Expression of vascular endothelial growth factor and matrix metalloproteinase-2 in uterine leiomyoma and correlation with angiogenesis

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Özet

Uterin leyomiyomlarda vasküler endotelyal büyüme faktörü ve matriks metalloproteinaz-2 ekspresyonu ve anjiyogenezle ilişkisi

Amaç: Leyomiyom uterusun en sık görülen tümörüdür. Uterin leyomiyomun büyümesinde anjiyogenik faktörler ve proteazlar rol oynayabilir. Çalışmamızın amacı uterin leyomiyomlarda vasküler endotelyal büyüme faktörü (VEGF) ve matriks metalloproteinaz-2 (MMP-2) ekspresyonunu araştırmak ve mikrodamar yoğunluğu (MVD) ile olası ilişkilerini belirlemekti. Gereç ve yöntemler: Çalışma materyali leyomiyom nedeni ile opere edilmiş 50 hastanın leyomiyom ve normal miyometriyumunu içeren parafin bloklardan oluşuyordu. VEGF, MMP-2 ve CD34 ekspresyonu immünohistokimyasal olarak çalışıldı. VEGF ve MMP-2' ye ait immünohistokimyasal yöntemle elde edilen bulgular semikantitatif analiz ile değerlendirildi. MVD ise, CD34 pozitif vasküler endotelyal hücreleri sayarak hesaplandı. Bulgular: Leyomiyomlardaki VEGF ve MMP-2 ekspresyonu normal miyometriyuma göre anlamlı olarak daha yüksek bulundu ($p=0.001$). CD34 boyaması MVD' nun leyomiyomlarda miyometriyuma göre daha az olduğunu gösterdi ($p<0.05$). VEGF ve MMP-2 boyanması ile MVD arasında korelasyon saptanmadı. Sonuç: Bu bulgular uterin leyomiyomların patogenezinde VEGF ve MMP-2' nin rol oynayabileceğini düşündürmüştür. Bu hastalığın gelişim sürecine anjiyogenez dışındaki bazı faktörler de katkıda bulunabilir.

Anahtar kelimeler: Uterus, leyomiyom, VEGF, MMP-2, mikrodamar yoğunluğu

Abstract

Objective: Leiomyoma is the most common tumor of the uterus. Angiogenic factors and proteases may play role in the growth of uterine leiomyoma.The aim of our study was to investigate the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) in uterine leiomyoma and their possible relation to microvessel density (MVD). Material and methods: Material for the study comprised parafin-embedded tissue sections of uterine leiomyomas and corresponding myometrium derived from 50 hysterectomized women. VEGF, MMP-2 and CD34 expression was investigated immunohistochemically. Semiquantitative analysis of the immunostainings for VEGF and MMP-2 was performed. MVD was calculated by counting of CD34 positive vascular endothelial cells. Results: Expression of VEGF and MMP-2 was significantly higher in leiomyomas than corresponding myometrium ($p=0.001$). The CD34 labeling showed decreased MVD in leiomyomas compared with myometrium ($p<0.05$). There was no correlation between VEGF and MMP-2 staining and MVD. Conclusion: These data suggested that VEGF and MMP-2 may play role in the development of uterine leiomyomas. Mechanisms other than promotion of angiogenesis may contribute to this disease process.

Key words: Uterus, leiomyoma, VEGF, MMP-2, microvessel density

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Introduction

Leiomyomas are the most common uterine neoplasms (1). They are a source of pain, bleeding, and infertility (2). They are seen in 20%-30% of women at reproductive age (3).

Leiomyoma-related menorrhagia is a significant medical problem. It is suggested that there is a fundamental alteration in the vascular structures of the myomatous uterus. Although ectasia of the venules is the best characterized vascular abnormality in the myomatous uterus, multiple defects of arterioles, veins and the extracellular matrix (ECM) surrounding them are probably responsible for this heterogeneous disorder. Growth factors that stimulate angiogenesis are other candidates to cause abnormal uterine bleeding in these women (4).

Vascular endothelial growth factor (VEGF) is the most important growth factor in adult tissues undergoing physiologic angiogenesis as well as pathologic angiogenesis (5). Matrix metalloproteinases-2 (MMP-2) is a proteolytic enzyme effectively degrading collagen type IV as well as collagen types I, V, VII, X and XI, gelatine, elastic, fibronectin, laminin, entactin and proteoglycans (6-8). MMPs not only degrade extracellular matrix, but also enhance angiogenesis (9). Hence, we aimed to investigate the expression of VEGF and MMP-2 in uterine leiomyoma and their possible relation to microvessel density (MVD) to define the pathogenesis of this important disorder.

Material and Methods

Subjects

The material for our study included parafin-embedded tissue sections of uterine leiomyomas and corresponding myometrium derived from 50 hysterectomized women, who were treated at the obstetrics and gynecology clinics of our hospital. All procedures were approved by the ethical review board of our university.

All women were premenopausal with ages between 37 to 55 years (mean, 47±0.6) years. None of the women received any hormonal medication within at least three months before hysterectomy. The patients who had coexisting gynecologic diseases including adenomyosis and endometrial polyp were excluded. All endometrial samples were grouped according to the menstrual cycle phases: proliferative (days 1 to 14 of the cycle), and secretory phase (days 15 to 28 of the cycle). The day of the menstrual cycle was established from the women' s menstrual history and was confirmed by endometrial dating using the criteria of Noyes et al (10). Twenty five patients were in the proliferative phase, 25 patients were in the secretory phase.

Immunohistochemistry

The representative blocks were sectioned and mounted on poly-L-lysin-coated slides. The streptavidin-biotin-peroxidase method was performed using the primary clonal antibodies against VEGF (monoclonal, 1/50, Neomarkers, USA), MMP-2 (polyclonal, prediluted, Neomarkers, USA), and CD34 (monoclonal, 1/300, Neomarkers, USA). For positive controls, a staining of colon carcinoma tissue for VEGF, and breast carcinoma tissue for MMP-2 were used. Normal identifiable vessels within the sections provided an internal control for CD34.

Assessment of VEGF and MMP-2 staining: Cytoplasmic staining was defined positive. Semiquantitative analysis of the immunostainings for VEGF and MMP-2 was performed for each case. An estimate of the percentage of immunoreactive cells was determined using a score of 0-3 (0: 0-4% cells stained; 1: 5-29% cells stained; 2: 30-59% cells stained; 3: 60-100% cells stained). The staining intensity was scored as 0-3 (0, negative; 1, weak; 2, moderate; 3, strong). Values for the quantity and staining intensity scores were then multiplied giving results that ranged from 0 to 9. The expression levels of VEGF and MMP-2 were reported according to the following scoring criteria: grade 0 (score 0); grade 1 (scores 1 to 3); grade 2 (scores 4 to 6); grade 3 (scores 7 to 9) (11).

Assessment of vascularity: Blood vessels were highlighted by staining endothelial cells with antibody against CD34. Cytoplasmic staining was defined positive. Microvessel counts were determined by using the method of Weidner et al (12). First the area with highest vascularity was identified at low power (40X and 100X). Microvessel counts were then made on a 200X field (0.15 mm^2). Any endothelial cell or cell cluster positive for CD34 and clearly separate from an adjacent cluster was considered to be a single, countable microvessel (12). Large vessels with thick muscular walls and large vessels lumina greater than approximately eight red blood cells were excluded from the count (13). The maximum number of microvessels staining positive at 200X was graded as: 1+, <20 vessels; 2+, 20-29 vessels, 3+, ≥30 vessels.

Statistical analyses

The immunohistochemical data were reported as the mean ± standard error of mean (SEM). Statistical analysis of the data was performed using Wilcoxon and Mann-Whitney U tests. Bivariate correlation between variables was determined by Spearman' s correlation coefficients. A p value <0.05 was considered significant.

Results

The size of tumors ranged from 0.5 to 17 cm. In 19 cases tumors were solitary, in 31 cases they were multiple. The results of immunohistochemical analysis are summarized in Table 1.

Table 1. Comparisons of immunohistochemical scores.

	Myometrium	Leiomyoma	p value
Vascular endothelial growth factor	1.0± 0.08	1.5± 0.08*	0.001**
Matrix metalloproteinase-2	0.6±0.1	1.1±0.08*	0.001**
Microvessel density	2.7± 0.08	2.4 ± 0.1*	0.013**

*Values are presented as mean ±SEM
**p<0.01

MMP-2 and VEGF staining was detected in the cytoplasm of smooth muscle cells in myometrium and leiomyoma (Fig. 1, Fig. 2). VEGF staining was granular (Fig. 3, Fig.4). Immunoreactivity was also observed in vascular endothelial cells. Expression of VEGF and MMP-2 was significantly higher in leiomyomas than corresponding myometrium (p=0.001). The CD34 labeling (Fig. 5) showed decreased MVD in leiomyomas compared with myometrium (p=0.013). There was no correlation between VEGF and MMP-2 staining and MVD.

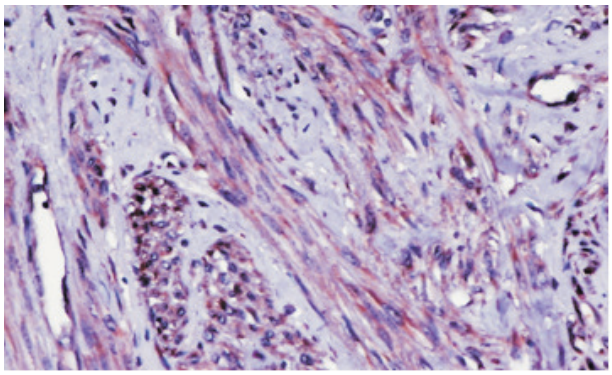

Fig. 1: Weak cytoplasmic MMP-2 staining of smooth muscle cells in leiomyoma (,x200).

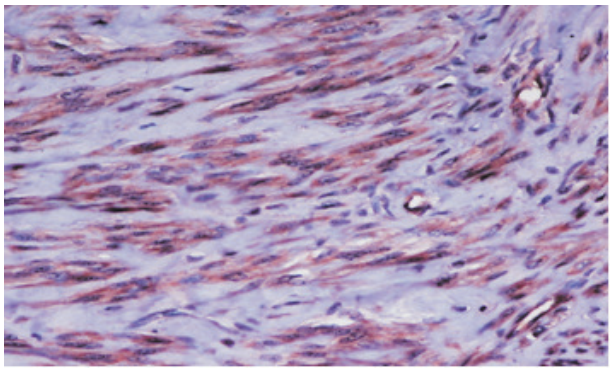

Fig. 2: Moderate cytoplasmic MMP-2 staining of smooth muscle cells in leiomyoma (,x200).

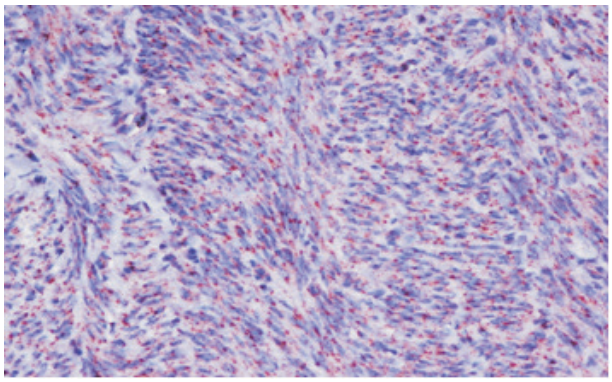

Fig. 3: Moderate cytoplasmic granular VEGF staining of smooth muscle cells in leiomyoma (,x200).

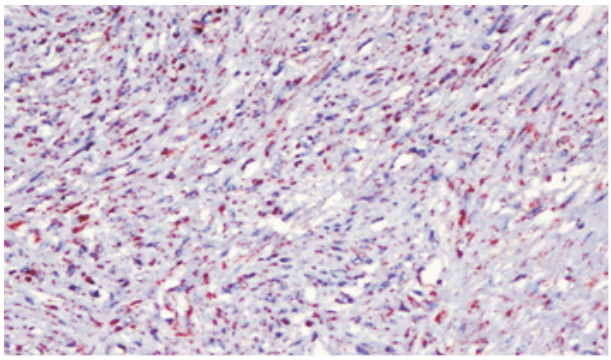

Fig.4: Strong cytoplasmic granular VEGF staining of smooth muscle cells in leiomyoma (,x100).

There was no obvious change of MVD and expression of VEGF and MMP-2 during the menstrual cycle. Regarding immunohistochemical findings, there was no statistically significant difference between solitary and multiple tumors. We did not find any correlation between immunohistochemical findings and tumor diameter.

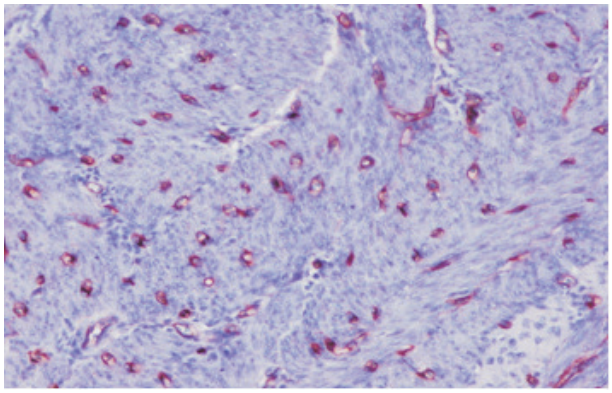

Fig. 5: Immunohistochemical staining for endothelial cells with CD34, representative example of MVD grade 3, in myometrium (,x200).

Discussion

The constant cyclical process of angiogenesis, the formation of new blood vessels, in the ovary and uterus is unique and not well understood (4). Angiogenesis is usually seen in pathological processes such as wound healing or tumor formation (14). The female reproductive tract provides a unique opportunity to study the physiology of this process. It involves an interaction between the blood vessels themselves and their surrounding ECM (4). Specific biological effects of ovarian steroids are known to be mediated through the actions of polypeptide growth factors, some of which are angiogenic (15). VEGF is an angiogenic growth factor which may be important in the pathogenesis of uterine leiomyoma. In a related study, VEGF messenger ribonucleic acid (mRNA) was evaluated in smooth muscle cells of myometrium and leiomyomata by in situ hybridization (16). In normal myometrium, levels of VEGF mRNA were significantly higher in the secretory phase than in the proliferative phase of the cycle. Leiomyomata did not have significantly different levels of VEGF mRNA compared with normal myometrium. In untreated leiomyomata, there was no significant difference between VEGF mRNA levels in the proliferative and secretory phases of the cycle. Leiomyomata from women treated with a GnRH analog did not have significantly different levels of VEGF mRNA from untreated leiomyomata.

Dixon et al found no statistically significant difference for VEGF in myometrium and leiomyoma immunohistochemically (17). Similarly, Poncelet et al found no difference in VEGF expression between leiomyomas and healthy myometrium, and no correlation between VEGF expression and MVD (18). Özçakır et al investigated the immunohistochemical expression of VEGF in leiomyomas and normal myometrium in women who were hysterectomized due to myoma uteri. They found that mean VEGF scoring was higher in normal myometrium than leiomyomas and the difference was statistically significant between the groups. They concluded that hemodynamic alterations within preexisting tumor vessels, rather than promotion of new blood vessel formation may play a role in the pathogenesis of leiomyoma (19).

Gentry et al investigated the expression of VEGF-A in leiomyoma tissue and adjacent myometrium in 36 pre-menopausal women undergoing hysterectomy for leiomyomas, with or without prior treatment with GnRH analog. VEGF-A was expressed in 77.8% leiomyoma sections from women without GnRH analog pretreatment, and in 83% of those from women with prior treatment. VEGF-A expression in the adjacent myometrium was much lower in both treated and untreated groups (20). We included only untreated patients, and our results were consistent with their findings.

More than half of the leiomyoma dry tissue mass is composed of ECM (18,19). The content of ECM is markedly higher in fibroids than in corresponding myometrium, with collagen type 1 being the most prevalent extracellular protein (21). Recently, it has been shown that growth of uterine leiomyomas may be related to increased activity of MMP-2 (22), a proteolytic enzyme effectively degrading different types of collagen (6-8). Bogusiewicz et al evaluated activity of MMP-2 by zymography and content of its tissue inhibitor by enzyme-linked immunosorbent assay in uterine leiomyoma and corresponding myometrium. They found that activity of MMP-2 was significantly higher in leiomyomas than myometrium. Content of tissue inhibitor of metalloproteinase TIMP-2 was similar in both tissues. They concluded that MMP-2 may be implicated in pathogenesis of leiomyoma (23). Our data seem to confirm their findings.

Wolañska et al studied the amounts and activities of MMPs in human myometrium and uterine leiomyomas in various stages of growth. They found that both myometrium and the investigated tumors contained MMP-1, MMP-2, MMP-3 and MMP-9. MMP-2 was the most abundant. In control myometrium only 10% of this enzyme existed in an active form, whereas in tumors, especially in large ones, the values reach 30%. It was suggested that the high activity of MMP-

2 was responsible for remodelling of ECM in the growing tumors (24).

There are studies revealing the differences in vasculature between leiomyomas and normal myometrium. Similar to our results, Poncelet et al showed decreased MVD in leimyomas with CD34 labeling. They demonstrated that vascular luminal area was increased in myomas compared with myometrium (18). Casey et al compared vascular parameters between fibroid and myometrium. They also compared small fibroids (<0.5 cm) with large fibroids. They showed that myometrium had a greater vascular area than small and large fibroids. MVD was higher in myometrium than in the fibroids. There were significantly larger diameter vessels in myometrium and large fibroids compared with small fibroids using CD34 and von Willebrand' s factor. They concluded that quantitative differences in vasculature existed between fibroids and myometrium. In general, the myometrium was more vascular than fibroids of differing sizes (25).

Walocha et al examined the vascular system of intramural leiomyomata collected upon autopsy by corrosion casting and scanning electron microcopy. The smallest (1-3 mm) fibroids were avascular, in larger ones (<1 cm) a few small vessels invaded the lesion from the periphery. The largest tumors (>1 cm) contained irregular networks of blood vessels with density similar to or lower than that of normal myometrium. Such tumors were surrounded by an extremely dense vascular layer ('vascular capsule') which was the source of larger vessels supplying and draining the tumor. They concluded that during the development of leiomyoma, the pre-existing blood vessels undergo regression and new vessels invade the tumor from the periphery, where intense angiogenesis, probably promoted by growth factors secreted by the tumor, leads to the formation of a 'vascular capsule'responsible for supply of blood to the growing tumor (26). We did not find any correlation between MVD and tumor diameter. We found that expression of VEGF and MMP-2 was significantly higher in leiomyomas than corresponding myometrium, but the CD34 labeling showed decreased MVD in leiomyomas compared with myometrium. There was no correlation between VEGF and MMP-2 staining and MVD. In conclusion, our data suggest that VEGF and MMP-2 may play role in the development of uterine leiomyomas. Mechanisms other than promotion of angiogenesis may contribute to this disease process. MMP-2 may be responsible for remodelling of extracellular matrix during the proliferation of uterine smooth muscle cells.

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