Angionesis and the inception of pregnancy

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8

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

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Introduction

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

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

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

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

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

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

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

Materials and methods

Materials

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

Human embryo conditioned medium

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

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

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

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

Cell culture

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

Incorporation of ^{3}H-thymidine

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

In vitro angiogenesis model

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

Enzyme-Linked Immunosorbent Assays (ELISA)

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

Statistics

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

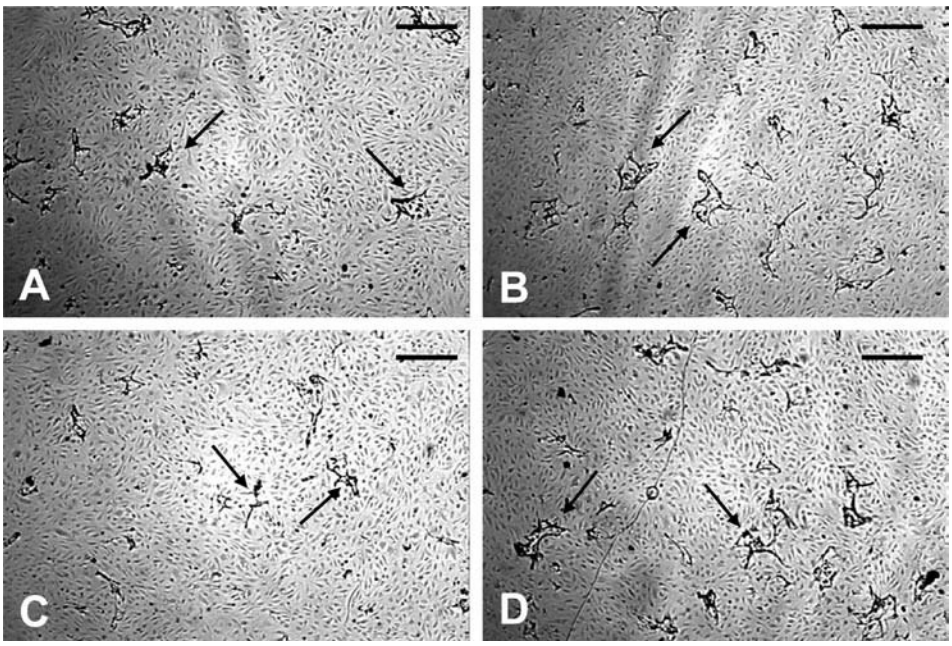

Figure 1. Conditioned medium of early stage embryos stimulate *in vitro* hEMVEC tube formation.
Phase-contrast pictures were taken of hEMVEC cultured on top of a three-dimensional fibrin matrix under control conditions (A), after stimulation with VEGF-A (10 ng/mL, B), 5% (v/v) control IVF medium (C),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

Results

Early stage embryo conditioned medium enhances hEMVEC proliferation and tube formation

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

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

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

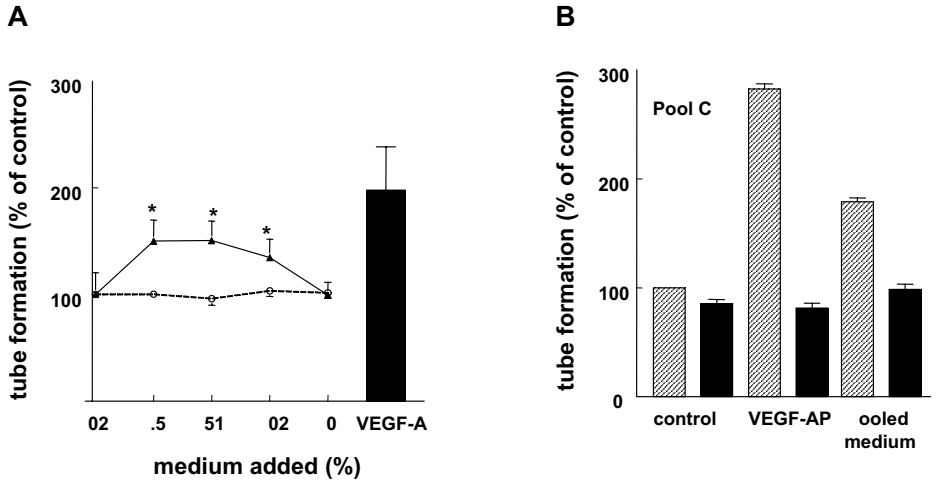

Figure 2. Conditioned medium of embryos stimulate *in vitro* hEMVEC tube formation, an effect which is VEGF-A-mediated.

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

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

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

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

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

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

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

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

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

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

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

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

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

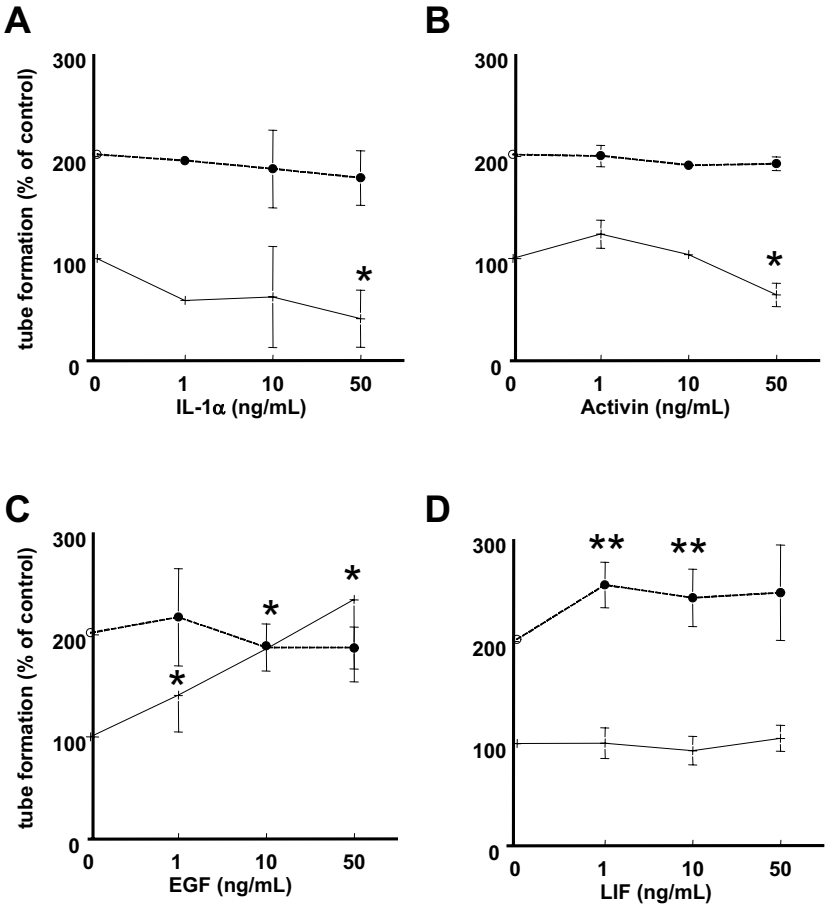

Figure 3. The influence of recombinant cytokines and hCG on the formation of capillary-like structures by hEMVEC.

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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