Effects of peritoneal fluid from endometriosis patients on the release of vascular endothelial growth factor by neutrophils and monocytes

Yong-Jin Na¹, Seung-Hong Yang¹, Dae-Won Baek², Dong-Hyung Lee¹, Ki-Hyung Kim¹, Young-Min Choi³, Sung-Tack Oh⁴, Young-Seoub Hong², Jong-Young Kwak²,⁵ and Kyu-Sup Lee¹,⁶

¹Department of Obstetrics and Gynecology, Pusan National University, ²Medical Research Center for Cancer Molecular Therapy, Dong-A University, Busan, ³Department of Obstetrics and Gynecology, Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University, Seoul and ⁴Department of Obstetrics and Gynecology, College of Medicine, Chonnam National University, Kwang-Ju, Korea

To whom correspondence should be addressed at: Medical Research Center for Cancer Molecular Therapy, Dong-A University, Busan 602-714, Korea. E-mail: jykwak@dau.ac.kr
To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Medical Research Institute, Pusan National University, Busan 602-739, Korea. E-mail: kuslee@pusan.ac.kr

BACKGROUND: An increase in the level of the vascular endothelial growth factor (VEGF) production has been reported in the peritoneal fluid (PF) of endometriosis patients. This suggests that changes in the vascular permeability and angiogenesis play an important role in the pathophysiology of this disease. This study examined the effects of the PF obtained from endometriosis patients on the release of VEGF by neutrophils and monocytes.

METHODS: Neutrophils and monocytes were obtained from young healthy volunteers and cultured with the PF obtained from either endometriosis patients (EPF) (n = 18) or a control group (CPF) (n = 4). A human monocyte/macrophage cell line, THP-1, was cultured with either 10% EPF or 10% CPF. The PF and culture supernatants were assayed for VEGF using ELISA. Real-time PCR and Western blotting were used to measure the VEGF mRNA and protein expression level, respectively.

RESULTS: The VEGF levels were higher in the EPF than in the CPF (591 ± 75 versus 185 ± 31 pg/ml, P < 0.05). However, the level of VEGF released by THP-1 cells in CPF and EPF was similar. The EPF induced the release of VEGF by neutrophils, but no VEGF was released by monocytes. The VEGF mRNA expression levels in the neutrophils were higher in the EPF, which was abrogated by cycloheximide, suggesting that the EPF induces the production of VEGF in neutrophils. Neutralizing antibodies against IL-8 and TNF-α did not completely prevent the EPF-induced release of VEGF by the neutrophils, even though these growth factors stimulated the release of VEGF by neutrophils. There was a positive correlation between the VEGF and IL-10 concentrations in the EPF (correlation coefficient = 0.549, P = 0.012, n = 18), but the neutralizing antibody of IL-10 did not affect the release of VEGF by the EPF-treated neutrophils.

CONCLUSION: The EPF induced the production and release of VEGF by neutrophils, suggesting that neutrophils may be a source of peritoneal VEGF. In addition, neutrophil-derived VEGF might be a marker for diagnosing endometriosis.

Key words: endometriosis/monocytes/neutrophils/peritoneal fluid/VEGF

Introduction

Endometriosis is a disease in women, in whom the endometrial tissue is found outside the uterus. Although the aetiology of endometriosis is unclear, evidence suggests retrograde menstruation with the subsequent transplantation and invasion of exfoliated cells (Arici and Oral, 1997). Activated lymphocytes and macrophages are the hallmark of the inflammatory response induced by endometriosis. Growth factors and cytokines, which are secreted by these activated immune cells, have been implicated in the implantation and growth of endometrial cells outside the uterus (Lebovic et al., 2001). Inflammation and neovascularization have been observed in and around the ectopic endometrial implants (Khorram et al., 1993). Moreover, inflammatory leucocytes have been found in these lesions (Khorram et al., 1993) as well as in the peritoneal fluid (PF) of women with endometriosis (Hill et al., 1988). However, neutrophils may play a role possibly in the earliest stages. It has been reported that the degree of peritoneal...
inflammation, as assessed by the total number of PF polymorphonuclear leukocytes, decreases with the increasing stage of the endometriosis (Haney et al., 1991).

The vascular endothelial growth factor (VEGF) is a homodimeric peptide growth factor with various biological effects, such as the formation of vascular tubes and the permeability of blood vessels (Keck et al., 1989; Leung et al., 1989). Many types of cells, including macrophages (McLaren et al., 1996a), neutrophils (Taichman et al., 1997) and T cells (Freeman et al., 1995), produce VEGF. The induction of VEGF is stimulated by various growth factors, cytokines and hypoxia (Neufeld et al., 1999). VEGF has been suggested to play a role in the progression of endometriosis, because this growth factor stimulates the angiogenic activity in endometriosis (Fasciani et al., 2000). Moreover, elevated VEGF levels have been found in the PF of endometriosis patients (McLaren et al., 1996b), and a positive correlation has been reported between the severity of endometriosis and the VEGF concentrations in the PF (Shifren et al., 1996).

On attraction, neutrophils are activated and secrete VEGF (Gaudry et al., 1997; Taichman et al., 1997; Webb et al., 1998). Neutrophils in the endometrium are a source of intra-vascular VEGF for vessels undergoing angiogenesis (Gargett et al., 2001), and the increased chemotactic activity for neutrophils has been demonstrated in the PF of endometriosis patients (Leiva et al., 1993). Previous studies have shown that the PF and endometrial macrophages are significant sources of VEGF (McLaren et al., 1996b). However, the biological significance of VEGF in neutrophils during the pathological process of endometriosis is unclear. Therefore, this study examined the effect of the PF on the release of VEGF by neutrophils and monocytes.

Materials and methods

Reagents

The recombinant human VEGF, interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), epithelial neutrophil-activating peptide-78 (ENA-78), granulocyte–macrophage colony-stimulating factor (GM-CSF) and neutralizing antibodies against IL-10, IL-8 and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The RPMI-1640 medium was purchased from Gibco-BRL (Gaithersburg, MD, USA). The specific antibodies against VEGF165 and β-actin were purchased from Santa-Cruz Biotech (Santa Cruz, CA, USA). The activating anti-Fas antibody (clone CH11, IgM) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). The Histopaque-1077, dextran (500 000 Da), lipopolysaccharide (LPS), cycloheximide, pertussis toxin (PTX) and other chemicals were acquired from Sigma Chemical Co. (St Louis, MO, USA).

Patients

The endometriosis patients (n = 18) in this study had either stage III or IV endometriosis (Kwak et al., 2002). The patients were staged according to the revised American Fertility Society scoring system (The American Fertility Society, 1985). The presence of endometriosis was assessed at the time of surgery and was later confirmed histologically. Women taking oral contraceptives and gonadotrophin-releasing hormone analogues were excluded. The PF from the endometriosis group (EPF) was collected in a sterile environment before any surgical manipulations. The control PF (CPF) (n = 4) was obtained from fertile women undergoing laparoscopic surgery for various gynaecological indications other than endometriosis. These conditions included a dermoid cyst and carcinoma in situ of the uterine cervix. Patients with infertility without endometriosis were not included in the control group because there are many underlying causes of the infertility, and the patient selection in this study was not based on the presence or absence of infertility. The PF was centrifuged at 800 × g for 10 min, and the supernatants were stored at −70°C.

Figure 1 is a schematic drawing of the study design. Each CPF and EPF sample was assayed for the presence of VEGF and VEGF production in the THP-1 cells. Among the nine EPF samples containing a high level of VEGF, five EPF samples, which were sufficient for all experiments in this study, were used to determine the level of VEGF production in the neutrophils and monocytes (blanked triangle in Figure 2A). The mean VEGF concentrations in the CPF mixture and EPF mixture were 185 and 905 pg/ml, respectively (Figure 2A). Informed consent was obtained from all subjects, and the Institutional Review Board at Pusan National University Hospital approved this study.

Neutrophil isolation and culture

The peripheral blood neutrophils were isolated from 10 young healthy donors using a method involving dextran sedimentation and differential centrifugation through a Ficoll–Hypaque density gradient (Kwak et al., 2002). Briefly, the blood was mixed with a solution containing 3% dextran and 0.9% NaCl and stored for 45 min at 25°C. The neutrophil-rich upper layer of the suspension was collected, the residual erythrocytes were removed by hypotonic lysis, and the remaining pellet was suspended in HEPES-buffered saline. The suspension was centrifuged on a Histopaque solution at 4°C. The isolated neutrophils (5 × 10⁶/ml) were maintained in a RPMI-1640 medium supplemented with or without 10% EPF, or 10% CPF in the presence of 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin in 24-well flat-bottomed plates at 37°C in a humidified atmosphere containing 5% CO₂ (Kwak et al., 2002). Neutrophils with lobed nuclei and neutrally stained cytoplasm and granules were found to be 95% morphologically pure by Wright–Giemsa staining.

Monocyte isolation

The peripheral blood mononuclear cells were prepared by density-gradient centrifugation of the whole blood (n = 10) over a peripheral blood mononuclear cell separation medium (Histopaque-1077). The
mononuclear cells were suspended in RPMI-1640 medium containing 10% FBS and incubated for 1 h at 37°C to allow the monocytes to attach to the culture dish. The cells were washed five times with warm medium to remove the lymphocytes, and the remaining cells were collected. The purity of the isolated monocytes was confirmed by flow cytometric analysis using the fluorescein-isothiocyanate-conjugated anti-CD14 antibody (BD Pharmingen, NJ, USA) with >90% cell purity.

**THP-1 cell culture**

The THP-1 cells (Korean Cell Line Bank, Seoul National University Hospital, Korea), which were derived from a human monocyte/macrophage cell line, were cultured in RPMI-1640 medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM l-glutamine, 20 mM HEPES and 10% FBS. The THP-1 cells were plated at 5 × 10⁶/ml in 24-well plates and treated with either 10% EPF or 10% CPF. After 24 h at 37°C and 5% CO₂, the THP-1 cells were centrifuged. The supernatants were then removed and assayed for VEGF.

**Real-time PCR**

The total cellular RNA from the neutrophils was isolated using the Tri reagent (Sigma). All RNA samples were treated with DNase I, and the quality of the RNA was determined by measuring the optical density at 260 nm (OD₂₆⁰). For each sample, 2 μg of the total RNA was first converted to cDNA using the reverse transcriptase and oligo (dT) primers. The real-time–PCR amplification reactions were then performed on aliquots of the cDNA using Assays-on-Demand TaqMan Gene Expression Products (p/n, 4335626) (Applied Biosystems, Foster City, CA, USA) and by continuous fluorescence monitoring (ABI PRISM® 7000; Sequence Detection System, Applied Biosystems). The following PCR reactions were used: an initial denaturation step (95°C for 120 s), followed by 40 cycles of denaturation at 95°C (15 s), annealing at 58°C (30 s) and extension at 72°C (45 s). The primers and probes for VEGF and β-actin were proprietary oligonucleotides purchased from Applied Biosystems ‘Assay-on-Demand’, and a 5’-end labelling with a reporter fluorochrome [6-carboxyfluorescein reporter] was used.

**ELISA assay**

The neutrophils and monocytes were pretreated with or without 1 μg/ml of PTX for 4 h, or with 0.5 ng of the neutralizing antibodies against TNF-α, IL-8 or IL-10 for 1 h. To determine whether the production of VEGF is mediated by protein synthesis, we also pretreated the neutrophils with an inhibitor of the translation, cycloheximide (10 μg/ml) for 1 h. The cells were cultured in RPMI-1640 media containing 10% EPF, 10% CPF, 10 ng/ml of IL-8, 10 ng/ml of ENA-78 or 20 ng/ml of TNF-α for the indicated times. The IL-10 and VEGF concentrations in the PFs and supernatants were measured in triplicate using standard ELISA kits (R&D Systems). The VEGF detected in this study using ELISA represents the VEGF₁₆₅ and VEGF₁₂₁ as specified by the manufacturer. The sensitivity of the ELISA was ≤5 pg/ml. Standard cytokine preparations were used as the internal controls.

**Western blot analysis**

Freshly isolated neutrophils and cells (1 × 10⁷), which were cultured in the presence or absence of 10% CPF, 10% EPF or 1 μg/ml of LPS for the indicated times, were pretreated with diisoulocorphosphate for 20 min and resuspended in a buffer containing 20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100 and protease inhibitors. The lysates (75 μg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at 25°C with a blocking buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100 and protease inhibitors). The lysates (75 μg) were incubated with the primary antibodies directed against either VEGF₁₆₅ or β-actin (1:1000 dilution) in a blocking buffer overnight at 4°C. The secondary antibodies conjugated to horseradish peroxidase were diluted 1:5000 in a blocking buffer and incubated for 1 h. The signal was detected using ECL chemiluminescence.

**Neutrophil apoptosis assay**

The neutrophils, which had been incubated in the presence or absence of GM-CSF (10 ng/ml), LPS (1 μg/ml) or agonistic anti-Fas antibody (1 μg/ml) for 24 h, were spun down on a glass slide in a cytospin (Shandon, Pittsburgh, PA, USA). The cells were stained with a Giemsa staining solution (Fluka, Bushs, Switzerland), and the percentage of apoptotic cells showing condensed and fragmented nuclei with a diminished cell volume was determined by counting at least 300 cells per slide (Kwak *et al.*, 2002).

**Statistical analysis**

The results are presented as mean ± SE. Mann–Whitney *U*-test was used to compare the individual groups. The correlation coefficients were calculated using the Spearman’s correlation test. A *P*-value <0.05 was considered significant. The number of samples is represented by *n*. Each experiment was carried out either in duplicate or in triplicate. The analyses were performed using the Statistics Package for Social Sciences version 10.1.

**Results**

**VEGF levels in EPF and supernatants from cultured THP-1 cells with EPF**

The VEGF concentration was found to be significantly higher in the EPF than in the CPF (591 ± 75 versus 185 ± 31 pg/ml,
The culture supernatants were harvested after the peripheral and neutrophils. Effect of EPF on the release of VEGF by monocytes cultured with PF (data not shown). The THP-1 cells were then examined to determine whether they could release VEGF after being stimulated with the EPF. In this experiment, 10% EPF was used because this concentration did not affect the detection of the secreted VEGF contained in EPF (260–1350 pg/ml). In addition, previous experiments showed that 10% EPF can affect the neutrophils, e.g. cell survival. As shown in Figure 2B, the level of VEGF released by the THP-1 cells in the CPF and EPF groups was similar (452 ± 13 versus 503 ± 30 pg/ml, Mann–Whitney U-test; P = 0.67). Moreover, there was no correlation between the VEGF levels in the PF and the supernatants from the THP-1 cells cultured with PF (data not shown).

**Effect of EPF on the release of VEGF by monocytes and neutrophils**

The culture supernatants were harvested after the peripheral blood neutrophils and monocytes had been treated with or without 10% EPF or 10% CPF for up to 24 h. The supernatants were then analysed by ELISA for the presence of VEGF. The same batches of neutrophils and monocytes from the same donors were used in each experiment. Separate experiments used the cells from different donors (n = 10). The basal VEGF concentration in the neutrophils cultured for 24 h was 78 ± 25 pg/ml, and the maximum VEGF concentration in the 10% EPF sample was 150 pg/ml. However, the VEGF concentrations in the supernatant of the neutrophils treated with 10% EPF was significantly higher than in 10% EPF. Therefore, this study examined whether some EPF (n = 5) and CPF (n = 4) samples have a different effect on the release of VEGF by neutrophils and monocytes. The EPF significantly increased the level of VEGF released by neutrophils compared with the CPF (70 ± 12 pg/ml, median 70 versus 508 ± 155 pg/ml, median 400 pg/ml, Mann–Whitney U-test; P = 0.014), whereas the level of the VEGF was similar in the monocytes cultured with the CPF and with EPF (64 ± 13 pg/ml, median 60 versus 191 ± 94 pg/ml, median 105 pg/ml, Mann–Whitney U-test; P = 0.27) (Figure 3A). However, each of the four single CPFs showed a similar level of VEGF released by neutrophils. Five EPF samples were then mixed at an equal ratio (each 20%). Incubation of the isolated neutrophils with the 10% EPF mixture for 24 h resulted in an increase in the amount of VEGF released by the neutrophils compared with the CPF mixture (90 ± 5 versus 580 ± 32 pg/ml, Mann–Whitney U-test; P = 0.003) (Figure 3A). When 10% of the CPF or EPF mixture was added to the monocytes and cultured for 24 h, there was a two-fold increase in the VEGF level in the conditioned medium compared with the CPF, but the difference was not statistically significant (85 ± 45 versus 206 ± 72 pg/ml, Mann–Whitney U-test; P = 0.06), even though the level of VEGF released by monocytes in the presence of EPF mixture was higher than the basal VEGF concentration in the cultured monocytes for 24 h (45 ± 35 pg/ml). Figure 3B shows the time-course release of VEGF after stimulating the neutrophils with EPF. A significant amount of extracellular VEGF was detected after 6 h stimulation, which was further increased after 24 h. In contrast, EPF had no significant effect on the release of VEGF after treating the monocytes for either a brief or a prolonged period (data not shown).

**Effects of EPF on the VEGF mRNA levels in neutrophils**

This study examined whether the EPF could alter the VEGF mRNA level in the neutrophils. The RNA was harvested from the neutrophils incubated with the 10% EPF mixture, 10% CPF mixture or the medium alone for the indicated times and reverse transcribed. Real-time PCR was performed using the primers specific to the VEGF transcripts, and the relative quantity
of each transcript was determined after normalization to the \( \beta \)-actin level. The fold change in the expression level of these transcripts in the PF-treated neutrophils compared with that in the media-treated neutrophils is shown (Figure 4A). Stimulating the neutrophils with the EPF for 2 and 6 h resulted in a 2.5- and 4.2-fold increase, respectively. The EPF-induced release of VEGF by the neutrophils was abrogated by pretreating these cells with 10 \( \mu \)g/ml of cycloheximide for 1 h (Figure 4B). These results suggest that the release of VEGF is the result of \textit{de novo} synthesis by a certain PF factor in neutrophils. Western blot analysis of the VEGF in the neutrophil lysates showed that the 2 h EPF treatment decreased the protein level of the 43 kDa band, but the amount of the intracellular VEGF proteins was maintained for up to 12 h (Figure 4C). In comparison, the 2 h LPS treatment decreased the protein level of VEGF in the cell lysates. The release of VEGF by neutrophils in the presence of the EPF was not abrogated by adding an LPS inhibitor, polymyxin B, indicating that this effect was not the result of LPS contamination (data not shown). This suggests that the EPF induces both the production and secretion of VEGF by neutrophils.

**IL-8 and TNF-\( \alpha \)-independent release of VEGF by EPF**

CXCL8 (IL-8) was recently reported to induce VEGF secretion by human neutrophils (Cullen et al., 2000), and the IL-8 concentration in the EPF was shown to be higher than that in the CPF (Ryan et al., 1995; Arici et al., 1996). Therefore, this study examined whether the effect of EPF on the release of VEGF by neutrophils is mediated by this cytokine. The CXC chemokines activate neutrophils via an interaction with the chemokine receptors CXCR1 and CXCR2. Therefore, the neutrophils were pretreated with the PTX, which inhibits signalling through the Gi and Gq family of G-proteins, and cultured in a medium containing either EPF or IL-8. As shown in Figure 5A, there appears to be an approximately 20% reduction in VEGF in the conditioned medium in the cells treated with PTX, but there was no significant difference, whereas the release of IL-8-induced VEGF was significantly inhibited by PTX. The neutralizing antibody of IL-8 caused a marked reduction in the ability of IL-8 to induce the release of VEGF by neutrophils (data not shown), but it had no effect on the EPF-induced release of VEGF by neutrophils (Figure 5B). This indicates that the IL-8-induced activation of the PTX sensitive receptor stimulates the release of VEGF in neutrophils. However, IL-8 in the EPF may be insufficient for releasing VEGF in neutrophils, or other unknown factor(s) in EPF down-regulate the release of VEGF by the EPF-treated neutrophils.

Next, the cells were treated with 20 ng/ml of TNF-\( \alpha \) for 24 h. As expected, TNF-\( \alpha \) induced the release of VEGF by neutrophils (Figure 5A). In contrast, TNF-\( \alpha \) was unable to stimulate the release of VEGF by monocytes. It is possible that this cytokine is the factor in EPF responsible for inducing the release of VEGF by neutrophils because there is a high level of TNF-\( \alpha \) in the EPF. As shown in Figure 5B, the TNF-\( \alpha \)-neutralizing antibody marginally inhibited the release of VEGF by the EPF mixture-treated neutrophils. A significantly lower level of VEGF was observed as a result of the combined treatment with

\[ \beta \-actin \]
Effects of EPF on the release of VEGF

The neutralizing antibody of the TNF-α and IL-8 inhibited the release of VEGF more than the treatment with each antibody (598 ± 32 versus 380 ± 25 pg/ml, Mann–Whitney U-test; *P < 0.05). However, treating the neutrophils with both antibodies simultaneously did not completely inhibit the release of VEGF. As a control, these two neutralizing antibodies significantly blocked the IL-8- and TNF-α-induced release of VEGF by the neutrophils (data not shown). This suggests that factors other than IL-8 and TNF-α in the EPF may also be responsible for the release of VEGF by neutrophils.

Effects of IL-10 on EPF-induced release of VEGF by neutrophils

A comparison was made between the IL-10 and VEGF levels detected in the EPF. As shown in Figure 6A, the IL-10 level in the EPF from the 18 patients correlated with the VEGF level (correlation coefficient = 0.549, *P = 0.012). Therefore, this study examined whether the neutralizing antibody of IL-10 could either down-regulate or up-regulate the release of VEGF by neutrophils and monocytes. The IL-10-neutralizing antibody alone had little or no effect on the release of VEGF by the EPF-treated cells (Figure 6B).

Effect of neutrophil survival on the VEGF release

This study examined whether neutrophil survival could affect the release of VEGF by neutrophils. This is because the number of neutrophils in the culture condition decreased as a result of apoptosis. However, aged cells might affect the release of cytokines, and the EPF reduces the rate of apoptosis.
of human neutrophils (Kwak et al., 2002). As shown in Figure 7B, GM-CSF and LPS inhibited the apoptosis of neutrophils. In contrast, the anti-Fas antibody enhanced the apoptosis of neutrophils. On the contrary, the release of VEGF by neutrophils was enhanced by LPS but not by GM-CSF (Figure 7A). In addition, Fas-stimulation had no effect on the release of VEGF. This suggests that the survival rate of these cells has no influence on the release of VEGF by neutrophils.

**Discussion**

The establishment of a new blood supply is essential for the survival of an endometrial implant and the development of endometriosis. Recent studies have shown that the level of several angiogenic factors, including VEGF, hepatocyte growth factor and IL-8, was higher in the EPF than in the CPF (Lebovic et al., 2001; Taylor et al., 2002), and the concentration of the anti-angiogenic factor, interferon-γ-induced protein-10 (IP-10), was lower in the EPF than in the CPF (Yoshino et al., 2003). This suggests that the peritoneal environment of these women is favourable to the enhancement of angiogenesis. However, there has been no detailed study of the changes in the factors that regulate the release of VEGF in endometriosis, even though McLaren et al. (1996a,b) reported that VEGF expression in the tissue macrophages, which are distributed throughout the stroma, and macrophages in the PF are the principal sources of VEGF. Estradiol has been reported to promote the production of VEGF by the PF macrophages (McLaren et al., 1996a). Therefore, the level of VEGF secretion by neutrophils in the absence of FBS and phenol red-free media was also measured to exclude the effect of any basal estrogen in the culture. EPF in the serum-free media was found to have a similar effect on VEGF production by neutrophils as EPF in the serum-containing media, and 17β-estradiol alone failed to increase the amount of VEGF released by the neutrophils (data not shown). Neutrophils can synthesize and release VEGF rather than release a pre-synthesized intracellular pool (Gaudry et al., 1997). This study demonstrated that the EPF increased both the VEGF mRNA expression level and the release of VEGF by neutrophils, suggesting that certain protein factor(s) in the EPF induces the release of VEGF by neutrophils during the process of endometriosis and that neutrophils may also be a source of the angiogenic growth factor in endometriosis. This may be related to other reports showing that growth factors secreted from the peritoneal granulocytes are involved in the pathogenesis of endometriosis (Garcia-Velasco and Arici, 1999). However, the specific mechanism by which this angiogenic factor is produced by the EPF-treated neutrophils will require further investigation.

This study found a discrepancy between the amounts of VEGF released by the EPF-treated neutrophils and monocytes. A false-positive result from real-time PCR and ELISA using purified neutrophils might have occurred as a result of mononuclear contamination. However, monocytes contain negligible amounts of VEGF (Kusumanto et al., 2003, Figure 3A). Moreover, this factor was neither expressed nor released by the EPF-treated monocytes. IL-10 was observed to down-regulate the secretion of VEGF by monocytes (Bottomley et al., 1999), and a high level of this cytokine has been detected in the EPF (Ho et al., 1997 and Figure 6A). However, neutralizing antibody of IL-10 could not up-regulate the release of VEGF in monocytes. It was shown that LPS stimulates the release of

![Figure 7](image-url)

**Figure 7.** Effect of apoptosis on the release of vascular endothelial growth factor (VEGF) by cultured neutrophils. (A) The neutrophils were cultured with or without granulocyte–macrophage colony-stimulating factor (GM-CSF) (10 ng/ml), lipopolysaccharide (LPS) (1 μg/ml) or the anti-Fas antibody (1 μg/ml) for 24 h. The VEGF concentrations in the supernatants were measured using an ELISA kit. (B) Neutrophils (2 × 10^6 cells) from healthy women were cultured for 24 h in the presence of the compounds as shown in panel A. The apoptotic cells showing condensed and fragmented nuclei as the solid arrows indicate were identified by their morphology using optical microscopy (upper panel) and were counted as described in ‘Materials and methods’. The data are shown as the percentage of cells showing the morphologic features of apoptosis (lower panel). The results are shown as mean ± SE of three independent experiments. *Mann–Whitney U-test; P < 0.01 versus media.
VEGF as a result of a contact co-culture of the monocytes with endothelial cells in the presence of a lipoprotein, but not in the absence of a contact co-culture (Pakala et al., 2002). Therefore, normal monocytes/macrophages might be sensitized or primed by a certain factor in the PF and then be able to secrete VEGF by other growth factors.

VEGF exists as one of four different molecular species, 121, 165, 189 and 206, which are produced by alternative exon splicing of a single VEGF gene. Real-time PCR (Webb et al., 1998) and Northern blotting (Scapini et al., 1999) showed that neutrophils express the mRNA species encoding the two most common VEGF splice variants, VEGF121 and VEGF165. The regulation of VEGF expression has been shown to occur in other cells via both a transcriptional and a post-transcriptional mechanism (Cohen et al., 1996; Levy et al., 1997). VEGF has been shown to be synthesized, be present in specific granules and be released by neutrophils (Gaudry et al., 1997; Taichman et al., 1997; Webb et al., 1998). This study demonstrated the up-regulation of VEGF expression at the mRNA level as well as the release of the VEGF protein in neutrophils cultured with the EPF. Moreover, the time–course experiments showed that the increased release of VEGF occurred for up to 24 h. Webb et al. (1998) reported that majority of the VEGF production occurred within the first hour of stimulation with TNF-α, and only relatively small amounts of VEGF were subsequently released. However, the mechanism involved in the expression of VEGF mRNA in neutrophils is unknown.

A series of cytokines known to stimulate the release of VEGF by neutrophils were examined to determine the primarily responsible molecule(s) for the release of VEGF. VEGF expression is regulated by various cytokines (Ferrara and Davis-Smyth, 1997). Several growth factors that may have angiogenic activity such as IL-8 and TNF-α have been found to be elevated in the EPF (Taylor et al., 2000). These factors also stimulate for the production and release of VEGF by neutrophils (Webb et al., 1998; Cullen et al., 2000), and a combination of VEGF and TNF-α might have an additive effect on angiogenesis. This study focused on IL-8 and TNF-α as candidate molecules in the EPF to stimulate neutrophils. This is because monocytes lack the required CXCR2 receptors, cannot respond to IL-8 and do not secrete VEGF in the presence of TNF-α. CXCL5 (ENA-78), which binds CXCR2 with a high affinity, did not induce the release of VEGF by neutrophils, whereas IL-8 has a high affinity for both CXCR1 and CXCR2 (Murphy et al., 2000). This suggests that the release of VEGF by neutrophils in the presence of IL-8 might be due to CXCR1 activation and not due to CXCR2 activation. However, neither PTX nor the neutralizing antibody of IL-8 significantly affected the EPF-induced release of VEGF by the neutrophils. Therefore, it is possible that this cytokine may not be the major VEGF-releasing factor in the EPF. The TNF-α-treated neutrophils showed considerable accumulation of VEGF (Scapini et al., 1999), but TNF-α failed to stimulate the production of VEGF by monocytes (Pakala et al., 2002). Webb et al. (1998) reported that the TNF-α-induced secretion of VEGF is independent of the de novo synthesis of VEGF and acts on a preformed pool of the VEGF molecule. In contrast, Ryuto et al. (1996) reported that the TNF-α-dependent induction of VEGF is mediated via the activation of the transcription factor in human glioma cells. Because the EPF-stimulated release of VEGF by neutrophils was partially but not completely abrogated by the combined treatment of the neutralizing antibodies of TNF-α and IL-8, it is possible that several growth factors or cytokines may act in concert to stimulate the release of VEGF by neutrophils.

The induction of VEGF release from neutrophils by PF might play a role in the inflammatory process of endometriosis. It was demonstrated that neutrophils augment the extravasation of tumour cells via the neutrophil-induced transmigration of tumour cells across the endothelial cell monolayers (Wu et al., 2000). The observation that the EPF induces the release of VEGF from the neutrophils suggests a role for neutrophil-derived VEGF in modulating the endothelial permeability and angiogenesis. Moreover, VEGF was shown to induce monocyte chemotaxis and transmigration via the endothelial monolayers (Clauss et al., 1990; Heil et al., 2000). Therefore, the chemotaxtactant properties of the neutrophil-derived VEGF might reinforce the recruitment of monocytes to the focal endometrial site in the peritoneum following the initial activation of neutrophils.

VEGF provides an attractive target for therapeutic intervention in endometriosis. Monocyte/macrophages, polymorphonuclear leucocytes and natural killer (NK) cells are found in the peritoneal cavity of normal subjects as well as in patients suffering from various disorders (Melichar and Freedman, 2002). However, it has been suggested that peritoneal monocytes and NK cells play an important role in endometriosis (Lebovic et al., 2001). It has been reported that various blood or ovarian factors have an influence on deep endometriosis and cystic ovarian endometriosis, whereas the PF concentrations have an influence on endometrial and superficially implanted cells (Konincx et al., 1998). This means that a superficial endometrial implant might be influenced by the PF factor(s).

Because neutrophils are conspicuous in the peripheral blood and stimulated neutrophils release a significant amount of VEGF, the neutrophil-derived VEGF might be sufficient to account for the VEGF levels in the PF or in local lesions of endometriotic implants, even though there is a lower number of infiltrating neutrophils in the PF than mononuclear cells. It is possible that increased vascular permeability contributes to endometrial angiogenesis, and the increased level of the PF components might occur through the release of VEGF-containing granules to the vessels by activated endometrial neutrophils (Williams and Solomkin, 1999), or through the action of the adherent junction proteins in neutrophils (Tinsley et al., 1999).

Recently, Shimoya et al. (2000) reported that the secretary leucocyte protease inhibtor concentration was higher in the PF of endometriosis patients than in the control women, which has an anti-inflammatory effect on the EPF through the inhibition of elastase-induced cytokine production via the peritoneal macrophages as a result of the consequent prevention of neutrophil accumulation in the PF. Marginating and adherent neutrophils in the endometrium provide a source for VEGF in the endometrial vessels undergoing proliferation (Gargett et al., 2001; Heryanto et al., 2004), and neutrophils have been shown to be a source of VEGF in the PF. Therefore, blocking the
activation of neutrophils and modulating the functions of macrophages will be an attractive target for an anti-angiogenic strategy.

Acknowledgements

This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GN13-0002).

References


Effects of EPF on the release of VEGF


Submitted on May 4, 2005; resubmitted on October 7, 2005, December 15, 2005, February 1, 2006; accepted on February 21, 2006