

# Differential expression of angiopoietins 1 and 2 and their receptor Tie-2 in human endometrium

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**Angiogenesis, the growth of new capillaries from pre-existing blood vessels, is a physiological process involved in both normal menstrual cycling and implantation of the embryo. So far, very little is known about the expression of angiopoietins, growth factors involved in angiogenesis, in human endometrium. Both angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands for the endothelial cell-specific receptor tyrosine kinase Tie-2. In this study we determined the mRNA expression of Ang-1, Ang-2 and Tie-2 by quantitative competitive RT/(QC)-PCR (including specifically designed competitor cDNA) in biopsied human endometrium throughout the menstrual cycle. We detected the mRNA for the angiopoietins in 30 out of 32 endometrial biopsies (94%), covering early proliferative ( $n = 4$ ), mid proliferative ( $n = 12$ ), late proliferative ( $n = 3$ ), early secretory ( $n = 3$ ), mid secretory ( $n = 5$ ) and late secretory ( $n = 3$ ) phases. Analysis of the target/competitor ratios (QC-PCR) revealed that Ang-1 mRNA expression was significantly up-regulated ( $P = 0.027$ ) during the secretory phase of the menstrual cycle. In contrast, the expression levels of both Ang-2 mRNA and Tie-2 mRNA showed only minor variations at different cycle stages. These findings were confirmed by the relative expression ratio of Ang-1 versus Ang-2 in a multiplex PCR. The expression of Ang-1, Ang-2 and Tie-2 mRNA was detected in both isolated endometrial epithelial and stromal cell fractions. Immunohistochemical localization of the proteins revealed qualitative differences in both cell type and cycle stage expression. In conclusion, the enhanced Ang-1 expression during the secretory phase might serve to stabilize the newly developed blood vessels.**

*Key words:* angiogenesis/angiopoietin/endometrium/gene expression

## Introduction

Angiogenesis is the formation of new blood vessels from pre-existing ones, a physiological process that is normally seen in fetal development, wound healing and in the female reproductive tract (Findlay, 1986; Folkman and Shing, 1992). The critical role of angiogenesis in ovarian and endometrial function was demonstrated when the angiogenesis inhibitor, AGM-1470, substantially retarded growth of both the corpus luteum and the endometrium when administered chronically to non-pregnant, cycling mice (Klauber *et al.*, 1997). In human endometrium, angiogenesis occurs periodically as part of the cyclical growth and shedding which take place during the menstrual cycle. During menstruation, repair of the ruptured blood vessels must occur; in the proliferative phase the rapid growth of endometrial tissue must be accompanied by angiogenesis; and finally in the secretory phase there is development and coiling of the spiral arterioles and growth of the subepithelial capillary plexus (Rogers and Gargett, 1998). While circulating estrogen and progesterone primarily regulate the overall control of endometrial growth and regression, it appears unlikely that steroids are direct regulators of vascular growth in this tissue.

Several groups, including ourselves, have previously shown that vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, and its receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR) and soluble Flt (for review see Neufeld *et al.*, 1999), are expressed in

human endometrium in a cycle-dependent fashion throughout the menstrual cycle (Shifren *et al.*, 1996; Torry *et al.*, 1996; Krüssel *et al.*, 1999; Möller *et al.*, 2001).

So far, very little is known about the expression of angiopoietins, another group of angiogenic growth factors, in human endometrium. Both angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands for the endothelial cell-specific receptor tyrosine kinase Tie-2. The prominent roles of angiogenic growth factors and their receptors have become evident from gene knock-out experiments in mice. Inactivation of Tie-2 and Ang-1 leads to disrupted vessel structure and embryonic lethality (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996). This occurs at a later developmental stage and with a phenotype distinct from those associated with inactivation of the vascular endothelial growth factor receptor tyrosine kinases (VEGFR-1 and VEGFR-2) which are also involved in vasculogenesis (Fong *et al.*, 1995; Shalaby *et al.*, 1995). Ang-1 is important in maintaining vessel integrity. The Ang-2 negative signal, acting through inhibition of Tie-2 signalling, leads to a loosening of cell–matrix and cell–cell contacts, allowing access to angiogenic inducers, e.g. VEGF. Thus, co-expression of Ang-2 and VEGF leads to angiogenesis. In the absence of angiogenic growth or survival signals, Ang-2 action results in a regression of vessel structures (reviewed in Hanahan, 1997; Yancopoulos *et al.*, 2000). In mice and humans, Ang-2 is selectively expressed in ovary, uterus and placenta (Maisonpierre *et al.*, 1997),

**Table I.** Primers used for RT-PCR

mRNA	Accession no. of cDNA	Size of amplified fragment (bp)	Type of primer	Position on cDNA	Sequence of primer
Ang-1 target	U83508	444	5'	1296–1315	5' → TCGTGAAGATGGAAGTCTAG → 3'
			3'	1720–1739	5' → TGCCACTTTATCCCATTTCAG → 3'
Ang-1 competitor		220	5' float	1296–1315 + 1540–1559	5' → TCGTGAAGATGGAAGTCTAGACA GCAGGAAAACAGAGCAG → 3'
Ang-2 target	NM_001147	754	5'	1048–1066	5' → AATAGTGAAGTCCACGGTG → 3'
			3'	1783–1801	5' → GAGCGAATAGCCTGAGCCT → 3'
Ang-2 competitor		344	5' float	1048–1066 + 1477–1495	5' → AATAGTGAAGTCCACGGTGAGAG ACTGGGAAGGGAATGAG → 3'
Tie-2 (TeK) target	NM_00459	395	5'	2413–2432	5' → TGGAATGACCTGCCTGACTG → 3'
			3'	2786–2806	5' → GATGATGTTTGGATGGTGTCC → 3'
Tie-2 (TeK) competitor		320	5' float	2413–2432 + 2507–2526	5' → TGGAATGACCTGCCTGACTGAACG TGAGGGAAGAACCAGC → 3'

the three tissues subject to physiological angiogenesis, whereas Ang-1 is widely expressed both in the embryo and in the adult (Davis *et al.*, 1996).

The aim of our study was to detect the relative mRNA expression levels of Ang-1, Ang-2 and the receptor Tie-2 in human endometrium throughout the menstrual cycle. Additionally, we separated a number of endometrial biopsies into stromal and epithelial compartments to analyse the cellular mRNA distribution. Furthermore we analysed by immunohistochemistry the protein distribution within the endometrium.

## Materials and methods

### Endometrial biopsies

Patients who underwent hysteroscopy for non-malignant reasons, mainly for unexplained infertility, were asked to participate in this study. All patients included had regular menstrual cycles and normal hormonal profiles; patients with polycystic ovaries were excluded. Each participating patient signed an informed consent approved by the local ethical board. Biopsies were taken by curettage before the hysteroscopy. By using NaCl distention medium instead of CO<sub>2</sub> for the hysteroscopy, we were able to flush the uterine cavity, such that the biopsy did not diminish the visibility. Specimens were washed briefly in phosphate-buffered saline (PBS) to remove contaminating blood and mucus. One part of the tissue was fixed in 4% paraformaldehyde; the other part was directly processed for RNA extraction. Menstrual phase was determined by patient's history and dating was verified by histological examination of the endometrium according to the criteria of Noyes *et al.* (1950). Subjects were from the early proliferative (days 4–7, *n* = 4), mid proliferative (days 8–11, *n* = 12), late proliferative (days 12–14, *n* = 3), early secretory (days 15–18, *n* = 3), mid secretory (days 19–22, *n* = 5) and late secretory (days 23–28, *n* = 3) phases. Endometrial samples (mid proliferative phase) from six additional patients were digested with collagenase for 2–4 h and separated into epithelial and stromal cell fractions by passing the cell suspension through a 40 µm nylon cell strainer (Falcon, Becton Dickinson) essentially as described before (Irwin *et al.*, 1989). The RNA was extracted from both cell types immediately after harvesting. The purity of the cell fractions was typically 92–96% as determined by immunohistochemical analysis of aliquots of cells plated on Labtec chamber slides (Falcon) with antibodies against cytokeratin (Dako) or vimentin (Dako).

### RNA extraction

Total RNA was isolated from tissue specimens using the Trizol reagent (Gibco BRL) according to the manufacturer's instructions. The amount and purity of the extracted RNA was quantified by spectrophotometry using an Eppendorf Biophotometer.

### Reverse transcription

For each mRNA sample, 10 µl RT mastermix was prepared [4 µl 25 mmol/l MgCl<sub>2</sub> solution, 2 µl 10×PCR buffer II, 1 µl dNTP mix (20 mmol/l each), 1 µl Oligo d(T)<sub>16</sub>, 1 µl RNase Inhibitor, 1 µl MuLV Reverse Transcriptase (all Perkin-Elmer)] and added to 1 µg total RNA diluted in 10 µl DEPC-treated H<sub>2</sub>O in a 0.5 ml PCR-Softtube (Biozym Diagnostik). RT mix was incubated at room temperature for 10 min, before starting the reaction in a Thermocycler (Uno II, Biometra) by using a program with the following parameters: 42°C for 1 h, 94°C for 5 min, 4°C for ∞. After the reaction was complete, samples were diluted with 80 µl DEPC-treated H<sub>2</sub>O and stored at –20°C.

### Primers for PCR

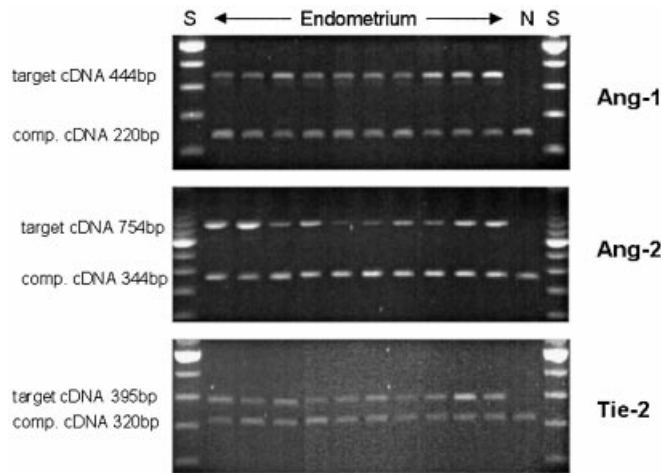
The cDNA sequences were obtained from the GenBank database of the National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH); <http://www.ncbi.nlm.nih.gov/>. Primer sequences were selected with the help of the program OLIGO 5.0 Primer Analysis Software (National Bioscience, USA). Primers were designed to cross intron/exon boundaries to ensure that the amplified product resulted from cDNA rather than contaminating genomic DNA. The identities of all PCR products were confirmed by cycle sequencing. The primer sequences, locations on the cDNA and the sizes of the amplified fragments are listed in Table I.

### Construction of the competitive cDNA fragments

Competitive cDNA fragments were constructed utilizing a method described previously (Krüssel *et al.*, 1998). A 'floating' primer was designed for each cDNA to introduce an internal deletion, resulting in a competitive fragment with the same primer binding characteristics as the target cDNA. Competitive cDNA was extracted from the agarose gel with an extraction kit (QIAEX II; Qiagen) and quantitated by spectrophotometry. The identities of all competitor fragments were confirmed by cycle sequencing. Sequences of the 'floating' primers and sizes of the resulting competitor fragments are listed in Table I.

### Competitive PCR

5 µl RT product (that is 1/20 of RT reaction) was mixed with 0.02 amol competitive cDNA for either Ang-1 or Ang-2 in 5 µl sterile H<sub>2</sub>O and with 40 µl PCR mastermix containing 5 µl 10× reaction buffer; 0.2 µl Taq DNA Polymerase (Pharmacia Biotech); 2 µl dNTP mix (50 mmol/l each, Eppendorf); 2 µl of 5' and 3' specific primers (5 µmol/l each) and 31.8 µl sterile H<sub>2</sub>O. The PCR was initiated by heating up to 94°C for 3 min, followed by 32 cycles denaturation at 94°C for 45 s, annealing at 53°C for Ang-1 or at 57°C for Ang-2 and extension at 72°C for 45 s. The reaction was terminated at 72°C for 5 min and cooled down to 4°C. PCR for Tie-2 was with 0.04 amol competitor, an annealing temperature of 55°C and 30 cycles. In each experiment a negative control reaction in which no cDNA was added was included. Preliminary experiments were performed to determine the optimal numbers of PCR amplification cycles for different primer pairs to ensure that the amplification was in the exponential phase and had not reached the plateau. PCR products



**Figure 1.** Representative competitive PCR products from endometrial biopsies from different patients (from left to right: cycle day 4, 5, 7, 8, 11, 15, 18, 21, 24 and 27). 2% agarose gels stained with ethidium bromide. S = standard (100 bp DNA ladder); N = negative control.

were visualized on a 2% agarose gel. The densitometrical analysis of bands was carried out on the GelDoc 1000 System (Bio-Rad Laboratories, USA) by using the Molecular Analyst Software (Bio-Rad). Densitometry values were used to calculate the ratios between target and competitor bands (*t/c* ratio).

#### Multiplex PCR

PCR was carried out for 35 cycles (45 s at 94°C, 45 s at 53°C, 45 s at 72°C) including 5'- and 3'-specific primers (5 µmol/l each) for both Ang-1 and Ang-2.

#### Immunohistochemistry

Immunohistochemistry was conducted using specific goat polyclonal antibodies raised against human Ang-1 and Ang-2 (sc-9360 and sc-7015; Santa Cruz Biotechnology, Inc., USA). Paraffin sections (5 µm) of endometrium from all stages of the menstrual cycle (*n* = 19) were dewaxed in xylene and rehydrated through descending grades of ethanol. Endogenous peroxidase activity was quenched by immersion in 3% H<sub>2</sub>O<sub>2</sub> for 10 min. Sections were then incubated with blocking solution containing 10% rabbit serum (Vector Laboratories, USA) and 1.5% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Primary antibodies were applied diluted at 1:100 in 1.5% BSA–PBS overnight at 4°C. Antibody localization was detected by sequential application of biotinylated rabbit anti-goat IgG (Vector) in PBS, and an avidin–biotin complex conjugated to horse-radish peroxidase (Vector). The substrate used was diaminobenzidine (Vector), which forms an insoluble brown precipitate, and nuclei were counterstained blue with Mayer's haemalum solution (Merck, Germany).

A similar protocol was utilized for detection of Tie-2 and von Willebrand Factor (vWF), which were localized using a rabbit polyclonal antibody (sc-324, Santa Cruz for Tie-2; and A-0082, Dako, Denmark for vWF), goat serum and a biotinylated goat anti-rabbit IgG (Vector).

A negative control was included for each tissue section by substitution of the primary antibody with a matching concentration of either goat or rabbit IgG.

#### Statistical analysis

All PCR experiments were repeated three times. Results were expressed as the mean ± SEM. Analyses were performed using SPSS, Version 10.1 (SPSS, USA). To determine if there were significant differences in mRNA expression levels at different stages of the menstrual cycle, non-parametric tests were used (Kruskal–Wallis, followed by Mann–Whitney *U*-test to detect differences between groups). A value of *P* < 0.05 was considered statistically significant.

## Results

We detected mRNA for the angiopoietins and the receptor Tie-2 in 30 out of 32 endometrial biopsies (94%), covering early proliferative (*n* = 4), mid proliferative (*n* = 12), late proliferative (*n* = 3), early

secretory (*n* = 3), mid secretory (*n* = 5) and late secretory (*n* = 3) phases. The expression levels of the angiopoietins were comparable, as judged from the same amount of competitor (0.02 amol) to be used in the competitive PCR reactions (Figure 1). Analysis of the target/competitor ratios revealed a significantly (*P* = 0.044, Kruskal–Wallis) different Ang-1 expression during the course of the cycle. Ang-1 expression was elevated in the early, mid and late secretory phase and the mid proliferative phase compared with the late proliferative phase (Figure 2). When we compare the data for the proliferative (*n* = 19) and secretory (*n* = 11) phases, there is a statistically significant (*P* = 0.027) increase of Ang-1 expression in the secretory phase (Figure 3).

Analysis of the expression levels of Ang-2 revealed a significant difference between early and mid secretory phase (Figure 2). Tie-2 expression showed only minor differences during the endometrial cycle (Figure 2), which did not reach statistical significance. There were, however, considerable individual variations in the mRNA levels within biopsies from the same phase of the menstrual cycle.

To confirm these results, another PCR experiment was performed, in which the relative expression levels of both angiopoietin genes were compared in a multiplex PCR reaction (Figure 4 and Figure 5). The lower Ang-2 versus Ang-1 ratios in the secretory phase were indicative of either reduced Ang-2 expression or, more likely in the light of our above result, an increased Ang-1 expression.

Figure 5 also shows a comparison of the multiplex and the competitive PCR methods used. The values for the Ang-2/Ang-1 ratio for the competitive PCR were obtained by comparing the mean target/competitor ratios for Ang-2 and Ang-1 for each individual biopsy. Both PCR experiments gave the same result, indicating the methodical accuracy.

By separating epithelial and stromal cell fractions from six additional endometrial biopsies, we could show that both the endometrial epithelial cells as well as the stromal cell fraction, including immune and endothelial cells, expressed mRNA for Ang-1, Ang-2 and Tie-2 (Figure 6). The minor differences in expression are not statistically significant.

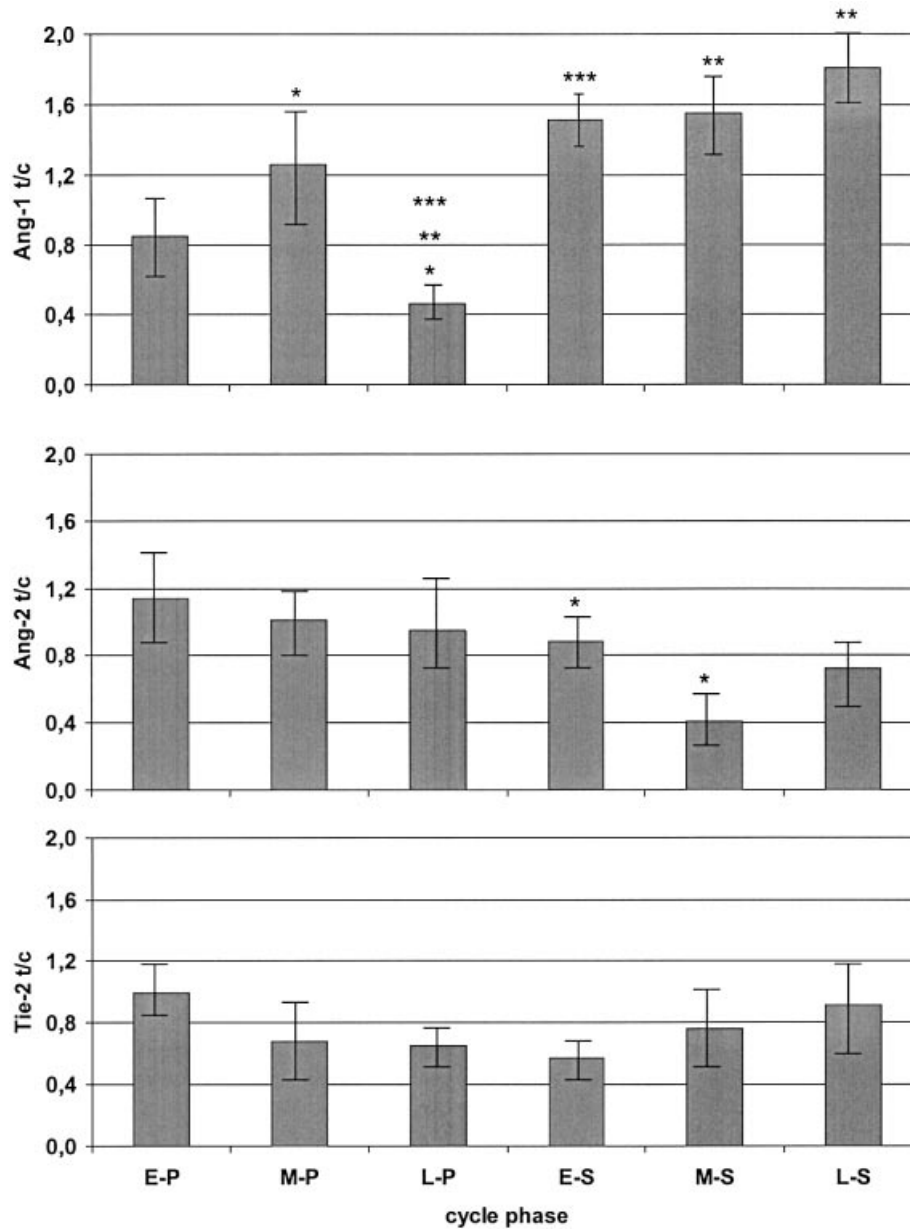
We examined the distribution of Ang-1, Ang-2 and Tie-2 protein in proliferative (*n* = 11) and secretory (*n* = 7) endometrium by immunohistochemistry (Figure 7). Staining showed interindividual differences, but nevertheless a clear pattern emerged. Staining for Ang-1 was detected in stromal cells, in glandular and luminal epithelium as well as in endothelial cells in both proliferative and secretory phase endometrium (Figure 7a, b).

Ang-2 immunoreactivity was detected in glandular epithelium, and in the stromal compartment of proliferative and secretory phase endometrium (Figure 7c, d). Both Ang-1 and Ang-2 proteins were found predominantly in the apical part of glandular epithelial cells.

Tie-2 staining was found in glandular epithelium and endothelial cells throughout the cycle, whereas staining of stromal cells was very low and heterogeneous (Figure 7e, f). As a control for staining of endothelial cells, we used an antibody against von Willebrand factor, which, as expected, selectively stained endometrial blood vessels (Figure 7g).

## Discussion

The regulation of human endometrial angiogenesis is still poorly understood, despite its unique role in the female reproductive cycle. Many studies have focused on the endometrial expression of vascular endothelial growth factor (VEGF) and its receptors (Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996; Torry *et al.*, 1996; Krüssel *et al.*, 1999; Sharkey *et al.*, 2000), but the functional significance in the endometrium is still uncertain, taking into account that the bulk of



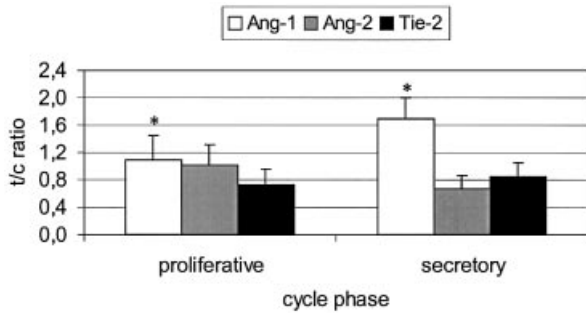
**Figure 2.** Histograms illustrating fluctuations in mRNA expression (mean values  $\pm$  SEM) for Ang-1, Ang-2 and Tie-2 across the menstrual cycle. Significantly elevated expression of Ang-1 was detected in the early, mid and late secretory phase and the mid proliferative phase compared with the late proliferative phase (\* $P = 0.043$ ; \*\* $P = 0.034$ ; \*\*\* $P = 0.048$  respectively, as determined by Mann–Whitney  $U$ -test). Ang-2 expression in the early and mid secretory phase are significantly different (\* $P = 0.034$  as determined by Mann–Whitney  $U$ -test). E-P, M-P, L-P = early ( $n = 4$ ), mid ( $n = 12$ ) and late ( $n = 3$ ) proliferative phase. E-S, M-S, L-S = early ( $n = 3$ ), mid ( $n = 5$ ) and late ( $n = 3$ ) secretory phase. t/c = target/competitor ratio.

VEGF is of glandular origin and secreted into the uterine cavity (Hornung *et al.*, 1998). The endometrium is a complex tissue with many different cell types in dynamic fluctuations. Therefore, Maas *et al.* (2001) studied the overall angiogenic activity of endometrium in the chick chorioallantoic membrane (CAM) assay. They found an angiogenic potential throughout the menstrual cycle, with a significantly increased VDI (vascular density index) in the early proliferative, early and late secretory phases as compared to the late proliferative phase. According to the hypothesis that changes in the balance of inducers and inhibitors of angiogenesis may activate the angiogenic switch (Hanahan and Folkman, 1996), it is highly unlikely that only one factor is responsible for the angiogenic potential of a specific tissue.

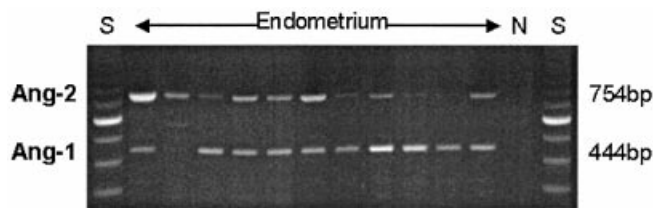
So far, there are only few studies dealing with angiotensin expression in the endometrium. In this study we found a significant

increase in Ang-1 mRNA expression during the secretory phase of the endometrial cycle. This finding was confirmed, although not quantitatively, at the protein level. On the other hand we found only minor differences in the mRNA and protein expression of Ang-2. Since we found neither mRNA nor protein levels of Tie-2 changed during the cycle, we assume that angiogenesis is regulated by the availability of the ligands rather than the receptor.

The observed Ang-1 mRNA increase during the secretory phase may reflect the need for both stabilization and maturation of the newly developed vessels. The secretory phase is characterized by a significant growth and coiling of spiral arterioles (Kaiserman-Abramof and Padykula, 1989), which supply the subepithelial capillary plexus, where the blood flow reaches a maximum during the early and mid-secretory phases in preparation for the implanting embryo (Gannon *et al.*, 1997). Ang-1 is assumed to function by



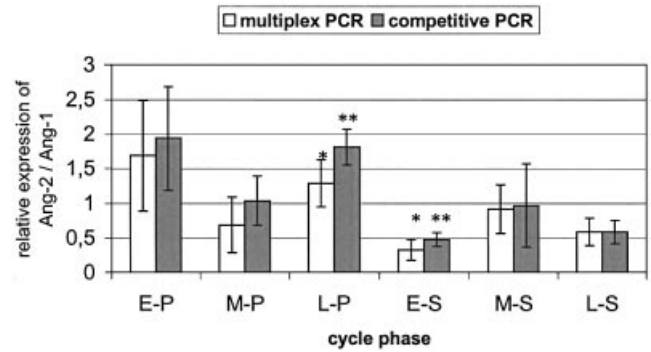
**Figure 3.** mRNA expression of Ang-1, Ang-2 and Tie-2 in the proliferative phase ( $n = 19$ ) and in the secretory phase ( $n = 11$ ). The Ang-1 expression is significantly different between the phases ( $*P = 0.027$  as determined by Mann-Whitney  $U$ -test). Results are shown as the mean  $\pm$  SEM.  $t/c$  = target/competitor ratio.



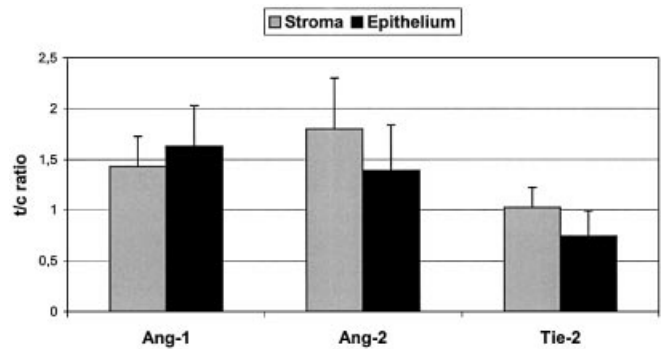
**Figure 4.** Multiplex PCR products from endometrial biopsies from different patients (from left to right: cycle day 4, 5, 7, 8, 11, 13, 15, 18, 21, 24 and 27). 2% agarose gels stained with ethidium bromide. S = standard (100 bp DNA ladder); N = negative control.

mediating the dialogue between the endothelium and surrounding pericytes and vascular smooth muscle cells, so as to promote the stability of blood vessels (Yancopoulos *et al.*, 2000). We propose that the Ang-1/Tie-2 system is important for endometrial vessel development during the postovulatory phase, when the spiral arterioles are developing. Our results are supported by a recent publication by Hewett *et al.* (2002), who found that endometrial Ang-1 expression (mRNA and protein) was down-regulated in patients with menorrhagia. Our data concerning the Ang-1 protein distribution are mostly in good agreement with their results. Ang-1 staining was detected in the glandular epithelium, stroma and blood vessels in the proliferative endometrium. The staining was more intense in the secretory phase, especially in the stroma surrounding the blood vessels (Hewett *et al.*, 2002). Contrary to our result, they found Ang-1 to be down-regulated in glandular epithelium during the secretory phase.

Whether our observation, that Ang-2 mRNA expression is significantly different in the early versus the mid secretory phase (Figure 2), is of any physiological importance, remains to be shown. We were not able to confirm this finding at the protein level. From our data it seems that there is no major difference in Ang-2 protein expression levels during the cycle. In accordance with our results, Hewett *et al.* (2002) found Ang-2 mRNA to be expressed at similar levels as assessed by RNase protection assay normalized to  $\beta$ -actin, in both normal and menorrhagic endometrium. In contrast, Krikun *et al.* (2000) showed by immunohistochemistry that expression of Ang-2 and Tie-2 was absent in the glands, low in stromal cells, and intense in the endothelial cells. Furthermore, they found no effect of the stage of menstrual cycle on the expression of Ang-2 or Tie-2. Their data, however, do not clearly show how many biopsies they have examined and how they reach this conclusion. Li *et al.* (2001) found Ang-2 expression, assessed by in-situ hybridization, predominantly in natural killer cells in the late secretory phase, which strongly implicates a role of these



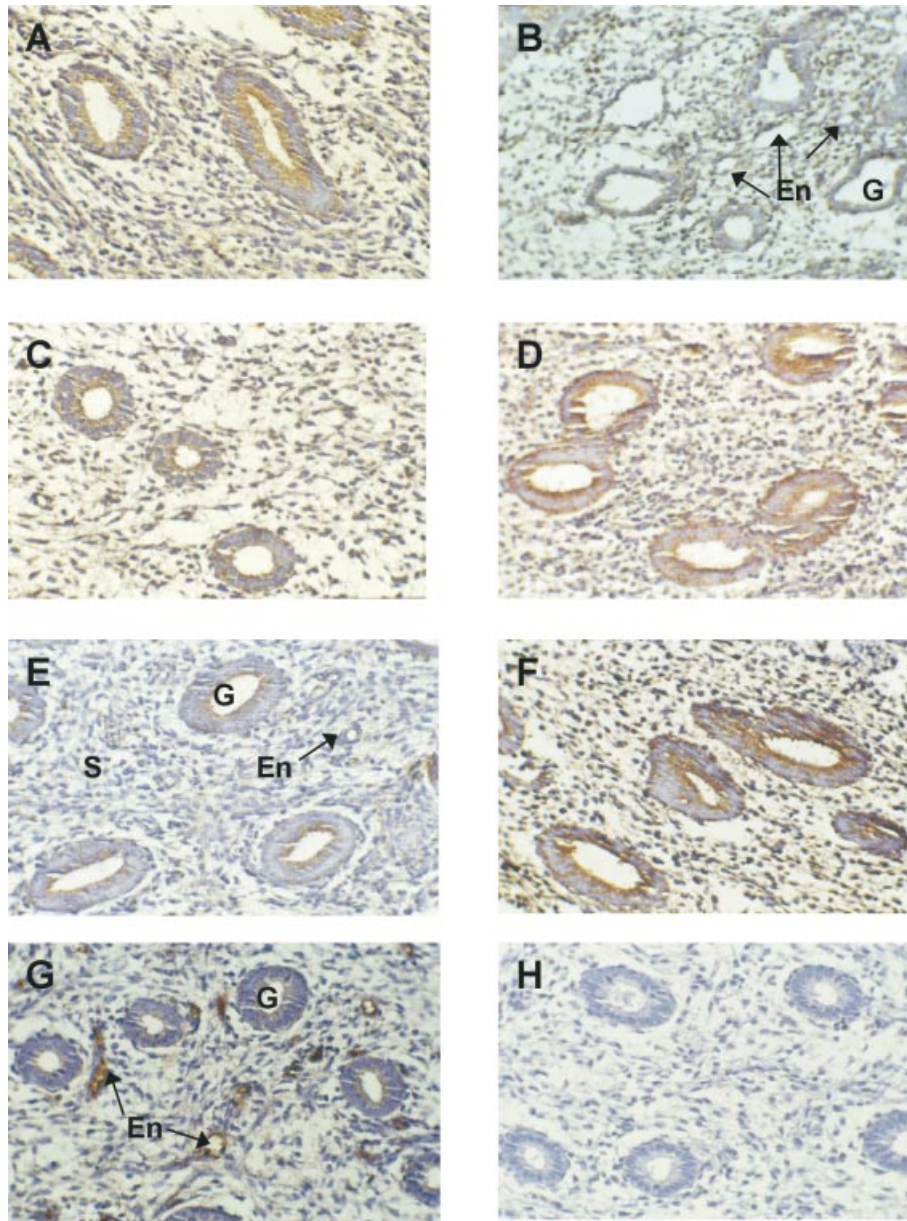
**Figure 5.** Relative ratio of angiopoietin mRNA expression in a multiplex PCR (white bars) compared to the results of the competitive PCR (gray bars). The values for the Ang-2/Ang-1 ratio for the competitive PCR were obtained by comparing the mean target/competitor ratios for Ang-2 and Ang-1 for each individual biopsy. Results are shown as the mean  $\pm$  SEM. The Ang-2/Ang-1 expression ratio is significantly different between the late proliferative and the early secretory phase ( $*P = 0.034$  and  $**P = 0.028$  as determined by Mann-Whitney  $U$ -test). E-P, M-P, L-P = early ( $n = 4$ ), mid ( $n = 12$ ) and late ( $n = 3$ ) proliferative phase; E-S, M-S, L-S = early ( $n = 3$ ), mid ( $n = 5$ ) and late ( $n = 3$ ) secretory phase.



**Figure 6.** Ang-1, Ang-2 and Tie-2 mRNA expression in human endometrium separated into epithelial (black bars) and stromal cell (gray bars) fractions ( $n = 6$ ). Statistical analysis revealed no significant difference. Results are shown as the mean  $\pm$  SEM.  $t/c$  = target/competitor ratio.

cells in vascular remodeling. In agreement with our semiquantitative RT-PCR data these authors found relatively steady mRNA levels of Ang-2 throughout the cycle. Contrary to our results, they found steady Ang-1 levels, but significantly altered Tie-2 expression during the course of the endometrial cycle. This might be explained by the different methodologies; they used a semiquantitative RT-PCR method comparing the expression level of the target gene with levels of  $\beta$ -actin. Our method used with specifically designed competitors is generally considered more accurate, since it eliminates tube-to-tube variation and the possible influence of different primers (Krüssel *et al.*, 1998). More importantly, in our study the multiplex PCR resulted in nearly the same Ang-2 versus Ang-1 expression ratio as the competitive PCR. This adds further significance to our findings, since these are independent methods.

From our Ang-2 data we speculate that there is a baseline Ang-2 level that is sufficient for angiogenesis to occur, provided that there are other inducers available in sufficient amounts (Hanahan and Folkman, 1996). Alternatively, it may be the local increase of Ang-2 availability, as suggested from the 'patchy' Ang-2 expression found in natural killer cells (Li *et al.*, 2001), rather than a generalized up-regulation, which results in angiogenesis. With respect to the local availability of a factor, the work of Gargett *et al.* (2001) is of importance. In a recent



**Figure 7.** Immunohistochemical staining of endometrium with Ang-1 (a, b), Ang-2 (c, d), Tie-2 (e, f) and vWF (g). Proliferative and secretory phase are the left and right columns respectively. (a and b) Staining for Ang-1 was detected in stromal cells, in glandular and luminal epithelium as well as in endothelial cells (b). (c and d) Ang-2 immunoreactivity was moderate in glandular epithelium, and weak in the stromal compartment of proliferative and secretory phase endometrium. (e and f) Moderate Tie-2 staining was detected in glandular epithelium, whereas stroma and endothelial cells were stained only weakly. (g) Strong vWF staining in blood vessels of secretory endometrium. (h) Control section demonstrating a lack of background staining when the primary antibody was replaced with a matching concentration of either goat or rabbit IgG. G = epithelial gland; S = stromal cells; En = endothelium. Magnification  $\times 250$ .

immunohistochemical analysis of full thickness endometrium, they were for the first time able to demonstrate a strong correlation between focal VEGF, found in marginating and adherent neutrophils inside the vessel lumen, and endothelial cell proliferation. We and others (Krikun *et al.*, 2000; Hewett *et al.*, 2002) were, however, not able to find spatially restricted Ang-2 expression loci, which one would assume to be in the vicinity of blood vessels.

We could show that both the endometrial epithelial cells as well as the stromal cell fraction expressed the mRNA for Ang-1, Ang-2 and Tie-2. Surprisingly, mRNA expression levels showed only minor differences between the cell types, since e.g. Tie-2 protein expression seemed much higher in the glandular epithelium than in the stromal cells. This may be explained by the fact that the Tie-2-expressing

endothelial cells may contribute to the stromal cell fraction. Alternatively, post-transcriptional regulatory events are resulting in a differential protein expression. It would be important to clarify whether the glandular expression of angiopoietins results in an apical secretion into the endometrial cavity, as has been shown before for VEGF (Hornung *et al.*, 1998), or if the proteins are secreted towards the underlying stroma, possibly acting on the subepithelial capillary plexus. The predominantly apical localization of the proteins, however, is suggestive of an apical secretion.

Despite extensive examination of the distribution of mRNA and protein of a wide variety of angiogenic factors, including the angiopoietins, no pattern has emerged that allows a conclusive explanation of endometrial angiogenesis. Certainly further in-vivo and

in-vitro experimentation is required to demonstrate the specific role of angiopoietins in endometrial angiogenesis and to elucidate the mechanisms involved.

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