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Vascular endothelial growth factor and platelet-derived endothelial cell growth factor expression are implicated in the angiogenesis of endometrial cancer

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Abstract

Although many angiogenic factors have been described, it is not well defined which factors are expressed in endometrial cancer. The object of this study was to examine mRNA levels of the two angiogenic factors, vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) in endometrial cancer tissues and their association with clinicopathological features including microvessel density. The level of VEGF and PD-ECGF mRNAs was assessed by semi-quantitative reverse transcription-polymerase chain reaction using β-actin as an internal standard in 38 patients with endometrial cancer. Microvessel counts were also assessed by immunostaining for factor VIII-related antigen in the most vascularised area of the specimen. VEGF/β-actin ratios of non-endometrioid tumours were significantly higher than those of endometrioid tumours ($P=0.013$). VEGF/β-actin ratios of cases with lymph–vascular space involvement were significantly higher than those of cases without lymph–vascular space involvement ($P=0.021$). Although it was not statistically significant, PD-ECGF/β-actin ratios in grade 3 tumours were higher than those in grade 1 and 2 tumours ($P=0.066$). The microvessel density was significantly correlated with the level of VEGF and PD-ECGF mRNA expression ($P=0.041$ and $P<0.0001$, respectively). Our findings provide evidence that the expression of both VEGF and PD-ECGF is involved in the promotion of angiogenesis in endometrial cancer. In addition, VEGF and PD-ECGF might contribute to the aggressive potential of high grade tumours or certain histological subtypes with unfavourable prognosis through the induction of angiogenesis.

Keywords: Vascular endothelial growth factor; Platelet-derived endothelial cell growth factor; Angiogenesis; Endometrial cancer; RT-PCR

1. Introduction

Endometrial cancer is the most common disease among gynaecological malignancies. The survival of patients is dependent on several factors, such as the presence of extra-uterine disease, histological grade, depth of myometrial invasion, lymph–vascular space involvement and cervical invasion [1]. It is well known that angiogenesis, the development of new blood vessels, is essential in tissue development, reproduction and wound healing [2]. It is also well recognised that solid tumours require angiogenesis in the process of progression and metastasis. In fact, tumour growth beyond 1–2 mm is strictly dependent on angiogenesis [3]. Angiogenesis also contributes to the metastatic process, carrying cancer cells into the circulation [4]. Recent studies have shown that quantification of angiogenesis measured by microvessel counts can be used as a prognostic factor for endometrial cancer [5–8].

Tumour tissues are thought to secrete angiogenic factors that activate neovascularisation around tumours [2]. Although many angiogenic factors have been described, it is not defined which factors play a major role in the angiogenesis of endometrial cancer. Their relationship to clinicopathological factors or microvessel densities have scarcely been examined. Vascular endothelial growth factor (VEGF) was originally detected in the conditioned medium of bovine pituitary folliculostellate cells. Vascular permeability factor (VPF) was first identified in tumour ascites and was subsequently found to be identical to VEGF when it was isolated and purified from pituitary glands [9,10]. VEGF is detectable in a number of tumour cell lines and tumour tissues and is thought to be a selective growth factor for endothelial cells [11]. Four molecular

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isoforms of VEGF mRNAs are generated by alternative splicing, composed of 206-, 189-, 165- and 121-amino acid residues [12]. The two shorter isoforms, VEGF165 and VEGF121, are secreted proteins which may act as diffusible agents, whereas the longer isoforms remain cell-associated [13, 14]. In contrast, PD-ECGF was first isolated from platelets. The transfection of the PD-ECGF gene into ras-transformed NIH 3T3 cells results in a dramatic increase of angiogenicity in a nude mouse model [15]. Recently, it was reported that PD-ECGF is identical to dThdPase, an enzyme involved in nucleic acid metabolism [16, 17]. PD-ECGF stimulates chemotaxis of endothelial cells *in vitro* and exhibits angiogenic activity *in vivo* [18]. In accordance with this, it has been demonstrated that the enzymatic activity of dThdPase is essential for angiogenesis [19].

In the present study, we examined the expression of these two angiogenic factors, VEGF and PD-ECGF mRNAs in 38 endometrial cancer tissues by semi-quantitative RT-PCR and addressed the association with clinicopathological features including microvessel density.

2. Patients and methods

2.1. Tissue samples

Our patient population consisted of 38 consecutive patients with a diagnosis of endometrial cancer at the Department of Obstetrics and Gynaecology of Okayama University Medical School, Okayama, Japan who underwent surgery during the period from 1996 to 1997. All specimens were obtained at the time of surgery. Each specimen was divided into two equal parts. One portion was snap frozen and stored at −80°C until required for RNA extraction, and the other was fixed in 10% formaldehyde solution for histopathological examination. Specimens which did not contain enough cancer cells (>70%) by histopathological diagnosis were excluded from the study. Surgical staging and pathological grade were reviewed based on the International Federation of Gynaecology and Obstetrics criteria [20].

2.2. RNA preparation of samples

Total RNA was prepared from each specimen with RNeasy Total RNA kit (Qiagen, CA, USA) according to the manufacturer's protocol. Tissues with RNA displaying high quality 18S and 28S bands on ethidium bromide-stained gels were selected.

2.3. Semi-quantitative RT-PCR

RT-PCR was carried out according to the RNA PCR Kit (Takara, Kyoto, Japan) protocol for reverse transcription of 1 μg total RNA with subsequent amplification of cDNA. Transcribed products were subjected to PCR for VEGF (sense primer: 5′-CGAAGTGGTG-AAGTTCATGGTG-3′, antisense primer: 5′-TTCTG-TATCAGTCTTTCCTGGTGAG-3′) [13], PD-ECGF (sense primer: 5′-CTAAGCACCGACCTCAAGTTG-3′, antisense primer: 5′-GAGAGCCCTCTGCCTCATA-GT-3′) [21] or β-actin (sense primer: 5′-CTCACCATG-GATGATGATAT-3′, antisense primer: 5′-TGGGTC-ATCTTCTCGCGGTT-3′) [22].

The details of PCR reaction mixtures and condition have been described elsewhere [23, 24]. Final PCR products were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. Ultraviolet (UV)-illuminated gels were photographed using Polaroid Type 667 films. Photographs were quantitated with an image scanner GT-9500 (EPSON, Suwa, Japan), and analysed with Basic Quantifier software (Bio Image, Ann Arbor, MI, USA). The intensity of β-actin amplification was used as an internal standard.

In order to verify semi-quantitative PCR of the target mRNA, the number of amplification cycles was altered and band intensities from the final products were examined in representative cases. The absolute linear range was obtained at 26 cycles for VEGF, 28 cycles for PD-ECGF and at 23 cycles for β-actin. Then, the relative ratio of PD-ECGF/β-actin PCR products (P/A ratio) and the relative ratio of VEGF/β-actin PCR products (V/A ratio) were calculated with these cycle numbers.

2.4. Immunohistochemical staining for microvessels

Expression of factor VIII-related antigen was assessed in 38 formalin-fixed, paraffin-embedded sections with the ABC procedure as previously described [25]. Briefly, anti-factor VIII-related monoclonal antibody (Dakopatts, Copenhagen, Denmark) was used as a primary antibody. The entire tumorous lesion was scanned under low-power magnification to select areas with the most intense vascularisation. The number of microvessels was recorded by counting any positively stained endothelial cells or endothelial cell clusters as a single, countable microvessel in a 100× microscopic field (0.618 mm^2), selecting at least the ten most neovascularised areas. Vessel counts per field were converted to vessels/mm^2. The mean of the top three counts was used as the microvessel density for each case. The number of microvessels was determined by an investigator who had no knowledge of the level of VEGF and PD-ECGF mRNAs.

2.5. Statistical analyses

Relationships between levels of VEGF and PD-ECGF mRNAs and clinicopathological features

including microvessel density were evaluated by a Mann–Whitney *U*-test. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1. Expression of VEGF and PD-ECGF mRNAs and clinicopathological features

Although VEGF206 transcripts were not amplified, VEGF189, 165 and 121 were routinely detected in this series of endometrial cancers. The V/A ratios calculated from total intensities of VEGF and β-actin products ranged from 0.1 to 3.3 with a median value of 0.9 and a mean of 1.1 ± 0.8. Fig. 1 shows representative photographs of the final RT-PCR products for VEGF and β-actin and the corresponding values of the V/A ratios. The P/A ratios calculated from intensities of PD-ECGF and β-actin products ranged from 0.1 to 4.7 with a median value of 1.2 and a mean of 1.5 ± 1.3. Fig. 2 shows representative photographs of the final RT-PCR products for PD-ECGF and β-actin and the corresponding values of the P/A ratios.

V/A ratios of non-endometrioid tumours were significantly higher than that of endometrioid tumours ($P = 0.013$) (Table 1). V/A ratios in cases with lymph–vascular space involvement were significantly higher than those in cases without lymph–vascular space involvement ($P = 0.021$) (Table 1). There was no apparent correlation between patterns of VEGF isoforms and clinicopathological factors. Although it was not statistically significant, P/A ratios in grade 3 tumours were higher than those in grade 1 and 2 tumours ($P = 0.066$) (Table 2).

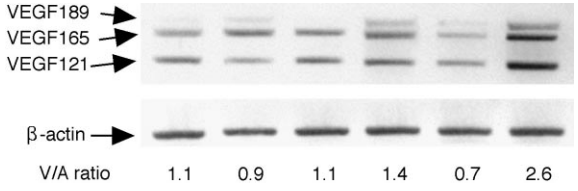

Fig. 1. RT-PCR analysis for determination of the level of VEGF mRNA. V/A, VEGF/β-actin ratio.

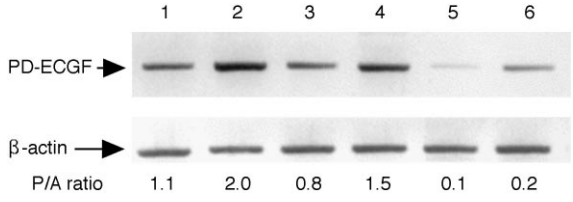

Fig. 2. RT-PCR analysis for determination of the level of PD-ECGF mRNA. P/A, PD-ECGF/β-actin ratio.

There was a weak correlation between V/A ratios and P/A ratios ($r = 0.40$, $P < 0.05$). The observation period was too short for the survival analysis in this study.

3.2. VEGF and PD-ECGF mRNA expression and microvessel density

Microvessel counts varied from 23.0 to 154.5 counts/mm^2 with a median value of 54.9 and an average of 66.0 ± 29.8. Microvessel density was significantly higher in grade 3 tumours in this series of endometrial cancers (data not shown). There was no correlation between microvessel densities and other clinicopathological factors. The patients were stratified into two subgroups with high (≥ 0.9) or low (< 0.9) V/A ratios and with high (≥ 1.2) or low (< 1.2) P/A ratios on the basis of the median value for each. The microvessel densities in patients with high V/A ratios were higher than those in patients with low V/A ratios ($P = 0.041$) (Table 3). There was a strong association between high P/A ratios and increasing microvessel density ($P < 0.0001$) (Table 3).

Table 1
Association between VEGF/β-actin ratio and clinicopathological factors

Variables	No.	Mean ± S.D.	*P* value[a]
Stage			NS
I and II	20	1.0 ± 0.8	
III and IV	18	1.2 ± 0.8	
Menopausal status			NS
Pre	10	1.0 ± 0.6	
Post	28	1.1 ± 0.9	
Myometrial invasion			NS
Inner 1/2	21	1.1 ± 0.9	
Deep 1/2	17	1.1 ± 0.7	
Cervical involvement			NS
Negative	25	1.0 ± 0.8	
Positive	13	1.2 ± 0.9	
Peritoneal cytology			NS
Negative	27	1.0 ± 0.8	
Positive	11	1.1 ± 1.0	
Histological cell type			$P = 0.013$
Endo	34	1.0 ± 0.7	
Non-Endo	4	2.1 ± 0.9	
Histological grade			NS
G1 + G2	32	1.1 ± 0.9	
G3	6	1.3 ± 0.6	
LVS involvement			$P = 0.021$
Negative	27	0.9 ± 0.8	
Positive	11	1.5 ± 0.8	
Extra-uterine disease			NS
Negative	27	1.0 ± 0.7	
Positive	11	1.3 ± 1.0	

Endo, endometrioid; NS, not significant; LVS, lymph–vascular space; S.D., starndard deviation.

[a] Mann–Whitney *U* test.

Table 2
Association between PD-ECGF/β-actin ratio and clinicopathological factors

Variables	No.	Mean ± S.D.	*P* value[a]
Stage			NS
I and II	20	1.5 ± 1.3	
III and IV	18	1.5 ± 1.4	
Menopausal status			NS
Pre	10	1.1 ± 1.1	
Post	28	1.6 ± 1.3	
Myometrial invasion			NS
Inner 1/2	21	1.5 ± 1.5	
Deep 1/2	17	1.5 ± 1.1	
Cervical involvement			NS
Negative	25	1.4 ± 1.3	
Positive	13	1.6 ± 1.4	
Peritoneal cytology			NS
Negative	27	1.5 ± 1.2	
Positive	11	1.5 ± 1.6	
Histological cell type			NS
Endo	34	1.4 ± 1.3	
Non-Endo	4	2.2 ± 0.6	
Histological grade			NS
G1 + G2	32	1.3 ± 1.2	
G3	6	2.5 ± 1.5	
LVS involvement			NS
Negative	27	1.3 ± 1.2	
Positive	11	1.9 ± 1.4	
Extra-uterine disease			NS
Negative	27	1.6 ± 1.3	
Positive	11	1.3 ± 1.2	

Endo, endometrioid; NS, not significant; LVS, lymph–vascular space; S.D., standard deviation.
[a] Mann–Whitney *U* test.

4. Discussion

Several angiogenic factors have been described, such as acidic and basic fibroblast growth factor, transforming growth factor-α, transforming growth factor-β, vascular endothelial growth factor (VEGF), placenta growth factor, interleukin-8, tumour necrosis factor-α and platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) [26–30]. In endometrial cancers, however, the association between the expression of these angiogenic factors and clinicopathological factors including microvessel densities has not been established. It is important to determine which angiogenic factor predominantly mediates angiogenesis as this could offer novel opportunities for therapeutic intervention of this disease. VEGF and PD-ECGF were detected at both the mRNA and protein levels in the human endometrium throughout the menstrual cycle [31, 32]. In this study, we explored whether the levels of VEGF and PD-ECGF mRNAs correlate with vascularisation and clinicopathological features in endometrial cancers. Analysis of mRNA levels in tumours by semi-quantitative RT-PCR requires cautious interpretation, because solid tumours are composed not only of cancerous cells but also of stromal cells.

Table 3
Association between microvessel count and VEGF/PD-ECGF mRNA expression

VEGF/PD-ECGF mRNA expression	No.	Mean ± S.D. (/mm^2)	*P* value[a]
VEGF ratios			
Low (< 0.9)	19	56.1 ± 25.4	$P = 0.041$
High (≥ 0.9)	19	75.6 ± 30.7	
PD-ECGF ratios			
Low (< 1.2)	19	47.4 ± 16.3	$P < 0.0001$
High (≥ 1.2)	19	83.7 ± 28.6	

S.D., standard deviation.
[a] Mann–Whitney *U* test.

Guidi and colleagues reported that no differences were observed in the level of VEGF mRNA expression by *in situ* hybridisation between high- and low-grade tumours, neither in tumours in the early or advanced stages of the disease [33]. However, the number of high-grade tumours and the number of patients with high-stage disease were relatively small. The present study showed that VEGF mRNA levels evaluated by semi-quantitative RT-PCR were significantly high in non-endometrioid subtypes. It is well known that certain histological types of endometrial cancer provide important information about the malignant potential of the neoplasm. Non-endometrioid histological subtypes account for approximately 10% of endometrial cancers and carry an increased risk of recurrence and distant metastasis [34, 35]. We presume that the elevated levels of VEGF mRNA in non-endometrioid tumours contribute to the aggressive potentials of these tumours. However, since there were only 4 cases with non-endometrioid tumour in this study further investigation is needed to clarify this issue. This study also showed that VEGF mRNA levels were significantly higher in cases with lymph–vascular space invasion.

Fujimoto and colleagues reported that the levels of PD-ECGF were significantly higher in endometrial cancers of well-differentiated adenocarcinoma with invasion to < 1/2 myometrium [36]. In contrast, our study showed that PD-ECGF mRNA levels were high in high-grade tumours. In fact, our immunohistochemical study demonstrated that the expression of PD-ECGF was predominantly observed in the stromal cells of the tumour and the immunoreactivity was significantly stronger in the high-grade tumours (unpublished data). Since histological grade is strongly associated with prognosis, the elevated PD-ECGF mRNA in high-grade tumours might contribute to the aggressive potential of these tumours. It is also noteworthy that expression of VEGF or PD-ECGF was not increased in cases with deep myometrial invasion. It

appears that increased levels of VEGF or PD-ECGF mRNA are not necessarily required for deep myometrial invasion. It is postulated, therefore, that other angiogenic factors may be upregulated for further myometrial invasion in endometrial cancer.

We also examined whether the level of VEGF and PD-ECGF mRNAs correlate with vascularisation in endometrial cancer. The neovascular hotspots appeared around the tumour margins in most cases. Our study demonstrated that the expression of the PD-ECGF gene is closely correlated with tumour vascularity. This is in accordance with the data of Fujiwaki and colleagues. They reported that PD-ECGF expression determined by immunohistochemistry was associated with an increased density of microvessels in endometrial cancer [37]. We also showed a significant difference in microvessel density between the two VEGF levels, although this difference was less than that for PD-ECGF. Recently, four new molecules have been added to the VEGF family; placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D [36]. Nicosis suggested that distinct repertoires of VEGF and VEGF-related molecules may stimulate angiogenesis in different tumours [38]. Further examination is necessary to clarify the possible role of VEGF-related molecules in the angiogenesis of endometrial cancer. It will be also interesting to investigate the relationship between mRNA levels for VEGF or PD-ECGF and the survival of patients. Unfortunately, the observation period was so short that survival of the patients could not be analysed in this study.

Anti-VEGF monoclonal antibody can inhibit both primary and metastatic growth of tumours without any side-effects in nude mice [39]. Thus, VEGF could be an important target of antitumour agents in endometrial cancer. 5-fluorouracil (5-FU) is routinely used for the treatment of a variety of tumours. 5′-deoxy-5-fluorouridine (5′-DFUR) and capecitabine are prodrugs of 5-FU and are converted to 5-FU by PD-ECGF which is preferentially expressed in tumour tissue [40, 41] compared with normal tissue. Thus, 5′-DFUR and capecitabine may be effective with less toxicity in patients with PD-ECGF-positive endometrial cancer.

In conclusion, our findings provide evidence that the expression of VEGF and PD-ECGF is involved in the promotion of angiogenesis in endometrial cancer. This study also suggests that VEGF and PD-ECGF, which are abundantly expressed in high grade tumours or certain histological subtypes with unfavourable prognosis, may promote tumour progression through the induction of angiogenesis.

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