Expression of the Hypoxically Regulated Angiogenic Factor Adrenomedullin Correlates with Uterine Leiomyoma Vascular Density

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ABSTRACT

Uterine leiomyomas are the most prevalent benign tumor type in women of reproductive age and are one of the most common indications for hysterectomy. The expression of five angiogenic factors, adrenomedullin (ADM), vascular endothelial growth factor (VEGF), acidic fibroblast growth factor, basic fibroblast growth factor, and platelet-derived endothelial cell growth factor/thymidine phosphorylase, were examined in 91 uteri collected throughout the menstrual cycle; 52 of which contained leiomyomata, and the remainder were normal controls. The microvascular density and endothelial proliferative indices were then determined for each of the uterine sections. ADM and VEGF were the most widely expressed angiogenic factors in the leiomyomas. Furthermore, the expression of ADM and VEGF in the endometrium and myometrium was up-regulated in leiomyoma-bearing uteri compared with controls. Although acidic fibroblast growth factor and basic fibroblast growth factor were expressed in leiomyomas and endometrium in all of the uterine samples examined, they were only expressed in the myometrium of leiomyomata-bearing uteri. Endothelial proliferation in leiomyomas was statistically greater than that of the myometrium and endometrium, both within and between uteri (P < 0.05). The vascular density in the myometrium but not the endometrium was significantly increased in leiomyoma-containing uteri (P < 0.05). Expression of ADM alone correlated directly with vascular density and endothelial cell proliferation index in leiomyomas and myometrium and may account for the high vascularity found in leiomyomas and the myometrium of leiomyoma-bearing uteri. As such, ADM is identified as a novel target for antiangiogenic therapy of these benign, clinically problematic uterine tumors.

INTRODUCTION

Uterine leiomyomas are the most common benign tumors affecting adult women. They are present in one of three women over the age of 30 (1, 2). Leiomyomas are a significant cause of menorrhagia, pelvic pain, infertility, and pregnancy loss and are one of the commonest indications for hysterectomy. Their growth is controlled by estrogen, because they only occur after puberty and regress after the menopause. The increased mitotic activity in leiomyomas during the luteal phase of the menstrual cycle suggests that their growth is stimulated by progesterone as well as estrogen (3, 4). Estradiol and progesterone receptors are present in leiomyomas and are often overexpressed when compared with that in adjacent myometrium (4–6).

Specific biological effects of ovarian steroids are known to be mediated through the actions of polypeptide growth factors (7), some of which are angiogenic (7). Growth of leiomyomas is known to be controlled by a series of hormone-regulated polypeptides. Thus, various growth factors have been identified in leiomyomas that include epidermal growth factor, VEGF, insulin-like growth factor, platelet-derived growth factor, bFGF, transforming growth factor-β, and an Mr 18,000 leiomyoma-derived mitogenic protein (8–14). bFGF shows a mitogenic effect on cultured leiomyoma cells (15). Other growth factors such as the peptide ADM and the angiogenic enzyme TP have been identified in uterine tissues but not systematically studied in leiomyomas (16–19). ADM was first identified as a hypertensive peptide isolated from a human pheochromocytoma cell line (20) and recently shown to be an endothelial cell growth and angiogenic factor (18). Expression of ADM in the endometrium has been shown to be induced by tamoxifen but not estrogen (18).

This study was undertaken to examine determine expression of the angiogenic factors ADM, VEGF, aFGF, bFGF, and PDECGF/TP in uterine leiomyomata. Vascular density and endothelial and smooth muscle proliferation indices were also examined. The overall aim was to clarify the role of angiogenesis in the etiology of uterine leiomyomata.

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The abbreviations used are: VEGF, vascular endothelial growth factor; ADM, adrenomedullin, aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PDECGF/TP, platelet-derived endothelial growth factor/thymidine phosphorylase; NHS, normal human serum; SS, swine serum; RAMP, receptor activity-modifying protein.
MATERIALS AND METHODS

Tissue Preparation. Formalin-fixed, wax-embedded specimens of endometrium, myometrium, and leiomyoma from hysterectomy samples were selected from the archival files of the Histopathology Department of the John Radcliffe Hospital. The 91 hysterectomy samples examined were obtained from women of ages 30–49 years. All women had a history of regular menstrual cycles (26–30 days) and had used neither oral nor intrauterine contraception or had not received any hormonal treatment for at least 6 months prior to surgery. The stage of the menstrual cycle at which the tissue was obtained was determined from the patient’s menstrual history and endometrial histology (21). The presence or absence of leiomyomas was confirmed by histopathological examination (S. M.). Specimens were obtained from 39 women without leiomyomas undergoing hysterectomy for a subjective complaint of menorrhagia. Eleven patients were menstruating (days 1–7), 14 patients were from the follicular phase, and 14 were in the luteal phase. Fifty-two uterine specimens containing leiomyomas were collected, of which 22 were menstrual, 16 were follicular phase, and 14 luteal phase. Sections of endometrium and myometrium were obtained from all specimens and were present from leiomyomas.

Immunohistochemistry. All sections were dewaxed using CitroClear (HS Supplies, Aylesbury, United Kingdom); rehydrated sequentially in absolute, 95, 70, and 20% ethanol and distilled water; and rinsed in TBS (0.05 M Tris base, 0.15 M NaCl, 10×; pH 7.6) prior to staining.

ADM. Immunohistochemistry was carried out as described previously (18). Briefly, slides were incubated with 5% goat serum (Dako) to reduce nonspecific background staining, followed by 1:800 anti-ADM (Peninsula Laboratories, Liverpool, United Kingdom). A second biotinylated swine anti-ADM antibody (Dako) at a dilution of 1:400 was then applied for 30 min, after which the slides were incubated with streptavidin 1:200 (Dako) for 30 min. The final color was developed with the New Fuchsin Substrate System (Dako).

VEGF. Immunohistochemical staining for VEGF was carried out using the streptavidin-biotin-alkaline phosphatase (ABC) method, as described previously (22). After dewaxing and rehydrating the sections, the slides were incubated in double-distilled water at 37°C for 10 min and in 200 ml of PBSA (0.13 m NaCl, 0.002 m KCl, 0.01 m Na2HPO4, 0.002 m KH2PO4) containing 25 mg of protease type 24 (Sigma, Poole, United Kingdom) at 37°C for another 10 min. The slides were then left in double-distilled water at room temperature for 30 min and finally washed in TBS. Prior to application of primary antibody, the sections were incubated with 5% NHS/TBS for 20 min to block nonspecific binding sites. The sections were then incubated with anti-VEGF antibody M293 (R & D Systems, Abingdon, United Kingdom) diluted to 12.5 mg/ml in TBS/5% NHS at 4°C. After incubation with the primary antibody, the sections were washed in TBS and incubated with biotinylated rabbit antimouse IgG diluted to 1:400 in TBS/5% NHS, washed again, and incubated with alkaline phosphatase-conjugated to streptavidin at dilution of 1:200 in TBS/5% NHS. The New Fuchsins Substrate System was used to visualize sections. In the negative controls, the primary antibodies were replaced with the same subtype of mouse immunoglobulin (Sigma) at the same concentration.

aFGF and bFGF. Immunohistochemical staining for aFGF was performed using the streptavidin-biotin-alkaline phosphatase (ABC) method using the Vectastain ABC kit for alkaline phosphatase (rabbit IgG) according to the manufacturer’s protocol using rabbit anti-bovine aFGF polyclonal antibody (R & D Systems) at a dilution of 1:200 in TBS/20% SS. The peroxidase-anti-peroxidase method was used for bFGF. Prior to the application of the primary antibody, the sections were incubated with 20% SS in TBS for 20 min. Rabbit anti-bovine bFGF polyclonal antibody (Sigma) diluted to 84 ng/ml in TBS/20% SS was added for 60 min. After washing in TBS, the sections were incubated with phosphate-conjugated swine antirabbit immunoglobulin (Dako, Cambridge, United Kingdom) at a dilution of 1:50 in TBS/20% SS for 30 min, washed again in TBS, and incubated with rabbit peroxidase anti-peroxidase at a dilution of 1:50 in TBS/20% SS for 30 min. Sections were washed in TBS twice, developed with diaminobenzidine tetrahydrochloride (Dako) at 0.6 mg/ml in TBS containing 3 μl/ml of hydrogen peroxide for 10 min. All slides were counterstained with hematoxylin (Sigma) and mounted in Apathy’s mounting medium (BDM Merck, Poole, United Kingdom). Negative controls had the same concentration of rabbit immunoglobulin in place of the primary antibody.

PD-ECGF/TP. Immunohistochemical staining for PD-ECGF/TP was performed using the alkaline phosphatase-anti alkaline phosphatase method, as described previously (17). Prior to application of the primary antibody, the sections were incubated with 20% normal rabbit serum to block nonspecific protein binding sites. Primary antibody (PGF44Cl) was added to the slides for 30 min. The slides were then washed twice in TBS and incubated for an additional 30 min with rabbit antimouse immunoglobulin at a 1:50 dilution. The sections were washed again in TBS/10% NHS. Chromogen development was performed using a New Fuchsins Substrate System (Dako) according to the manufacturer’s instructions by incubation for 5–10 min. Slides were counterstained with hematoxylin and mounted with Apathy’s mounting medium.

Ki67/CD34 Double Staining. Immunohistochemical staining for CD34 was performed using the streptavidin-biotin-alkaline phosphatase (ABC) method. Throughout the protocol, all antibody dilutions and washes were performed in TBS, with incubations being performed at room temperature. Antigen retrieval was performed by means of pressure cooking in 1.6 l. 0.01 m sodium citrate buffer (pH 6.0) for 90 s, followed by a 30-min rinse in distilled water and 5 min in TBS. Prior to the application of the primary antibody, the sections were blocked in 10% NHS. The sections were incubated at room temperature for 30 min with the primary CD34 antibody (Qbend 10, Novacastra, United Kingdom), diluted 1:25. Again, the sections were rinsed for 2 × 5 min, followed by a 30-min incubation with the secondary biotinylated rabbit antimouse IgG (Sigma), diluted 1:400. The sections were incubated with phosphate-conjugated streptavidin at a dilution of 1:200 for 30 min; the final color was developed with the New Fuchsins substrate (Dako).

The Vectastain ABC kit was used for the staining of Ki67. Prior to staining, double stain enhancer (Zymed) was added to the sections; endogenous peroxidase activity was quenched by...
the application of 0.3% hydrogen peroxide, diluted in distilled water. The sections were washed and incubated in horse serum, after which the primary Ki-67 antibody (BioGenex, San Ramon, CA) at a dilution of 1:10 was added for 30 min. The sections were washed and incubated with the secondary biotinylated ABC antibody for 30 min, with the final color being developed with diaminobenzidine tetrahydrochloride substrate (Sigma).

**Determination of Vascular Density and Endothelial Cell Proliferative Indices.** The vascular density of myometrium and leiomyoma was determined by Chalkley counting (23). The three most vascular areas where the highest number of discreet microvessels stained were chosen. Vascular density was then determined using a 25-point Chalkley eyepiece graticule at \( \times 250 \). The graticule was rotated in the eyepiece to where the maximal number of vessels were overlaid by graticule dots. Individual density was then obtained by taking the mean of three graticule counts.

The endothelial cell proliferative index was determined at \( \times 400 \). The endothelial cell proliferative index was calculated as the percentage of all Ki-67 positively stained endothelial nuclei that also had concomitant positive cytoplasmic staining in CD34-positive cells.

**Statistical Analysis.** Analysis of microvascular density, determined by Chalkley counts, and the endothelial cell proliferation index used the nonparametric Mann-Whitney test and Spearman Rank correlation coefficient. The intensity of immunostaining for angiogenic factors was scored on a scale of 0–3, with 3+ indicating a strong positive result, by two independent observers. Kendall Tau Analysis was used for correlations between microvascular density and angiogenic factor expression.

**RESULTS**

The expression of five angiogenic polypeptides, ADM, VEGF, aFGF, bFGF, and PDECGF/TP, were examined in uteri containing leiomyomas and in a control group bearing none (Fig. 1). The vascular density and endothelial proliferative index of all tissue sections were determined in parallel.

**Leiomyoma.** The most strongly expressed angiogenic factors were ADM and VEGF, both localizing principally to the endothelium and vascular smooth muscle. No change in expression was seen throughout the menstrual cycle. aFGF and bFGF were present in leiomyomas with expression localized primarily to the smooth muscle cells and not changing with the menstrual cycle. Expression of PDECGF/TP was not detected in leiomyomas.

**Myometrium.** Expression of ADM was found in the myometrium, with staining localizing to the smooth muscle of arteries and venules and endothelium with approximately equal intensity. The intensity of ADM expression in the myometrium was generally weaker than in leiomyomas. ADM expression was up-regulated in the myometrium of leiomyoma-bearing uteri. Macrophages, arterial walls, venules, and smooth muscle cells stained positive for VEGF, with increased expression in the presence of a leiomyoma. aFGF and bFGF were only detected in the myometrium of leiomyoma-bearing uteri. PDECGF/TP expression was of weak intensity, localizing to the smooth muscle cells.

**Endometrium.** ADM and VEGF localized to the epithelium and endothelium of the endometrium; stromal macrophages exhibited high levels of ADM, as shown previously (18). The presence of a leiomyoma appeared to up-regulate the expression of ADM and VEGF in the endometrium as compared with controls. The presence of aFGF and bFGF was noted in the epithelium but not the stroma or endothelium of the endometrium. The expression of aFGF and bFGF in the endometrium was unaffected by the presence of a leiomyoma. PDECGF/TP was detected in the endometrium throughout the menstrual cycle, localizing to epithelial, stromal, and endothelial cells.

**Vascular Density and Endothelial Proliferation Index.** The vascular density of the various tissues was analyzed at the various stages of the menstrual cycle, menstrual, proliferative, and secretory, by CD34 immunostaining, followed by Chalkley counting. The vascular density of the endometrium, myometrium, and leiomyomata did not change significantly during the menstrual cycle. The vascular density of the myometrium was significantly higher \( (P < 0.05) \) in leiomyoma-bearing uteri compared with controls. The vascular density of the endometrium was unchanged by the presence of a leiomyoma. The endothelial proliferation index in leiomyoma, myometrium, and endometrium did not vary with the various stages of the menstrual cycle. A significant correlation between vascular density and endothelial cell proliferation index was observed \( (P < 0.05) \). Table 1 shows that endothelial cell proliferation is higher in the leiomyoma and the endometrium compared with the myometrium. This was expected because the leiomyoma and endometrium are areas of active angiogenesis, whereas the myometrium is not.

Analysis by Kendall Tau correlations of the degree of staining for angiogenic factors ADM or VEGF with microvascular density and endothelial proliferation index showed a number of significant correlations (Fig. 2). The level of ADM expression directly correlated with both the vascular density and proliferation index in leiomyoma \( (P < 0.05) \). No such correlation was seen with VEGF, aFGF, or bFGF expression \( (P > 0.08) \). In myometrium, ADM expression correlated with the proliferative index. ADM expression in leiomyomas and myometrial vascular density were also significantly correlated \( (P < 0.01) \).

**DISCUSSION**

This study has shown that ADM and VEGF are the most widely expressed angiogenic factors in uterine leiomyomas and that these tumors have a higher vascular density and endothelial proliferative index than both the normal myometrium and endometrium. ADM and VEGF both share similarities in that they are induced by hypoxia (24, 25) and increase vascular permeability, in addition to being angiogenic. It is well documented that they can be hypoxically regulated via a hypoxic response motif present within their promoter sequences (26, 27). Similar to most tumors, uterine leiomyomata are generally considered to be hypoxic (21). The two factors differ in that VEGF exhibits estrogen-dependent induction (7, 25), but ADM does not (18). Also, although the mitogenic activity of VEGF is highly restricted (mainly to endothelial cells), ADM exhibits widespread effects in both benign and malignant tissues (28). Thus, ADM could be a key player in the growth of leiomyomas.

ADM expression is known to be up-regulated by the in-
Inflammatory cytokines interleukin-1, tumor necrosis factor-alpha, and tumor necrosis factor-beta in cardiac tissue (myocytes and nonmyocytes), rat endothelium, and rat and bovine vascular smooth muscle (29–31). Modulation of expression of these cytokines by estradiol and progesterone has been demonstrated in mouse and human uterine tissues (32, 33). However, little information is available concerning the expression of the interleukins and tumor necrosis factors in leiomyoma.

Leiomyomata have classically been thought to show poor vascularity (21). For the first time, we show this not to be the case and that the vascular density of leiomyomas is comparable with that of normal myometrium and endometrium. We have also shown that the vascular density of myometrium (but not that of endometrium) was higher in leiomyoma-bearing uteri compared with controls. This may be attributable to excess release of angiogenic stimuli by the leiomyoma stimulating angiogenesis in the surrounding myometrium. Alternatively, it may be that women with an unusually high myometrial vascular density or increased expression of ADM, VEGF, and aFGF and bFGF may have a predisposition for the development of uterine

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**Table 1** Comparison of the endothelial proliferative index of the endometrium, myometrium, and leiomyomata

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean endothelial proliferation rate</th>
<th>SE</th>
</tr>
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<tbody>
<tr>
<td>Endometrium</td>
<td>10.729</td>
<td>1.16</td>
</tr>
<tr>
<td>Myometrium</td>
<td>3.97</td>
<td>0.36a</td>
</tr>
<tr>
<td>Leiomyoma</td>
<td>11.21</td>
<td>1.175a</td>
</tr>
</tbody>
</table>

*Leiomyomas examined had a significantly higher endothelial cell proliferative index than the myometrium (P < 0.005). More than 100 fields were examined for each tissue type.*
leiomyoma. This is of interest in the light of the known familial predisposition to leiomyomas (34).

Vascular density and endothelial proliferative index were unaltered throughout the menstrual cycle in endometrium, myometrium, and leiomyoma. This concurs with previous studies of the endometrium and myometrium by others (35, 36). Vascular density correlated with the proliferative index in the endometrium, myometrium, and leiomyoma.

Fig. 2 shows the intensity of myometrial ADM immunostaining against median leiomyoma vascular density and median myometrial vascular density. A striking finding of this study was that expression of ADM but not that of VEGF or aFGF or bFGF correlated with the vascular density of the leiomyomas. This points to an important role for ADM in uterine leiomyoma angiogenesis. We have shown previously that ADM plays a key role in endometrial angiogenesis and hyperplasia seen in women receiving tamoxifen therapy for breast carcinoma (18).

This is the first report of expression of aFGF and bFGF in leiomyoma. In this context, this finding is of interest in view of the previous report of FGF receptor 1 in leiomyomas (37). The lack of correlation of aFGF and bFGF with vascular density and proliferation in leiomyomas means that their roles in angiogenesis in this tissue is unclear.

No expression of PDECGF/TP was detected in leiomyomas, but it was present in the endometrium and myometrium. Patterns of expression in the endometrium were in agreement with previous reports (17). Other groups have also failed to detect PDECGF/TP expression in leiomyoma (16). It is unusual for PDECGF/TP to not be up-regulated in neoplastic tissue (38). It is of interest that PDECGF/TP is also not expressed in endometrial malignancies (17).

Increased angiogenesis in leiomyomas raises the possibility of therapeutic intervention with antiangiogenic agents. Although at present many promising drugs are in development (39), they will need to be well tolerated before their use can be envisioned in benign tumors. Nevertheless, antiangiogenic strategies for the treatment of non-life-threatening pathologies are receiving considerable attention.

The high level of VEGF and ADM expression observed within leiomyomas presents a possible target for therapeutic intervention, using antibodies specific to VEGF and ADM to treat leiomyomas. Specific monoclonal antibodies capable of inhibiting VEGF are now available. Such antibodies have been shown to exert a potent inhibitory effect on both the growth of xenografted human tumors in mice (40) and in development of the vasculature of the primate corpus luteum (41). Indeed, abrogation of VEGF signaling has been shown to lead to vascular apoptosis and tumor regression (42). Use of blocking anti-VEGF antibodies has revealed remarkably few side effects of their use. The effect of blocking ADM action has yet to be evaluated.

It has been reported recently that the calcitonin receptor-like receptor can function either as a calcitonin gene-related peptide or ADM receptor, depending on which RAMPs are expressed. RAMPs are required to transport calcitonin receptor-like receptor to the plasma membrane. RAMP2-transported receptors are ADM receptors, whereas RAMP1-transported receptors are calcitonin gene-related peptide receptors. This would suggest that antibodies to RAMP2 may also have potential as a therapeutic tool. Thus, as for other solid tumors, antiangiogenesis may have potential in the future therapy of leiomyomas.

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