Angiopoietin-1, angiopoietin-2 and Tie-2 expression in eutopic endometrium in advanced endometriosis

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Endometriosis is one of the most common gynaecological disorders, but its aetiology and pathogenesis remain obscure. The refluxed menstrual debris in women with endometriosis may be more prone to implantation, invasion and growth in the peritoneum or ovary through the actions of extracellular proteolysis and angiogenesis. It has been hypothesized that the endometrium from women with endometriosis has higher angiogenic activity and expresses more angiogenic factors. Using quantitative competitive PCR (QC-PCR) combined with the reverse transcription of total RNA into cDNA, we investigated the expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and Tie-2 in the eutopic endometrium from 56 women with severe endometriosis and that from 64 women without endometriosis during the follicular and luteal cycles. The protein expression from the eutopic endometrium was analysed by western blotting. Results were analysed statistically by Kruskal–Wallis and Mann–Whitney U tests. The eutopic endometrium from women with endometriosis expressed higher levels of mRNA and protein of Ang-1 (P < 0.05) and higher levels of mRNA of Ang-2 than the endometrium from normal women (P < 0.05). Tie-2 mRNA and protein expression from the eutopic endometrium did not differ significantly between endometriosis patients and normal controls. These results suggest that the eutopic endometrium from endometriosis patients is more angiogenic and prone to growth because of greater Ang-1 mRNA and protein expression and higher Ang-2 mRNA expression than the endometrium from women without endometriosis. Thus, increased angiogenic activity may be responsible for the pathogenesis of endometriosis.

Key words: angiogenic factor/angiopoietin-1/angiopoietin-2/endometriosis/Tie-2

Introduction

Endometriosis, a benign gynaecological disorder, is defined as the presence of endometrial glandular and stromal cells outside the uterine cavity. The pathophysiology of endometriosis is controversial but is most widely attributed to the peritoneal seeding of viable endometrial cells during retrograde menstruation (Sampson, 1927; D’Hooghe et al., 1995). Endometriosis can develop because of aberrant abnormalities inherent to the eutopic endometrium (Sharpe-Timms, 2001). The refluxed menstrual debris in women with endometriosis may be more prone to implantation, invasion and growth in the peritoneum or ovary through the actions of extracellular proteolysis and angiogenesis (Chung et al., 2002a,b). Recent studies have shown the importance of neovascularization within the peritoneal cavity for endometriosis pathogenesis. The angiogenic dynamics would provide an adequate blood supply to the desquamated endometrium, enabling attachment and implantation on the mesothelial surface. Neovascularization, therefore, has been considered to be a key factor in the progression of endometriosis (Smith, 1997). This pathological condition has been defined as an angiogenic disease (Healy et al., 1998). Angiogenic and growth factors may play a significant role in the pathogenesis of endometriosis. The high content of vascular endothelial growth factor (VEGF), as demonstrated by several authors (Smith, 1996; Donnez et al., 1998), has led to the hypothesis that VEGF-induced angiogenesis is critical to the pathophysiology of endometriosis. VEGF, midkine (MK) and pleiotropin all show higher expression in the eutopic endometrium of endometriosis patients than in normal women (Donnez et al., 1998; Chung et al., 2002a; Tan et al., 2002). MK levels are increased in the peritoneal fluid of women with advanced endometriosis, suggesting that MK plays a role in the development of endometriosis (Hirota et al., 2005).

Angiopoietins are a family of recently described growth factors of the vascular endothelium that secrete angiogenic factors that function mainly by promoting vessel maturation and remodelling (Holash et al., 1998; Gale and Yancopoulos, 1999; Yancopoulos et al., 2000). The principal member of the angiopoietin family, angiopoietin-1 (Ang-1), binds to its endothelial cell-specific tyrosine kinase receptor, Tie-2 (Davis et al., 1996). In vivo, Ang-1 functions by promoting the interaction and adherence of endothelial cells to the surrounding supporting cells to promote the integrity and maintenance of formed vessels. Ang-1 may be needed to stimulate new vessel formation during ovulation when active angiogenesis occurs (Hazzard et al., 1999).

Angiopoietin-2 (Ang-2) leads to a loosening of cell–matrix and cell–cell contacts, allowing access to angiogenic inducers such as VEGF. Thus, the expression of either Ang-2 or VEGF may lead to angiogenesis. In the absence of an angiogenic growth factor, the action of Ang-2 results in a regression of vessel structures (Hanahan, 1997; Yancopoulos et al., 2000). Ang-2 blocks the activation of Tie-2 by Ang-1 by acting as a competitive antagonist, suggesting that it is a...
naturally occurring inhibitor of Ang-1 and Tie-2 activities (Thurston et al., 1999). In mice and humans, Ang-2 is selectively expressed in ovary, uterus and placenta (Gale and Yancopolous, 1999), which are 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).

In this study, we determined Ang-1, Ang-2 and Tie-2 mRNA and protein expression in eutopic endometrium from women with and without endometriosis by quantitative competitive PCR (QC-PCR) and western blot analysis. We hypothesized that the expression of Ang-1, Ang-2 and Tie-2 would be higher in eutopic endometrial tissue from women with endometriosis, consistent with higher angiogenic activity and increased growth.

Materials and methods

Tissue collection
Endometrial samples were obtained from 120 premenopausal women aged 29–45 years, undergoing laparoscopic surgery or hysterectomy for non-malignant lesions. Patients with pelvic inflammatory disease, adenomyosis and dysfunctional uterine bleeding were excluded. Patients had not taken any non-steroidal anti-inflammatory drugs, GnRH agonists and steroids for the 3 months prior to surgery. Patients with endometriosis stages III and IV diagnosed by both pathology and laparoscopic findings according to the revised American Fertility Society classification of endometriosis (American Fertility Society, 1985). Endometrial tissue from 64 control patients without endometriosis confirmed by laparoscopic surgery was also collected. The study protocol was approved by the Institutional Review Board on the Use of Human Subjects in Research at Ewha Womans University, and informed consent was obtained from each patient.

Endometrial samples were taken using a Novak curette in the operating room before the laparoscopic procedure; in patients undergoing hysterectomy, the uterine cavity was opened and endometrium obtained immediately after the uterus was removed. Tissue samples were classified by histological dating according to the method of Noyes et al. (1950) into two groups: proliferative phase (n = 62) and secretory phase (n = 58). The remaining tissue was washed in phosphate-buffered saline (PBS) solution to remove contaminating blood, and RNA was immediately extracted.

RNA extraction
The extraction of RNA from the tissue sample was carried out with the RNA-STAT-60 reagent (Tel-Test ‘B’, Friendswood, TX, USA). Briefly, tissue samples were washed three times in PBS (Gibco BRL, Grand Island, NY, USA) to remove blood contamination. One hundred milligrams of tissue was homogenized in 1 ml of RNA-STAT-60 reagent. Total RNA was separated from DNA and proteins by adding chloroform and was precipitated using isopropanol.

The precipitate was washed two times in 75% ethanol, air dried and re-diluted in diethylpyrocarbonate (DEPC)-treated dH₂O. The amount and purity of extracted RNA were quantified by spectrophotometry in a GenQuant RNA/ DNA calculator (Pharmacia Biotech, Cambridge, UK), and 10–100 μg of total RNA was routinely obtained.

RT–PCR
Specific sequences of oligonucleotide primers for Ang-1, Ang-2 and Tie-2 were obtained from Gene Bank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, http://www.ncbi.nlm.nih.gov/ cgi-bin/Genbank). One corresponding set of primers for Ang-1, Ang-2 and Tie-2 was found with the help from the program Oligo 5.0 Primer Analysis Software (National Bioscience, Plymouth, MN, USA) and synthesized by Biomed, Seoul, Korea. The primer sequences, locations on the mRNA and sizes of the amplified fragments are summarized in Table I.

For RT–PCR, the Gen Amp RNA PCR kit (Perkin-Elmer, Foster City, CA, USA) was used. Nineteen microlitres of RT-mastermix for each sample was prepared containing 5 mmol/l MgCl₂, 1× PCR buffer II, 1 mmol/l of each deoxy- NTP, 2.5 μl of oligo-(deoxynucleotidyl)hex (all from Perkin-Elmer), 100 IU of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and 1 μg of total RNA diluted in 1 ml of DEPC-treated H₂O and placed into 0.2-ml thin-wall PCR tube (Applied Scientific, South San Francisco, CA, USA). RT was carried out in the DNA Thermal Cycler 9600 (Perkin-Elmer) using a program with the following parameters: 42°C, 15 min; 99°C, 5 min; then quenched at 4°C. After the reaction was completed, samples were stored at −20°C until the PCR. As a negative control, 1 μl of DEPC-treated H₂O without RNA sample was subjected to the same RT reaction.

Construction of the competitive and target cDNA fragment for Ang-1, Ang-2 and Tie-2
A 612-, 518- and 395-base pair (bp) fragment of native Ang-1, Ang-2 and Tie-2 cDNA (the target) was obtained by PCR amplification of reverse-transcribed total RNA from endometrial biopsies with the regular 3′ and 5′ primers (Table I). The PCR product was visualized by agarose gel electrophoresis stained with ethidium bromide (EtBr); cDNA was extracted from the gel, purified with an agarose gel extraction kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry (Pharmacia Biotech).

To construct a competitive cDNA fragment, we designed a floating primer with a sequence complementary to cDNA between the 3′ and the 5′ primer-binding sites by attaching the complementary sequence of the binding site of the original 3′ Ang-1, Ang-2 and Tie-2. After PCR with the regular 5′ primer and the 3′ floating primer, the PCR product was visualized by agarose gel electrophoresis stained with EtBr. cDNA extraction, purification and concentration determination were performed as described above. These steps resulted in cDNA fragments of 276, 284 and 320 bp, each with 3′-end and 5′-end primer-binding sites on their ends, which were products of 336, 234 and 75 bp deletion from the target cDNA, respectively.

| Table I. Oligonucleotide primers for Ang-1, Ang-2 and Tie-2 mRNA amplification in eutopic endometrium |
|-----------------|-----------------|-----------------|
| mRNA           | Primer 5′-3′    | Size (bp)       |
| Ang-1 Upstream  | 5′-GCTTACCAGATTCACACTGTTCC-3′ | 612             |
| Downstream     | 5′-TTGCTACCTTGCCAACAACACTG-3′  | 276             |
| Competitor     | 5′-TTGCTACCTTGCCAACACACTGATTGCCTAAGGGAATGGACTG-3′ | 2382–2362 |
| Ang-2 Upstream  | 5′-TGGACAATTATTCAGCGACGTG-3′ | 518             |
| Downstream     | 5′-GCTGTCGGATCATCATGGTG-3′   | 284             |
| Competitor     | 5′-GCTGTCGGATCATCATGGTGTCCTGTGAGGAAATGGACTG-3′ | 1830–1809 |
| Tie-2 Upstream  | 5′-TGGAGATGACCCTGCTGACTG-3′ | 395             |
| Downstream     | 5′-GATGATGTTGGATTGGTGTTCC-3′  | 320             |
| Competitor     | 5′-TGGAGATGACCCTGCTGACTG-3′ | +2507–2526      |

S.E.Hur et al.
Results

RT–PCR of endometrial tissue during the follicular and luteal cycles

RT–PCR was employed to increase the sensitivity of detection, and the 838-bp sequence of α-actin, 612-bp sequence of Ang-1, 518-bp sequence of Ang-2 and 395-bp sequence of Tie-2 mRNA were expressed by all eutopic endometrial samples from women with and without endometriosis in both the follicular and luteal cycles of the menstrual cycle. α-Actin mRNA expression was also measured in all the samples studied, thus confirming the integrity of RNA and the RT–PCR process (data not shown).

Quantitative Ang-1 mRNA expression in eutopic endometrial tissue

Quantitative expression of Ang-1 mRNA in eutopic endometrium from endometriosis patients was examined during the follicular and luteal cycles and was compared with eutopic endometrial expression in control patients. During the follicular and luteal cycles, eutopic endometrium from endometriosis patients showed significantly increased Ang-1 mRNA expression compared with eutopic endometrium from controls ($P = 0.022$ and 0.002, respectively) (Figure 1).

Quantitative Ang-2 mRNA expression in eutopic endometrial tissue

During the luteal cycle, eutopic endometrium from endometriosis patients showed significantly increased Ang-2 mRNA expression compared with eutopic endometrium of luteal cycles from controls ($P = 0.002$) (Figure 2).

Quantitative Tie-2 mRNA expression in eutopic endometrial tissue

Quantitative expression of Tie-2 mRNA in eutopic endometrium of endometriosis patients was higher in the luteal phase compared with control group, and it was statistically significant ($P < 0.001$) (Figure 3).

Data analysis

Statistical analysis was performed by Kruskal–Wallis and Mann–Whitney $U$ test. The statistical analysis was carried out using the Statistical Package for Social Science version 11.5 (SPSS, Chicago, IL, USA) with $P < 0.05$ considered statistically significant.
Ang-2/Ang-1 mRNA expression ratio in eutopic endometrial tissue
During the luteal cycle, eutopic endometrium from endometriosis patients showed significantly increased Ang-2/Ang-1 mRNA expression ratio compared with eutopic endometrium from normal patients ($P < 0.001$) (Figure 4).

Discussion
The establishment and development of an effective blood supply and the development of angiogenesis are essential for the survival and growth of endometriotic implants and may also be related to abnormal angiogenesis (Folkman and Shing, 1992). Eutopic and ectopic endometria express various angiogenic factors (including members of the VEGF family) that may be related to the pathophysiology of endometriosis (Donnez et al., 1998; Chung et al., 2002a). We hypothesized that disturbed angiogenesis underlies the pathophysiology of endometriosis.

Angiopoietins are potent angiogenic factors that are expressed in the normal human endometrium. The expression of Ang-1 mRNA increases significantly during the secretory phase of the endometrial cycle (Hirchenhain et al., 2003). Few studies have investigated the association between endometriosis and angiopoietins, and the role of the angiopoietins/Tie system in the pathogenesis of endometriosis has not been fully clarified. An in vivo study found that Ang-1 was immuno-histochemically detectable in the peritoneal endometriosis tissue in

Western blot assay
Ang-1 expression in eutopic endometrium from endometriosis showed significantly increased Ang-1 expression compared with eutopic endometrium from controls during the follicular and luteal cycles ($P < 0.001$ and 0.001, respectively) (Figure 5).

Ang-2 and Tie-2 expression in eutopic endometrium from endometriosis showed similar expression pattern of results of western blot assay compared to QC-PCR data of mRNA, but there was no statistically significant difference between normal and endometriosis patients (Figures 6 and 7).
Ang-1, Ang-2 and Tie-2 expression in eutopic endometrium

In this study, we investigated the expression levels of Ang-1, Ang-2 and Tie-2 in eutopic endometria from women with or without endometriosis, with the aim of determining the factors that play a role in the pathogenesis of endometriosis.

Ang-2 strongly up-regulates the production of proteases such as matrix metalloproteinases (MMPs) in the presence of VEGF (Etoh et al., 2001), and the increased proteolytic activity via MMPs may help to explain the invasive factors that result in endometriosis (Chung et al., 2002b). In this study, the level of Ang-1 and Ang-2 mRNA expression was significantly higher in eutopic endometrium from patients with endometriosis than in those from controls, which suggests that these factors play an important role in the pathogenesis of endometriosis.

The increased expression of Ang-2 induces the expression of MMPs in eutopic endometrium, which also supports that a eutopic endometrium is the primary factor in the theory of regurgitated endometrium being the origin of endometriosis.

The prognosis of hepatocellular carcinoma patients is significantly worse in the presence of a high Ang-2/Ang-1 mRNA ratio (Mitsuhashi et al., 2003). An in vitro study found that the Ang-2/Ang-1 expression ratio was possibly shifted towards a more dominant role of Ang-2 in endometrial grafts that were implanted on chicken chorioallantoic membranes (Drenkhahn et al., 2004). In this study, we found that during the secretory phase, the Ang-2/Ang-1 mRNA expression ratio was significantly higher in eutopic endometria from endometriosis patients than in those from controls. The dominance of Ang-2 over Ang-1 through the Tie-2 receptor in the presence of VEGF may cause a constant immature neovascularization in endometriosis, such as in hepatocellular carcinoma (Mitsuhashi et al., 2003). Moreover, Ang-2

endothelial cells, periendothelial cells, in the glandular epithelium and in stromal structures and that there were no significant differences in the expression of pigmented and non-pigmented lesions (Gescher et al., 2004). There have been no reports on eutopic endometria or on the relationship between endometrial pathogenesis and angiopoietins.
promotes vessel sprouting only in conjunction with VEGF, by blocking the stabilization signal of Ang-1 at the level of Tie-2. Thus, the results from this study suggest that Ang-2 has a more important role in the angiogenesis of endometriosis development.

Ang-1 and Ang-2 bind to the Tie-2 receptor, with Ang-1 acting agonistically and Ang-2 acting antagonistically. Tie-2 becomes auto-phosphorylated upon Ang-1 binding (Davis et al., 1996), whereas short-term Ang-2 treatment is not capable of inducing Tie-2 auto-phosphorylation in endothelial cells (Maisonpierre et al., 1997). In this study, we found that the expression of Tie-2 mRNA in eutopic endometria did not differ between the endometriosis and the control groups. Hirchenhain et al. (2003) found that neither the mRNA nor the protein levels of Tie-2 changed during the menstrual cycle in the normal human endometrium, from which they concluded that angiogenesis is regulated by the availability of the ligands rather than the receptor. We also found that neither the mRNA nor the protein levels of Tie-2 changed during the menstrual cycle. Thus, this study suggests that Tie-2 has no role in the angiogenesis of endometriosis development and cyclic changes of the eutopic endometrium and that the ligands (Ang-1 and Ang-2) are factors in the pathogenesis. The first 360 amino acids [Ig-like domain plus epidermal growth factor (EGF)-like repeats] of the Tie-2 receptor are necessary and sufficient to bind both Ang-1 and Ang-2. Thus, we used an anti-Tie-2 Ab for western blotting; this has an 18 amino acid peptide sequence within the extracellular, N-terminus of the ligand-binding site. One of the limitations of our study is that we did not stain with an Ab against a phosphorylated and non-phosphorylated Tie-2 Ab, whereas it is important to know the phosphorylation status, as it shows whether the angiogenesis is in an active state.

In this study, we compared the expressions of Ang-1, Ang-2 and Tie-2 in the eutopic endometrium with and without endometriosis and found an increased expression of Ang-1 and Ang-2 in the former. The angiopoietins function at later stages of vascular development (i.e. during vascular remodelling and maturation), which suggests that, in endometriosis, an increased ability to generate new vessels is essential if the endometrium is to survive outside the uterus. These differences between normal subjects and endometriosis patients support the hypothesis that the endometrium did not differ from the endometrium of the normal population and thus that the original eutopic endometrium is important to the pathophysiology of endometriosis. Future studies should investigate the function of each angiopoietin and the effects of other angiogenic factors.

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References


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