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Research Article

The Expression of Osteopontin and Integrin αvβ3 in Endometriosis and their Effects on Angiogenesis

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Abstract

Endometriosis is a common disease of women. In the present study, the expression of osteopontin (OPN), integrin αvβ3 and vascular endothelial growth factor (VEGF) in tissue of endometriosis were studied in order to investigate their functions and the clinical value. The result of the present study revealed that OPN was expressed in the gland epithelium cells, and the expression of OPN in ectopic endometrium and eutopic endometrium of endometriosis were higher than that of eutopic endometrium of myoma. Ectopic endometrium could not change from proliferative to secretory stage; integrin αvβ3 was expressed in the cytoplasm of ectopic endometrium cells and membrane. The expression of integrin αvβ3 in endometriosis lesion was higher than that of eutopic endometrium and eutopic endometrium of myoma. VEGF was expressed in the cytoplasm of ectopic endometrium cells where brown granules were found. The expression of VEGF in endometriosis lesion was higher than that of eutopic endometrium and eutopic endometrium of myoma. The expression of OPN and integrin αvβ3 in ectopic endometrium showed a good linear relationship and the correlation coefficient was 0.7798. There was a significantly positive correlation between OPN, integrin αvβ3 and VEGF, and the correlation coefficients were 0.596 and 0.568, respectively, but there was no relationship among OPN, integrin αvβ3 and VEGF in eutopic endometrium of myoma. So we conclude that OPN, integrin αvβ3 and VEGF may correlate with adherence and aggression in endometriosis. It provides evidence for the cooperative roles of OPN, integrin αvβ3 and VEGF in angiogenesis, which can promote the evolvement of endometriosis.

Keywords: Osteopontin, Integrin αvβ3, Vascular endothelial growth factor, Endometriosis, Immunohistochemical assay

Introduction

Endometriosis is a common disease of women, while only 2% to 5% of the general populations are thought to have endometriosis; the prevalence in an infertile population may approach 40%. Endometriosis is associated with a decrease in cycle fecundity, the prevalence of endometriosis is increased in infertile women. The canceration rate is about 0.7-1.0%. Endometriosis has the characteristics of wide distribution and recurrence, which was similar to malignant tumor. One of the Arg-Gly-Asp (*RGD)*-containing ligands for αvβ3 is the glycoprotein, osteopontin (OPN). OPN, as a phosphorylated acidic glycoprotein, is defined to be both a multifunctional cytokine and an adhesion protein secreted by a variety of cells [1]. OPN is recognized to participate in a wide range of physiologic and pathologic processes, consisting of cell-mediated immunity, tissue repair, cellular migration, and remodeling [2,3]. OPN is highly expressed in a variety of malignant tumor tissue, and it is closely related with tumor invasion and metastasis [4]. Integrins are a ubiquitous class of cell membrane bound proteins critical for cell-cell and cell-substratum adhesion. They comprise two components, an alpha subunit and a beta subunit. Over 22 different integrins have been identified and they are present in all human cells except for erythrocytes [5-7]. They have been implicated in many aspects of reproduction, including fertilization and implantation [8,9]. Endometrial integrins are expressed in both epithelium and stroma. While most integrins are constitutively expressed in the endometrium, they have been identified as several cycle-dependent integrins, including the α1, α4, β3, and β5 integrin subunits [10]. These patterns have been confirmed by various techniques, including flow cytometry and PCR evaluation [11,12], and recently by *in situ* hybridization [13]. The reproducibility of integrin expression in the endometrium allows a complementary approach to histologic dating for the evaluation of uterine receptivity. Most interest has been focused on the integrin αvβ3 since this integrin appears in endometrial glands and luminal surfaces [7], and integrin αvβ3 is closely related with invasive growth and

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distant metastasis of cancer. Angiogenesis is considered as a major process in the pathogenesis of endometriosis. Many factors are involved in this complex mechanism, and the vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis; it is a potent endothelial cell mitogen, morphogen, and vascular permeability-inducing agent [14,15]. A large number of studies have observed that VEGF was significantly higher in women with endometriosis, which supported a key role for VEGF in the pathological angiogenesis in endometriosis [15,16].

In the present study, to investigate whether OPN and Integrin αvβ3 was involved in the pathogenesis of endometriosis, the expression of OPN, Integrin αvβ3 and VEGF were examined in eutopic endometrium of women with or without endometriosis, and the relationship and mechanism were also discussed.

Methods

Tissue collection

All the tissue of the study group was obtained from 112 patients (mean age 36.48 ± 6.93 years, range 20-46 years) with endometriosis undergoing laparoscopy or laparotomy. All the patients were surgically and histologically diagnosed with endometriosis at stages II to IV according to the revised American Society for Reproductive Medicine Classification (1985). They included 112 cases of ectopic endometriums and 40 cases of eutopic endometriums. In the control group, endometrial tissues were collected from 21 patients (mean age 38.35 ± 7.58 years, range 28-49 years) undergoing laparoscopy or laparotomy and histologically diagnosed with benign uterine myoma. Consent forms were signed by all of the participants. No patients in the present study received any hormonal preparations within 6 months before surgery.

Immunohistochemical assay

These tissues were fixed in l0% neutral buffered formalin and embedded into paraffin blocks. Tissue blocks were sectioned at 4 μm and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides. The following procedures were performed according to Elivision TM plus two-step method. Immunoreaction was visualized with peroxidase-3, 3'-diaminobenzidine (DAB). Antigen retrieval was performed by microwave, and endogenous peroxide activity was quenched with Peroxidase Block for 5 minutes. Sections were then incubated with monoclonal rabbit (EPR3688) anti-human OPN antibody (dilution 1:100, Abcam, USA), rabbit anti-human *VEGF* monoclonal antibody (dilution 1:200, Abcam, USA), and rabbit anti-integrin αvβ3 antibody (dilution 1:100, BD Biosciences, NJ, USA) for 30 minutes at room temperature. Peroxidase labelled polymer was incubated for 30 minutes at room temperature, and then treated with substrate-chromogen for 3 minutes. Sections were counterstained with harris hematoxylin. All stainings were performed with the same procedure but with the phosphate buffer saline (PBS) as the negative control.

The positive criteria: brown-yellow staining could be observed around the cell membrane/endochylema under high-power lens. The negative criteria: no brown-yellow staining could be observed around the cell membrane/endochylema under high-power lens. The positive results were defined as the following: "+" weakly stained cells were observed in more than 10% of the tumor cells; "++": moderately stained cells were observed in more than 30% of the tumor cells; and "+++": strongly stained cells were observed in more than 60% of the tumor cells. Immunostaining was evaluated by two independent pathologists through blind manner to identify the results.

Statistical analysis

SPSS 12.0 software was used for statistical processing. Data was expressed as means ± SD or percentages. Measurement data was analyzed using t-test, and count data was analyzed using the chi-square test. Difference of ranked data was compared by means of Radit analysis. The correlation of ranked data was performed with Spearman correlation analysis. A value of $p < 0.05$ was considered significant.

Results

The localization and expression of OPN and integrin αvβ3 in the tissue of endometriosis

As shown in Table 1, the stronger expression of OPN in ectopic endometrium and eutopic endometrium of endometriosis were higher than that of control endometrium (P=0.005). The stronger expression of integrin αvβ3 in ectopic endometrium was higher than that of eutopic endometrium and control endometrium (P=0.005). OPN was expressed in the gland epithelium cells, Integrin αvβ3 was expressed in the cytoplasm of ectopic endometrium cells and membrane (Figures 1-4).

The localization and expression of VEGF in the tissue of endometriosis

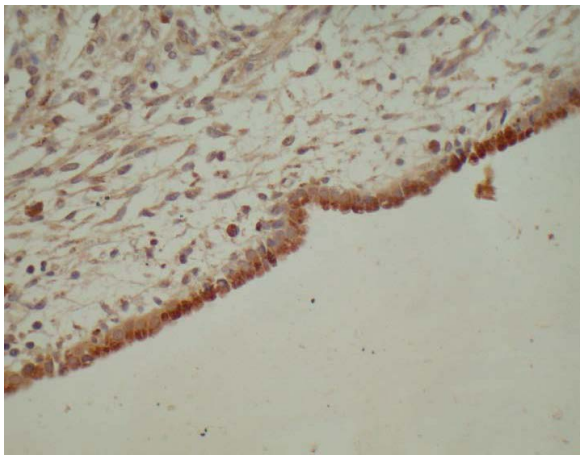
As shown in Table 2, the expression of VEGF in ectopic

Groups	The expression of OPN				The expression of integrin αvβ3			
	-	+	++	+++	-	+	++	+++
Ectopic endometrium	0	30	50	32	0	50	40	22
Eutopic endometrium	0	12	12	16	18	22	0	0
The control endometrium	7	10	3	1	20	1	0	0
P	*P*=0.005				*P*=0.005			

Table 1: The expression of OPN and integrin αvβ3 in the tissue of endometriosis (cases).

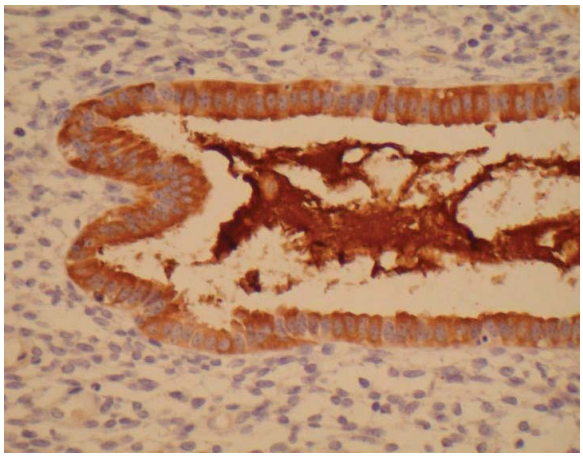
The expression of VEGF	Ectopic endometrium	Eutopic endometrium	The control endometrium	p
-	0	0	7	P=0.01
+	26	16	8	
++	56	20	5	
+++	30	4	1	

Table 2: The expression of VEGF in the tissue of endometriosis (cases).



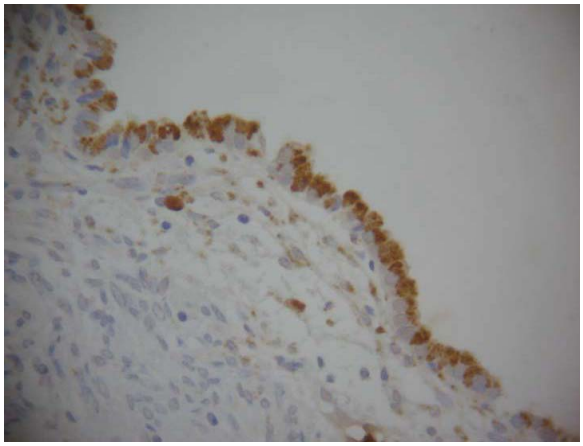
Positive expression of OPN in ectopic endometrium.

Figure 1: OPN immunohistochemistry staining result in ectopic endometrium (1*100).



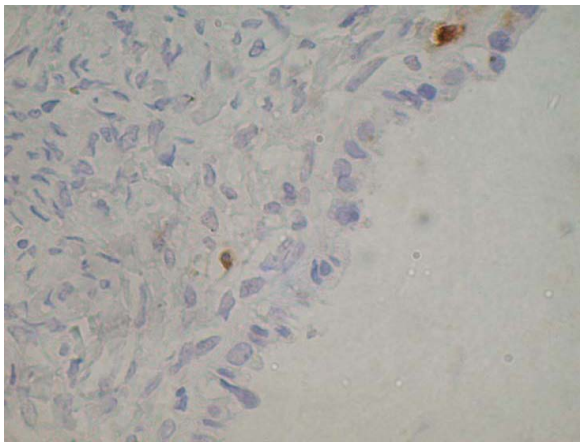
Strong positive expression of OPN in eutopic endometrium.

Figure 2. OPN immunohistochemistry staining result in eutopic endometrium (4*100).



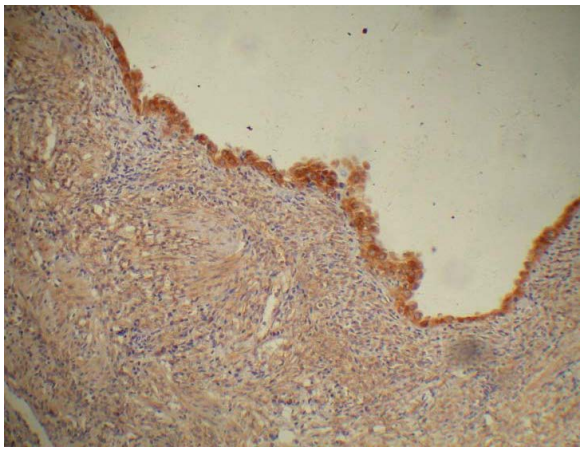
Strong positive expression of Integrin αvβ3 in ectopic endometrium.

Figure 3. Integrin αvβ3 immunohistochemistry staining result in ectopic endometrium (4*100).



Weakly positive expression of Integrin αvβ3 in control endometrium.

Figure 4. Integrin αvβ3 immunohistochemistry staining result in control endometrium (4*100).



Strong positive expression of VEGF in ectopic endometrium.

Figure 5. VEGF immunohistochemistry staining result in ectopic endometrium (1*100).

endometrium was higher than that of eutopic endometrium and control endometrium (*P*=0.01). VEGF was expressed in the cytoplasm of ectopic endometrium cells and brown granules (Figure 5).

The relationship among the expression of OPN, integrin αvβ3 and VEGF in tissue of endometriosis

There was a significantly positive correlation between the expression of OPN, integrin αvβ3 and VEGF in ectopic endometrium(r=0.596, *P*=0.000; r=0.568, *P*=0.001), and the expression of OPN and integrin αvβ3 also showed highly linear correlation (r=0.738, *P*=0.000).

Discussion

The expression and significance of OPN and integrin αvβ3 in the tissue of endometriosis

Endometriosis means the growth, infiltration and repeated bleeding of the functional endometrial tissue in a location outside of the uterine endometrium and myometrium, which can form nodules

and masses. The happening of endometriosis explains that the endometrial cells entering pelvic can survive, and they have strong capacity of planting, which needs the ability to adhere, invade and angiogenes. So it is suggested that the ectopic endometrium cells have the capacity of adhering, migrating and angiogenesis.

Integrins are adhesion receptors widely distributed on the cell surface, and they are related to cell and extracellular mesenchymal combining, cell adhering. They are a kind of transmembrane protein and comprised of α and β peptide. They are divided into extracellular domain, transmembrane domain and cytoplasmic region. Extracellular domain of α chain specifically recognizes and adheres to RGD sequence of ECM ligand, and intracellular region of β chain is connected to cytoskeletal proteins to communicate cells with ECM. Integrin αvβ3 may induce cell-extracellular matrix adhesion, stimulation of platelet aggregation, promoting tissue reparation and angiogenesis. OPN is a kind of cell secreted phosphoglycoprotein in ECM [17], containing a highly conserved specific cell adhesion function domain of RGD sequence in the structure, binding to cell surface integrins by the sequence, and the main receptor is αvβ3. OPN combined with αvβ3 release local stimuli such as hormones, and growth factors, which mediate cell adhesion to ECM to form adhesion plaque. Integrin can connect ECM with intracellular cytoskeletal proteins to affect the cell adhesion, motility and phagocytosis. In the structure of OPN, the RS site is a thrombin cleavage site, which can be cleaved into two pieces, 45 kDa and 24 kDa. The fragment of 45 kDa stimulates cell adhesion and migration easier [18]. Through PIK-4/AKT-NF-KB and C-SRC/EFR/BRK signal transduction system, OPN combined with integrin αvβ3 to enhance synthesis and secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP). Integrin αvβ3 can promote the growth, invasion and metastasis of tumors, because it can be combined with and activate MMP-2, get the activity of decomposition protein, cause the degradation of ECM, dissolve the extracellular matrix barriers, and enhance tumor cells' movement force. Siletti [19] found that a molecular TSRI265 could obviously inhibit tumor cell transference by blocking integrin αvβ3 and MMP-2.

The present study has demonstrated that integrin αvβ3 was expressed in all the cytoplasm of ectopic endometrium cells and brown granules were found. The expression of integrin αvβ3 in endometriosis lesion was higher than that of eutopic endometrium and the control group. Integrin αvβ3 in 11/20 of ectopic endometrium was expressed as weak positive, and that 9/20 was negative. Regidor [20] studied expression of integrin αvβ3 in ectopic endometrium and eutopic endometrium of 30 patients with the endomtriosis using immunohistochemical method, and he found that all the eutopic endometrium of endomtriosis did not express integrin αvβ3, while integrin αvβ3 was expressed in half of the ectopic endometrium cells. Continuously expression of integrin αvβ3 in the ectopic endometrium cells suggested that it probably participates in pelvic planting adhesion.

The significant role of OPN and integrin αvβ3 in the process of new angiogenesis of endomtriosis

The process of the endometrial invasion includes adhesion, invasion and angiogenesis, among which angiogenesis is the foundation of endometrial glands and stroma development, and the establishment and supportment of the blood supply is basic conditions for endometriosis implant survival and ectopic occurrence. Therefore, neovascularization is the key step of endomtriosis. VEGF is the cell mitogen of the vascular endothelial, and the main factor for angiogenesis, which plays an important role in the procoagulant endothelial proliferation and angiogenesis, by means of the binding to specific receptor of vascular endothelial cells [21]. VEGF can stimulate the proliferation of vascular endothelial cells, induce the migration and the formation of lumen-like structures, increase vascular permeability [22], and directly induce new blood vessel formation in tumors. It is proved that the VEGF could induce microvascular extravasation, activate thrombin and ultimately promote angiogenesis, through activating growth factors, adhesion molecules, proteolytic enzymes and receptors. While the structure of OPN contains thrombin cleavage site, OPN may be associated with integrin αvβ3 to activate proteolytic enzymes (including uPA and MMP) and induce the formation of new blood vessels through the cell signal transduction pathways. So we conclude that OPN and VEGF have common pathway to angiogenesis.

The results of this study demonstrated that expression of VEGF was positive in ectopic endometrium, in which positive ++~+++ expression was significantly higher than that in eutopic endometrium and the control endometrium. It revealed that VEGF might be involved in the formation of the endometriosis lesion, and promote new angiogenesis, which was beneficial to the implantation and growth of ectopic endometrium. The experiment also proved that the expression of OPN and integrin αvβ3 in ectopic endometrial tissue are significantly related to VEGF, the r_s were 0.596 and 0.568, respectively. OPN and integrin αvβ3 were significantly correlative, r_s=0.738, while in the control of endometrial tissue they were unrelated. The high expression and correlation between OPN, integrin αvβ3 and VEGF in ectopic endometrial tissue suggested that they both had synergistic effects in vascular formation of endometriosis. OPN was once used to transfect C1300 cells, and a significant angiogenesis had been observed without affecting proliferation and apoptosis of the cells. So OPN may be mediated by the induction of angiogenesis in tumor growth [23]. It was proved that OPN could stimulate the migration of endothelial cell, and promotes the newborn of vessels, and the action was closely correlated with VEGF [24]. The reason is that OPN can induce the migration of endothelial cell through chemotaxis, adhesion molecules such as glassy mucin (vitronectin, VN). Both OPN and glassy mucin have RGD sequences, and glassy mucin can activate the receptor of VEGF, VEGFR-2, to trigger angiogenic activity [25]. The integrin αvβ3 may enhance phosphorylation, and mitosis effects of VEGFR-2, and collaborate with VEGF to promote angiogensis. Rawlings [26] revealed that stain of VEGFR-2 showed overlap with integrin αvβ3, which suggested that there was positive synergy between integrin αvβ3 and VEGFR-2. Liu [27] also confirmed that VEGF synergized with integrin αvβ3 to promote angiogenesis via N-Ras and phosphatidylinositol 3- kinase (PI3) -K signaling.

In the process of tumor angiogenesis, the degradation of extracellular matrix pioneered gateway for endothelial cell's transference. It has been proved that the vascular endothelial cell's appearance expressed a great deal of integrin αvβ3 in the process of tumor angiogenesis which united and activated directly with MMP-2 and exerted extra cellar matrix's degradation action [28]. Extracellular matrix was very important for vascular endothelial cell's survival. Usually it only expressed few integrin αvβ3, but in the process of angiogenesis, the expression of integrin αvβ3 in vascular

endothelial increased evidently. If the combination of intergrin αvβ3 and extracellular matrix were interrupted, *apoptosis* of the vascular endothelial cells would be induced, and angiogenesis would be suppressed [29,30]. If the integrin αvβ3 combined with its ligand, it could make endothelial cell arise proliferation, differentiation and transference, and form new blood vessel by the MAPK's pathway. If the integrin αvβ3's function was restrained and couldn't combine with its ligand, the p53 gene would be activated, and p21 gene's expression would rise and induce the apoptosis of endothelial cell. The notable correlation of OPN and integrin αvβ3 also confirmed their association in the endomtriosis' nosogenesis and cooperative action in the process of new angiogenesis.

Conclusion

OPN, integrin αvβ3 and VEGF were overexpressed in ectopic endometrium, and this finding suggested that OPN, integrin αvβ3 and VEGF might be correlated with adherence and aggression in endometriosis. It provided evidence for the cooperative roles of OPN, integrin αvβ3 and VEGF in angiogenesis, which might promote the evolvement of endometriosis.

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