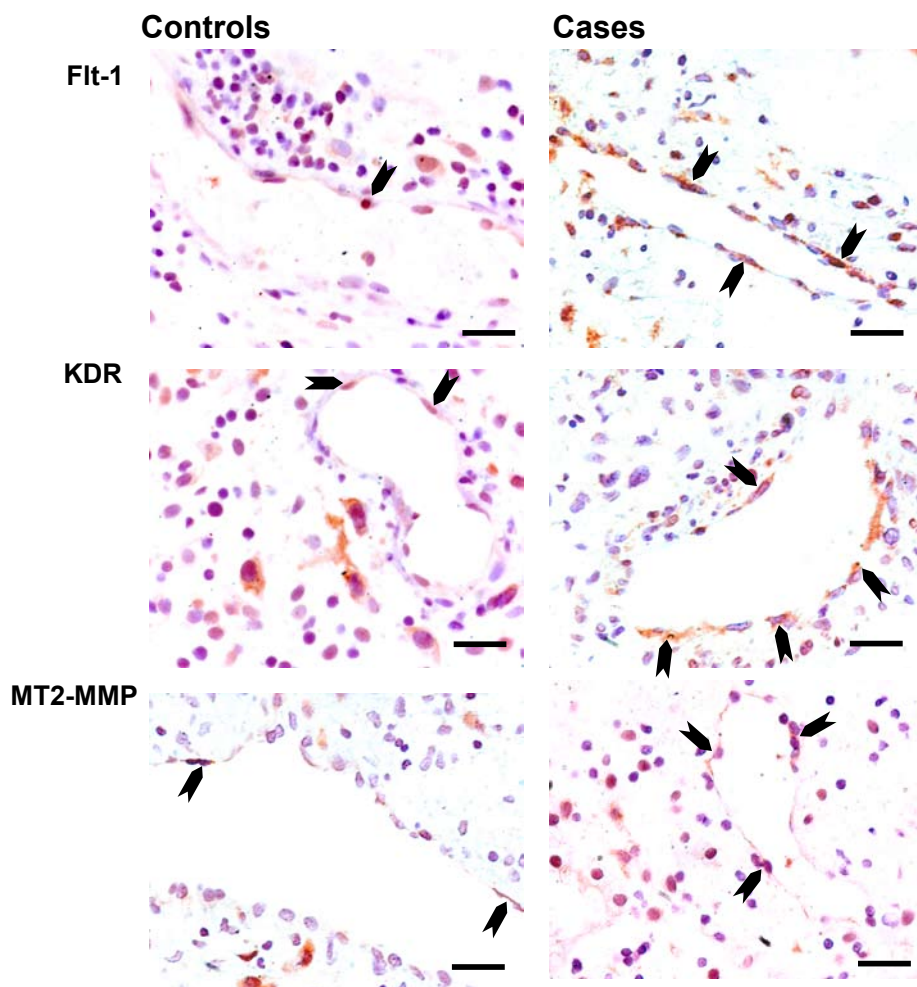


Figure 3. Antigen presence of angiogenic factors and proteases in endothelium in cases and controls. The protein expression of angiogenic factors and proteases was determined in DSE, DP and DB of cases and controls via immunohistochemistry. Examples of the differential endothelial expression (arrowheads) of flt-1, KDR and MT2-MMP between cases (right site of panel) and controls (left site of panel) are given. **Flt-1.** Elevated flt-1 expression in endothelial cells in DB of cases compared to controls. **KDR.** Elevated KDR expression in endothelial cells in DB of cases compared to controls. **MT2-MMP.** Elevated MT2-MMP expression in endothelial cells in DB of cases compared to controls. All panels bar = 50 μ m.