Quantification of vascular endothelial growth factor-A in leiomyomas and adjacent myometrium

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ABSTRACT

Although uterine leiomyomas constitute the commonest benign tumour in women, the regulation of their growth is poorly understood. It is believed that angiogenesis, the process by which new capillaries develop from pre-existing blood vessels, may be involved. We therefore investigated the expression of vascular endothelial growth factor-A (VEGF-A), a primary regulator of angiogenesis, in leiomyoma tissue and the adjacent myometrium in 36 pre-menopausal women undergoing hysterectomy for leiomyomas, with or without prior treatment with gonadotrophin-releasing hormone analogue (GnRHa). In 5 μm sections prepared from archival paraffin-wax blocks, VEGF-A was demonstrated by standard immunohistochemistry using a monoclonal antibody. VEGF-A was expressed in 14 of 18 (77.8%) leiomyoma sections from women without GnRHa pretreatment, and in 15 of 18 (83%) of those from women with prior treatment. VEGF-A expression in the adjacent myometrium was much lower, being noted in two of 18 (11.1%) sections from women without prior GnRHa treatment and in one of 18 (5.5%) sections from tissue that had been subject to prior down-regulation. Moreover, when VEGF-A expression was present, expression was strong in leiomyomas (>20 focal areas/cm²), but not in adjacent myometrium. The differential expression of VEGF-A antigen in leiomyomas compared with the adjacent myometrium indicates that local angiogenesis may be important in the development and growth of these tumours. GnRHa therapy does not appear to alter this pattern of VEGF-A expression.

INTRODUCTION

Uterine leiomyomas are the most common benign tumours of the female genital tract, and are frequently the cause of abnormal uterine bleeding and pressure symptoms. They occur in 20–30% of women over 30 years of age [1], but little is known of their biology, making it difficult to develop satisfactory non-surgical treatments. Oestrogen and progesterone are believed to be physiological regulators of leiomyoma growth [2], but the effect of progesterone is controversial and less well understood. Medical therapy (down-regulation) with gonadotrophin-releasing hormone analogues (GnRHa), which results in a medical menopause, reduces leiomyoma volume by over 40% [1], but the mechanism of action is unclear and the leiomyomas enlarge again on cessation of therapy.

Many growth factors have been implicated in the pathogenesis of leiomyomas [3,4]. Some growth factors stimulate the myometrium directly or act through oes-
trogen and progesterone to promote leiomyoma growth, while others inhibit such growth and proliferation. Shimomura et al. [5] demonstrated that progesterone up-regulates proliferating cell nuclear antigen and epidermal growth factor receptor in leiomyoma cells. Fibroblast growth factor [6] and platelet-derived growth factor [7] have also been studied, with varying expression in leiomyomas.

Since the establishment of nutrient blood vessels is fundamental to the growth of all tumours, it has been suggested that angiogenesis may play a role in the regulation of leiomyoma growth. Angiogenesis, however, is regulated by facilitators and inhibitors, which may act through specific receptors on target cells [8]. It is a complex multi-step process that is essential in development and reproduction. It occurs physiologically in the ovaries and in the uterus, but also in pathological states, such as tumour growth and progression. In the ovaries, angiogenesis is involved in corpus luteum formation, and in the uterus it is seen in the proliferative phase of the endometrium. Angiogenesis also contributes to wound healing. Evidence from studies on breast cancer and cutaneous melanoma [9,10] indicate that tumour growth is dependent upon the proliferation of blood vessels, and that the mechanism is controlled by a variety of angiogenic factors, in particular vascular endothelial growth factor-A (VEGF-A) and platelet-derived endothelial cell growth factor (PD-ECGF)/thymidine phosphorylase.

VEGF-A, also known as vascular permeability factor, is a potent endothelial mitogen that is expressed by macrophages and promotes vascular permeability [11]. It is a heparin-binding glycoprotein [12,13] with a molecular mass of 34–46 kDa. So far five isoforms of VEGF-A have been clearly defined, which vary in the number of amino acids (121, 145, 165, 189 and 206) [14]. In addition to VEGF-A, four other VEGF-related molecules have been identified, i.e. VEGF-B, VEGF-C, VEGF-D and VEGF-E, along with placental growth factor [15]. VEGF-C, in addition to its effect on angiogenesis, also regulates lymphangiogenesis [16]. The actions of the other molecules are, however, still under investigation.

In the present study, we investigated the expression of VEGF-A in uterine leiomyomas and the adjacent myometrium, because of its established role in promotion of tumour angiogenesis.

**METHODS**

**Tissue samples used**

Randomly selected paraffin-wax-embedded 5 μm sections of leiomyomas and adjacent myometrium from 36 pre-menopausal multi-ethnic women undergoing surgery for benign indications at the Royal Free Hospital, London, between 1996 and 1999, were used for the study. The women were non-obese and were aged between 30 and 55 years. GnRHα (Zoladex; AstraZeneca, Kings Langley, U.K.) was administered 4 weeks before surgery to 18 of the women, but the remaining 18 had no prior GnRHα therapy. The study was granted Ethical Committee approval.

**Identification of VEGF-A**

VEGF-A antigen was demonstrated by standard immunohistochemistry using a monoclonal antibody. The anti-VEGF-A antibody (clone 26503.11; R&D Systems, Abingdon, U.K.) used in our study is against the 165-amino-acid isoform, but as there is significant overlap between all VEGF-A isoforms, this antibody will also detect the other isoforms of VEGF-A. Antibody retrieval was carried out in warmed 12.5 mg/100 ml protease type 24 solution (Sigma, Poole, Dorset, U.K.) for 10 min. The slides were incubated overnight at 4 °C with the primary anti-VEGF-A antibody at 1:40 dilution. Each slide had a separate negative control in which sections were incubated with mouse IgG2b isotype control (clone 20116.11; R&D Systems) at 1:40 dilution with Tris-buffered saline. VEGF-A antigen reactivity was determined using the streptavidin/biotin detection system after incubation with secondary antibody. The process
was repeated four times to ensure consistency. A slide with positive VEGF-A expression in leiomyoma is shown in Figure 1(a), and a negative control slide is shown in Figure 1(b).

**Scoring**

VEGF-A expression was scored according to the number of focally stained areas. Any area with ten or more stained cells was regarded as focally stained. Positive VEGF-A expression was defined as $\geq 10$ focal areas/cm$^2$, and strong VEGF-A expression as $\geq 20$ focal areas/cm$^2$. Scoring was performed independently by two observers (C.C.G. and L.F.W.T.F.), then averaged. For sections where the number of focal areas differed by 5 or more between the observers, the staining was repeated and scored by a third observer (J.C.C.). All observers were blinded to the identity of the samples.

**RESULTS**

The individual VEGF-A scores (i.e. number of focally stained areas/cm$^2$) for all the specimens are shown in the upper panel of Figure 2, with a comparative box-plot of the mean scores for leiomyoma and for the adjacent myometrium in the lower panel. VEGF-A was expressed in 14 of 18 (77.8%) leiomyomas from women without GnRHα pretreatment, and in 15 of 18 (83%) leiomyomas from women with prior GnRHα pretreatment. VEGF-A expression, when present, was strong ($\geq 20$ focal areas/cm$^2$) in all the leiomyoma sections, independent of GnRHα treatment. VEGF-A expression in the adjacent myometrium was, however, lower in sections from GnRHα-treated women, being noted in two of 18 (11.1%) sections without GnRHα pretreatment and in one of 18 (5.5%) with pretreatment. VEGF-A expression

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**Figure 2** Expression of VEGF-A

Upper panel: quantification of VEGF-A antigen as focal areas in leiomyoma and in adjacent myometrium from 36 women (positive VEGF-A expression defined as $\geq 10$ focal areas/cm$^2$). Lower panel: mean VEGF-A scores (+ 95% confidence intervals) in leiomyoma and adjacent myometrium from 36 women.
in both leiomyoma and myometrium was independent of age, body weight and menstrual cycle day.

**DISCUSSION**

VEGF-A expression has been identified in a range of human tumours, including glioblastoma [17], renal [18], bladder [18] and ovarian [19] carcinoma. The present study has demonstrated a higher expression of VEGF-A antigen in leiomyomas than in the adjacent myometrium, indicating a possible role for VEGF-A in the pathobiology of leiomyomas. The specific role is unclear; however, since VEGF-A is not mitogenic in smooth muscles [20], it may be involved in leiomyoma growth by promoting the formation of new blood vessels that supply and feed the tumour. This action does not seem to be oestrogen-dependent, since down-regulation with GnRHα, which abolishes endogenous oestrogen production, did not suppress VEGF-A expression in leiomyomas. Moreover, there was no difference in VEGF-A expression between the oestrogen-dominant proliferative phase of the menstrual cycle and the progesterone-dominant luteal phase. Oestrogen is, however, believed to promote leiomyoma growth [21]. It would seem, therefore, that there is an alternative (oestrogen-independent) pathway for promoting leiomyoma growth.

This finding has interesting potential clinical applications. Neutralizing antibodies against VEGF and manipulation of VEGF receptors have been used to control angiogenesis [22], and have shown promising results in animal models [23]. The use of anti-VEGF-A antibodies to treat leiomyomas should therefore be explored. Similarly, anti-angiogenic drugs such as angiotatin and endostatin [24] may have a future role in the medical treatment of leiomyomas. In a recent case report [25], a large uterine leiomyoma was observed to shrink by 90% in a woman treated for hepatitis with interferon, an antiangiogenic agent [26]. This shrinkage persisted at 17 months after cessation of interferon therapy. It would be interesting to investigate whether these agents would reduce the rare neoplastic progression of leiomyoma to leiomyosarcoma, which occurs in less than 0.1% of cases [27,28].

Although we did not find any association between oestrogenic drive and VEGF-A expression in leiomyomas or adjacent myometrium in the present study, it is possible that any influence of oestrogens on leiomyomas is mediated through other, as yet unidentified, VEGF isoforms. It is also possible that oestrogen derivatives that influence vascularity, such as 2-methoxyoestradiol, act via haemodynamic alterations within pre-existing tumour vessels, and not by promoting new blood vessel formation (i.e. angiogenesis).

Our results differ from those of Harrison-Woolrych et al. [29], who examined both VEGF-A protein and mRNA, but found no differential expression of either VEGF-A protein or mRNA in the myometrium and leiomyomas of 29 women. Several reasons may account for this. Harrison-Woolrych et al. [29] used a polyclonal, rather than a monoclonal, antibody. They did not give a detailed analysis of the mRNA expression in their specimens, but rather counted all expression as positive. Our scoring was more robust than theirs. Our use of paraffin-wax sections instead of frozen sections (which they used) could have been associated with artefactual variation in VEGF-A expression between leiomyomas and myometrium, but we are unaware of any evidence that VEGF-A is less stable in myometrium than in leiomyomas. We are therefore confident of our results, given the robust criteria we utilized and the fact that we determined VEGF-A antigen directly.

In the present study, there were some variations in VEGF-A antigen reactivity between patients, but whether these were due to differences in the stage of tumour growth is unclear and should be investigated in future studies.

The differential expression of VEGF-A in leiomyomas compared with the adjacent myometrium indicates the need for further research into the role of other angiogenic growth factors. This could have a significant impact on our understanding and management of leiomyomas.

**ACKNOWLEDGMENT**

We are grateful to the North London Nuffield Hospital (London, U.K.) for financial support of C.C.G. for the duration of the study.

**REFERENCES**


Received 21 March 2001/2 July 2001; accepted 24 August 2001