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Uterine Smooth Muscle Cells Express Functional Receptors (flt-1 and KDR) for Vascular Permeability Factor/Vascular Endothelial Growth Factor

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SUMMARY: Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), is an angiogenic factor with important roles in tumor growth, wound healing, and inflammation. VPF/VEGF interacts with endothelial cells by way of two high-affinity receptor tyrosine kinases: flt-1 and KDR. The vast majority of published studies have described expression of the VPF/VEGF receptors only in endothelial cells, and the statement is frequently made that these receptors are endothelial-cell–specific. In this study, we detected mRNA for flt-1 and KDR by in situ hybridization in smooth muscle cells in sections of the wall of the uterus. To confirm these unexpected findings, smooth muscle cells from the uterus and, as a control, from the colon were isolated, characterized, and cultured. Both uterine and colonic smooth muscle cells in culture expressed VPF/VEGF, but only smooth muscle cells from the uterus expressed mRNA for flt-1 and KDR by Northern analysis and in situ hybridization. Cell culture extracts of uterine but not colonic smooth muscle cells were also positive for flt-1 by Western analysis. Moreover, cultured uterine but not colonic smooth muscle cells phosphorylated the flt-1 receptor and proliferated strongly in response to added VPF/VEGF. This is one of the first rigorous demonstrations that a normal cell type other than endothelial cells can express functional receptors for VPF/VEGF in vivo and in vitro, suggesting that VPF/VEGF may have important, previously unsuspected roles on cell types other than endothelium. (*Lab Invest 1997, 76:245–255*).

Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), is a potent inducer of microvascular hyperpermeability (Dvorak et al, 1979; Senger et al, 1983) as well as an endothelial cell mitogen (Ferrara and Henzel, 1989; Gospodarowicz et al, 1989; Keck et al, 1989; Leung et al, 1989). VPF/VEGF interacts with endothelial cells by way of two high-affinity receptor tyrosine kinases flt-1 and KDR (de Vries et al, 1992; Terman et al, 1992). VPF/VEGF and its endothelial cell receptors are strongly expressed in a number of conditions characterized by microvascular hyperpermeability and angiogenesis including tumors (Berse et al, 1992; Brown et al, 1993a, 1995b; Dvorak et al, 1991; Plate et al, 1992; Senger et al, 1986; Shweiki et al, 1992), wound healing (Brown et al, 1992b), and certain inflammatory conditions (Brown et al, 1995a, 1995b; Detmar et al, 1994; Fava et al, 1994; Koch et al, 1994).

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The vast majority of published studies have described expression of the VPF/VEGF receptors flt-1 and KDR only in endothelial cells, and the statement is frequently made that these receptors are endothelial-cell–specific. A few reports, however, have claimed that by various methods VPF/VEGF receptors may be expressed on other cell types, mostly tumor-derived cell lines. Melanoma cell lines (Gitay-Goren et al, 1993) and mononuclear phagocytes (Shen et al, 1993) have been reported to bind VPF/VEGF, and a melanoma cell line has been reported to express flt-1 mRNA (Cohen et al, 1995). KDR mRNA expression has been reported in certain ovarian carcinomas by in situ hybridization (ISH) (Boocock et al, 1995). These studies, however, did not demonstrate that the receptors were functional. Expression of flt-1 mRNA has been described in trophoblast, and a choriocarcinoma cell line has been shown to express both flt-1 and KDR by reverse transcriptase-PCR (RT-PCR) and proliferate in response to VPF/VEGF (Charnock-Jones et al, 1994). Human retinal pigment epithelial cells have been reported to express VPF/VEGF, flt-1, and KDR mRNA by RT-PCR and proliferate in response to VPF/VEGF (Guerrin et al, 1995).

In this report, we show that uterine myometrial smooth muscle cells express the VPF/VEGF receptors flt-1 and KDR, phosphorylate flt-1, and proliferate in response to VPF/VEGF.

Results

ISH studies were performed on sections of myometrium from 22 hysterectomy specimens (patients aged 40 to 72 years). In 9 cases, the hysterectomy was performed for endometrial carcinoma, and in 13 cases for other reasons, ie, leiomyomas, prolapse, etc. In all cases, myometrial smooth muscle cells labeled for VPF/VEGF mRNA (Fig. 1, a and b). Low but detectable levels of mRNA of the VPF/VEGF receptors flt-1 and KDR were seen in vascular endothelial cells in nearly all cases. Surprisingly, in four cases, the smooth muscle cells also labeled for flt-1 mRNA (Fig. 1, e and f), and in 11 cases (including the 4 cases that labeled for flt-1) for KDR mRNA (Fig. 1, g and h). In the remaining cases, labeling of uterine smooth muscle cells for VPF/VEGF receptor mRNA was equivocal. No specific cellular labeling was seen with sense control probes, and background levels were low (Fig. 1, c and d). No distinct correlations could be made between the pathologic diagnosis and the expression of VPF/VEGF and its receptors. In addition, no correlation could be made between patient age and the expression of VPF/VEGF and its receptors, although it must be noted that nearly all of the patients in the study were in the peri- or postmenopausal age range.

To confirm these unexpected ISH findings, tissue samples were collected from the muscular wall of the uterus (seven cases) and, as a control, from the muscular wall of the colon (five cases). Tissue samples of the muscular walls of the uterus and colon contained significant VPF/VEGF, flt-1, and KDR mRNA by Northern analysis (Fig. 2). However, whole tissue samples consist of multiple cell types, including not only smooth muscle cells but also endothelial cells, fibroblasts, neural cells, etc., and Northern analysis does not identify the specific cell type or types present in a complex tissue that synthesize the mRNA of interest.

Because our main goal was to determine whether uterine smooth muscle cells expressed VPF/VEGF receptor mRNA, we isolated and cultured smooth muscle cells from the seven samples of the muscular wall of the uterus and, as a control, from the five samples of the muscular wall of the colon, as described in "Materials and Methods." Isolated cells stained for both smooth muscle actin and calponin by immunocytochemistry (Fig. 3a). In addition, to exclude the possibility that endothelial cells were present in the

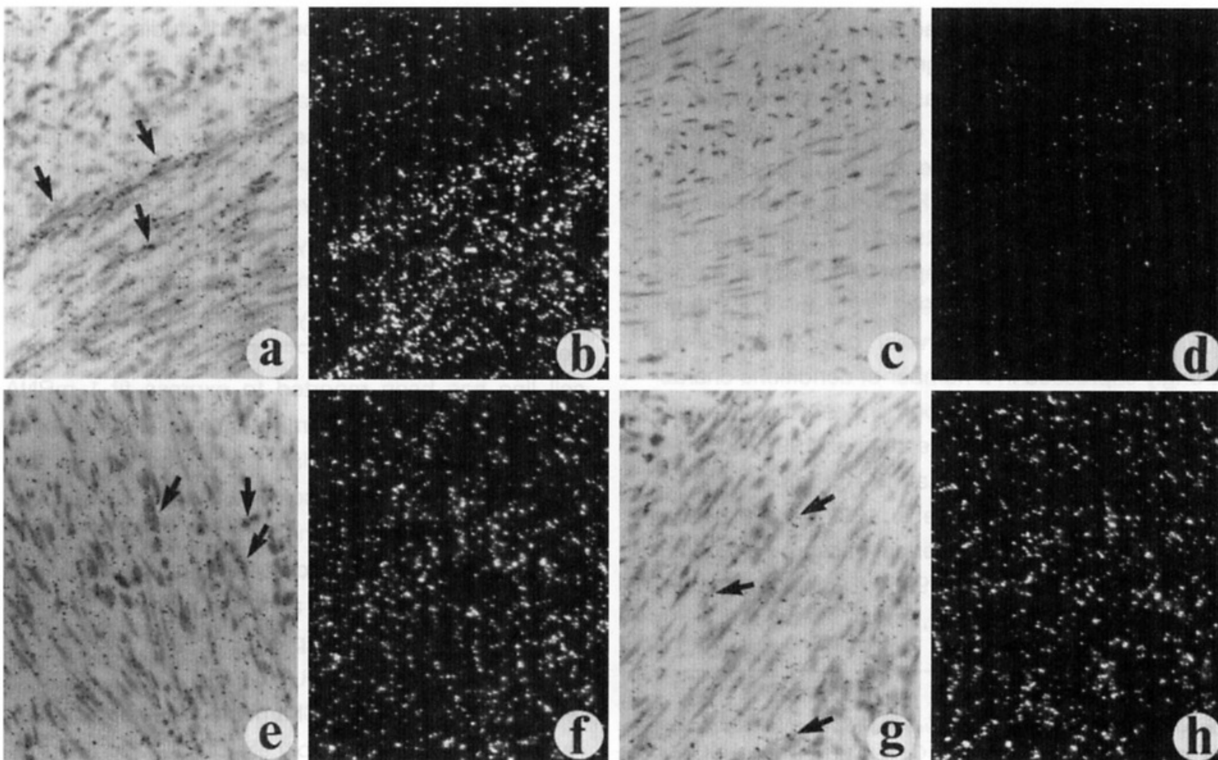

Figure 1.
A tissue section of uterine wall shows strong expression of mRNA for VPF/VEGF (a and b), flt-1 (e and f), and KDR (g and h) in myometrial smooth muscle cells. No labeling is seen with sense control probe (c and d). Original magnification, ×330.

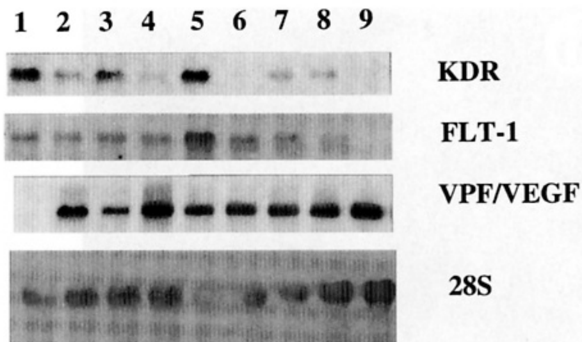

Figure 2.
Northern blot analysis of total RNA (10 μg/lane) extracted from human dermal foreskin microvascular endothelial cells (Lane 1), cultured myometrial-derived smooth muscle cells (Lanes 2 to 4), freshly isolated normal myometrium (Lanes 5 and 6), freshly isolated muscularis propria from normal colon (Lanes 7 and 8), and cultured colon-derived smooth muscle cells (Lane 9). The following human cDNA fragments were used as probes: KDR, flt-1, and VPF/VEGF. To control for transfer efficiency and loading levels, the blots were also hybridized to the 28S ribosomal subunit.

cultures, we showed that the cells did not stain for von Willebrand factor (VWF) (Fig. 3c) and did not take up acetylated low-density lipoprotein (LDL) (Fig. 3e). In contrast, control endothelial cells did not stain for smooth muscle actin (Fig. 3b) but were positive for VWF (Fig. 3d) and did take up acetylated-LDL (Fig. 3f). By ISH, cultured smooth muscle cells from myometrium strongly expressed VPF/VEGF mRNA (Fig. 4, a and b), flt-1 mRNA (Fig. 4, e and f), and KDR mRNA (Fig. 4, g and h). No specific cellular labeling was seen with sense control probe, and background levels were low (Fig. 4, c and d). These results were confirmed by Northern analysis of cultured uterine derived smooth muscle cells which labeled strongly but variably for VPF/VEGF, flt-1, and KDR mRNA (Fig. 2). In contrast, smooth muscle cells derived from the wall of the colon did not express detectable levels of either flt-1 or KDR mRNA, although they did express VPF/VEGF mRNA (Fig. 2). Cell culture extracts of uterine derived smooth muscle cells were positive for flt-1 protein by Western analysis, whereas those from colonic smooth muscle cells were not (Fig. 5a).

Immunoprecipitation of ^{35}S-methionine-labeled cell-conditioned medium demonstrated VPF/VEGF protein in the conditioned medium of both uterine- and colonic-derived smooth muscle cells as well as dermal fibroblasts but not in the conditioned medium of dermal microvascular endothelial cells (Fig. 5b). Using an RT-PCR approach with two specific primers, we were able to detect the three most common VPF/VEGF splicing variants, VPF/VEGF$_{121}$, VPF/VEGF$_{165}$, and VPF/VEGF$_{189}$, in both uterine and colonic smooth muscle cell cultures (Fig. 6).

To determine if the VPF/VEGF receptors expressed on uterine smooth muscle cells were functional, proliferation assays were performed on three separate cultures of uterine smooth muscle cells, three separate cultures of colonic smooth muscle cells, and control endothelial cells with the addition of either VPF/VEGF, basic fibroblast growth factor (bFGF), or 5% fetal calf serum (FCS) (Fig. 7). A strong proliferative response was noted in all three cell types with the addition of either 5% FCS or bFGF (2 ng/ml). When 10 ng/ml VPF/VEGF was added, however, a strong proliferative response was seen in endothelial cells and uterine smooth muscle cells but not in colonic smooth muscle cells. The proliferative effect of VPF/VEGF on myometrial smooth muscle cells was of the same order of magnitude as that of VPF/VEGF on control endothelial cells. This response was dose-dependent because 1 ng/ml VPF/VEGF did not induce a significant proliferative response. In addition, a marked increase in phosphorylation of the VPF/VEGF receptor flt-1 was seen when VPF/VEGF was added to cultured uterine smooth muscle cells, but no signal was detected when VPF/VEGF was added to colon-derived smooth muscle cells (Fig. 8).

Discussion

VPF/VEGF is an important angiogenic factor that induces microvascular hyperpermeability (Dvorak et al, 1979; Senger et al, 1983), acts as an endothelial cell mitogen (Ferrara and Henzel, 1989; Gospodarowicz et al, 1989; Keck et al, 1989; Leung et al, 1989), and interacts with endothelial cells by way of two high-affinity receptor tyrosine kinases flt-1 and KDR (de Vries et al, 1992; Terman et al, 1992). Many published studies state that VPF/VEGF receptors are endothelial-cell–specific, despite sporadic reports claiming that they may be expressed on other cell types (Boocock et al, 1995; Charnock-Jones et al, 1994; Cohen et al, 1995; Gitay-Goren et al, 1993; Shen et al, 1993).

We were intrigued by in situ hybridization studies that indicated that, in at least some samples of uterine tissue, mRNA for the VPF/VEGF receptors flt-1 and KDR were not only expressed by endothelial cells but also by smooth muscle cells of the uterine wall. Pure populations of smooth muscle cells were isolated and cultured from the wall of the uterus and, as a control, from the wall of the colon. Northern analysis and ISH demonstrated expression of mRNA of both flt-1 and KDR in smooth muscle cells isolated from the uterus but not in smooth muscle cells isolated from the colon. Furthermore, flt-1 protein could be demonstrated by Western blotting in extracts of cultured smooth muscle cells from the uterus but not the colon. Smooth muscle cells from the uterus proliferated strongly in

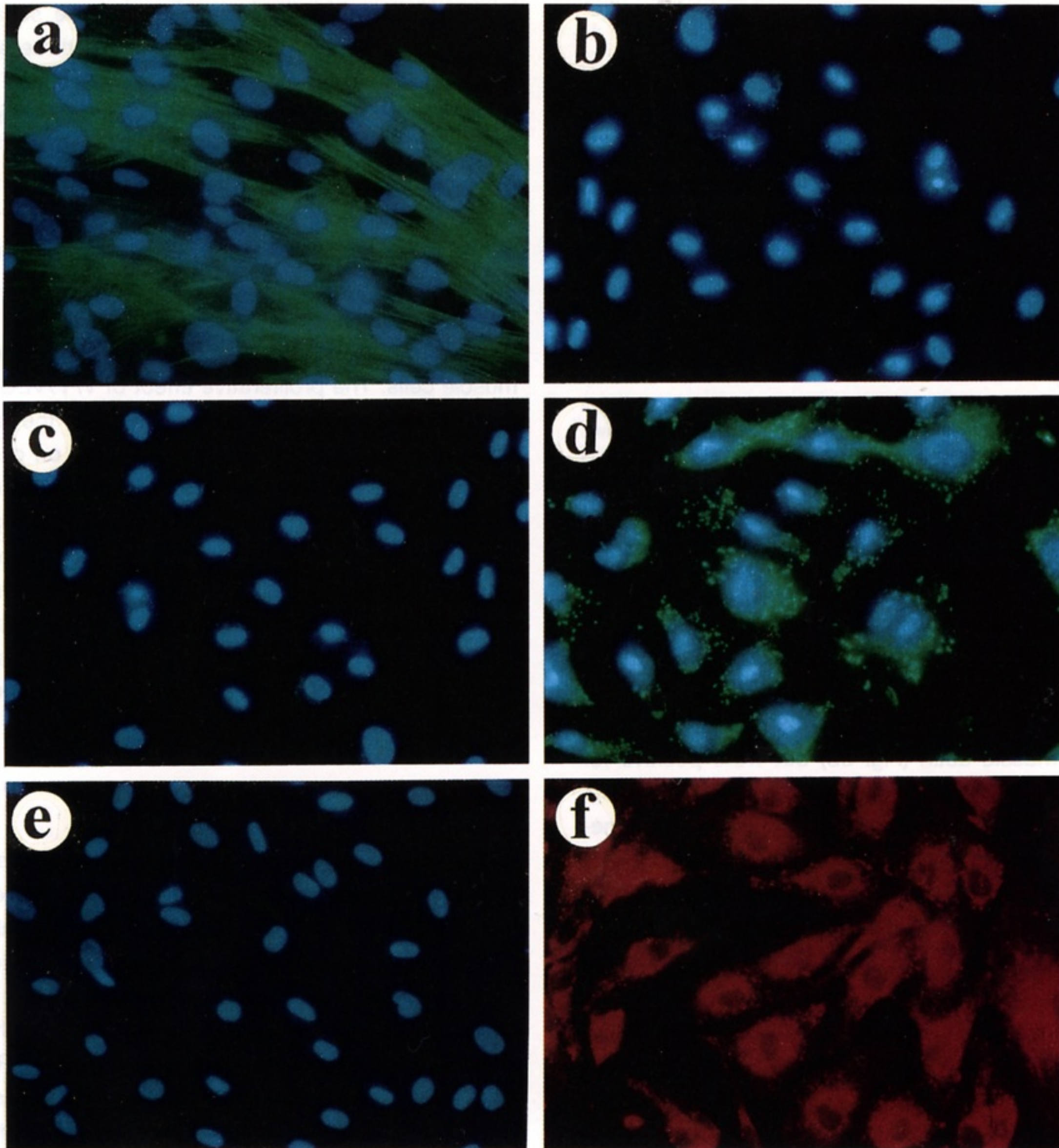

Figure 3.

Smooth muscle cells (a, c, and e), isolated as described in "Materials and Methods," were characterized for the presence of smooth muscle and endothelial cell markers; human dermal endothelial cells (b, d, and f) were included as controls. Primary antibodies included: anti- α-actin (a and b) and anti-VWF (c and d). Immune complexes were detected with biotinylated secondary antibodies followed by avidin-FITC. In addition, cultures were also incubated with acetylated-LDL for four hours to assay for the presence of LDL receptors in the cell surface (e and f). Nuclei were counterstained with Hoertch.

response to exogenous VPF/VEGF, whereas smooth muscle cells from colon did not. The proliferative effect of VPF/VEGF on myometrial smooth muscle cells was of the same order of magnitude as that of VPF/VEGF on control endothelial cells. The uterine smooth muscle cells increased phosphorylation of their flt-1 receptor in response to VPF/VEGF, whereas colonic smooth muscle cells showed no signal. Uterine and colonic smooth muscle cells were shown to express VPF/VEGF mRNA by ISH and Northern analysis, and VPF/VEGF protein by immunoprecipitation from culture medium. VPF/VEGF expression has recently been described in uterine smooth muscle cells (Harrison-Woolrych et al, 1995). This is one of the first reports to show, in a convincing fashion, that functional VPF/VEGF receptors are present on a normal cell type other than endothelial cells, thereby suggesting that VPF/VEGF serves important and previously unsuspected functions in additional cell types.

These findings raise the interesting possibility that VPF/VEGF is acting as an autocrine growth factor in uterine smooth muscle cells and furthermore that alterations in expression of VPF/VEGF or its receptors play a role in the pathogenesis of the very common, benign or rare, malignant smooth muscle tumors which arise in the uterus.

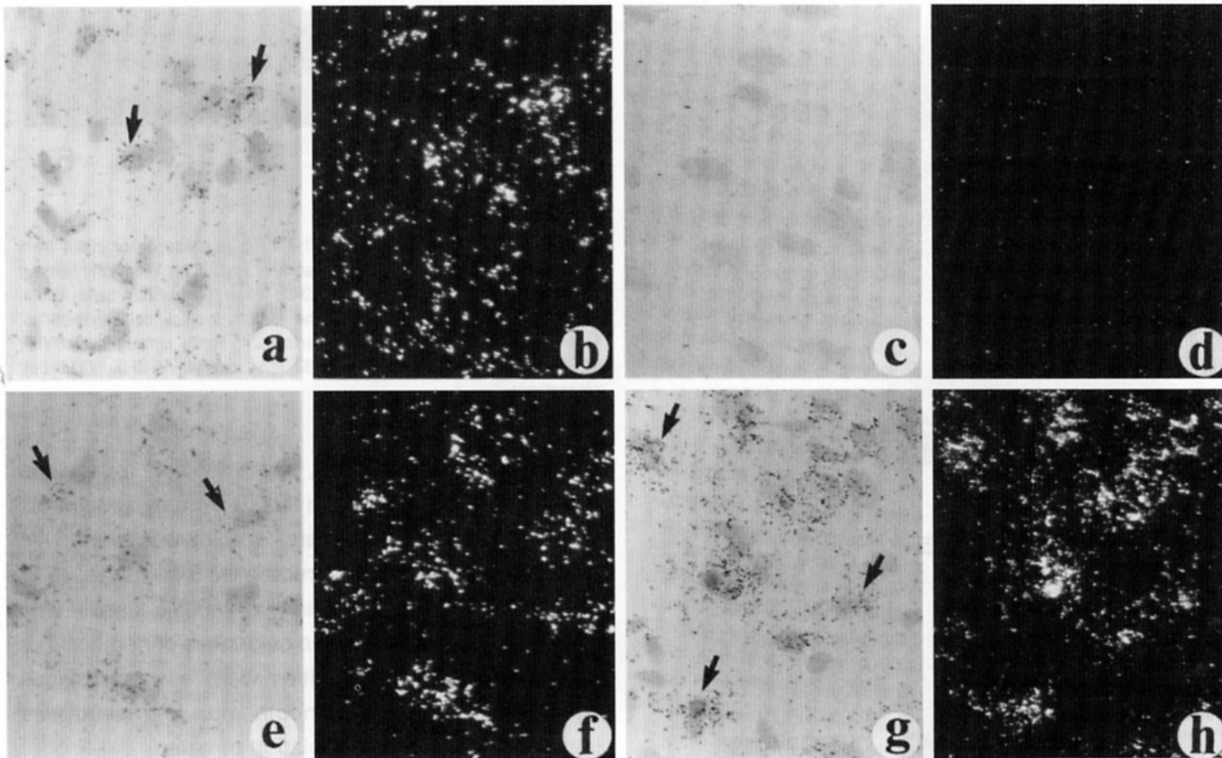

Figure 4.
ISH studies illustrating paired brightfield and darkfield photomicrographs of the same microscopic field. Cultured myometrial smooth muscle cells strongly express mRNA for VPF/VEGF (a and b), and for both of its receptors, flt-1 (e and f) and KDR (g and h). No labeling is seen with sense control probe (c and d).

The fact that uterine smooth muscle cells express VPF/VEGF receptors, whereas colon smooth muscle cells do not, is also of interest and provides evidence that smooth muscle cells derived from the muscular wall of different organs (colon and uterus) display important phenotypic differences.

The factors that regulate the expression of VPF/VEGF and its receptors in uterine smooth muscle cells are unknown. VPF/VEGF expression is under hormonal control in both the ovary (Kamat et al, 1995; Phillips et al, 1990) and uterine epithelium (Cullinan-Bove and Koos, 1993). Uterine smooth muscle cells express estrogen and progesterone receptors (Wilson et al, 1980), and it is possible that expression of VPF/VEGF and its receptors by smooth muscle cells in the uterus is related to activation of hormone receptors, but this question will require further study. Although no correlations were found between patient age or pathologic diagnosis and expression of VPF/VEGF and its receptors, the number of patients studied was relatively small, and nearly all were in the peri- or postmenopausal age range. It will be of interest to see if there is any correlation of expression of VPF/VEGF or its receptors by myometrial smooth muscle cells with the endometrial cycle in a larger group of normally cycling patients.

VPF/VEGF expression is also known to be regulated by hypoxia (Goldberg and Schneider, 1994; Koos and Olson, 1991; Shweiki et al, 1992) and by other growth factors in various cell types (ie, TGFα up-regulates expression in cultured keratinocytes (Detmar et al, 1994), and TGF-β and PDGF have been reported to up-regulate expression of VPF/VEGF in other cell types (Brogi et al, 1994; Dolecki and Connolly, 1991; Finkenzeller et al, 1992; Pertovaara et al, 1994)). The role of other growth factors in the regulation of the expression of VPF/VEGF and its receptors in myometrial smooth muscle cells remains to be determined.

In summary, we have demonstrated that a normal adult nonendothelial cell type, the uterine smooth muscle cell, expresses the VPF/VEGF receptors flt-1 and KDR and proliferates strongly in response to VPF/VEGF. Uterine smooth muscle cells also express VPF/VEGF. The precise role of VPF/VEGF and its receptors in uterine physiology and pathology will require further investigation. Although VPF/VEGF plays an important role in the angiogenesis and increased microvascular permeability associated with tumors, wound healing, and inflammatory conditions, our findings in uterine smooth muscle cells indicate that VPF/VEGF may also have additional important

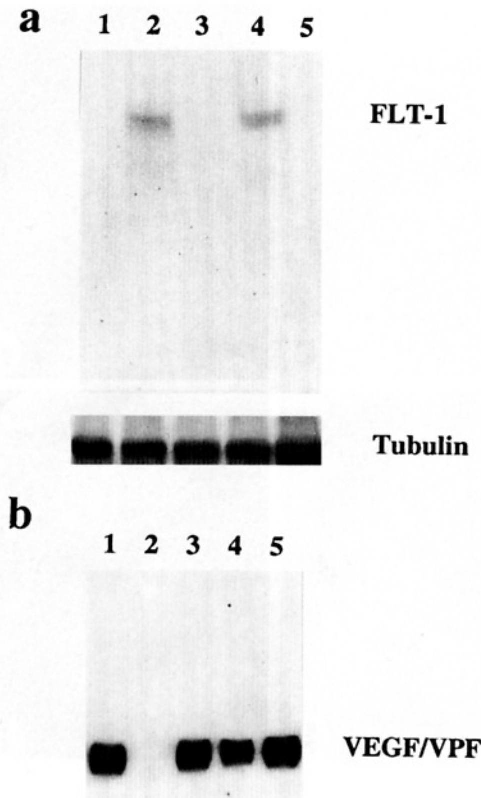

Figure 5.
a, Cultures of HaCat, dermal endothelial cells, dermal fibroblasts, and smooth muscle derived from colon and myometrium were scraped and solubilized into Laemmli buffer, and proteins were resolved by SDS-PAGE. The gel was subsequently transferred to nitrocellulose and probed with anti-flt-1 antibodies and with anti-tubulin (as a control for loading) followed by [^{125}I]-protein A for characterization of the immune complexes. Lanes: 1, HaCat; 2, dermal endothelial cells; 3, colon-derived smooth muscle cells; 4, myometrial-derived smooth muscle cells; and 5, dermal fibroblasts. b, Similar cultures to those indicated in A were grown in the presence of [35S]-methionine to radiolabel proteins metabolically for 48 hours. Conditioned media from the cultures were immunoprecipitated with anti-VPF/VEGF antibodies and subjected to SDS-PAGE analysis. Lanes: 1, HaCat; 2, dermal endothelial cells; 3, colon-derived smooth muscle cells; 4, myometrial-derived smooth muscle cells; and 5, dermal fibroblasts. An equal number of counts were loaded in Lanes 1 and 3 to 5. The whole sample was loaded in Lane 2 because no appreciable counts could be detected.

and previously unsuspected effects on cell types other than endothelial cells.

Materials and Methods

Human Tissues

In situ hybridization (ISH) studies were performed on sections of myometrium from 22 hysterectomy specimens (patients aged 40 to 72 years). In nine cases, the hysterectomy was performed for endometrial carcinoma and in 13 cases for other reasons, ie, leiomyomas, prolapse. The smooth muscle cells in the sections of myometrium studied by ISH were histologically typical and showed no evidence of neoplasia.

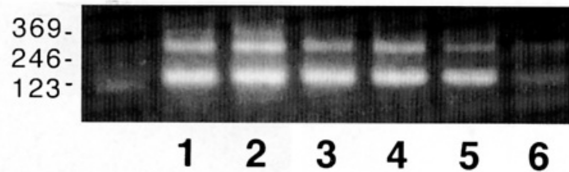

Figure 6.
Agarose gel showing PCR products from the amplification of cDNA from myometrial smooth muscle cells with primers specific for VPF/VEGF. Farthest left Lane, molecular weight marker (123-bp ladder, Gibco-BRL); Lanes 1 to 3, myometrial smooth muscle cells; Lane 4, human epidermal keratinocytes; Lanes 5 and 6, colonic smooth muscle cells. Bands correspond to VPF/VEGF$_{121}$ (171 bp), VPF/VEGF$_{165}$ (303 bp), and VPF/VEGF$_{189}$ (375 bp), respectively.

For cell culture studies, specimens of muscularis propria were obtained from five colectomy specimens (age range, 40 to 65 years) and myometrium was obtained from seven hysterectomies (age range, 35 to 65 years). Only grossly normal muscle tissue was chosen for the study, and no evidences of neoplasia or other histologic abnormalities were seen on microscopic sections of the smooth muscle. Tissue was immersed in DMEM for isolation of cells.

ISH

Tissues were fixed for 4 hours in 4% paraformaldehyde in PBS, pH 7.4, at 4°C and were then transferred to 30% sucrose in PBS overnight at 4°C. Tissue was then frozen in OCT (optimal cutting temperature) compound (Miles Diagnostics, Elkhart, Indiana) and stored at −70°C. Frozen 6-μm sections were subjected to ISH as previously described (ffrench-Constant et al, 1989). Briefly, slides were passed through xylene and graded alcohols; 0.2 M HCl; Tris/EDTA with 3 mg/ml proteinase K; 0.2% glycine; 4% paraformaldehyde in PBS, pH 7.4; 0.1 M triethanolamine containing 1/200 (v/v) acetic anhydride; and 2X SSC. Slides were hybridized overnight at 50°C with ^{35}S-labeled riboprobes in the following mixture: 0.3 M NaCl; 0.01 M Tris, pH 7.6; 5 mM EDTA; 50% formamide; 10% dextran sulfate; 0.1 mg/ml yeast tRNA; and 0.01 M dithiothreitol. Posthybridization washes included 2X SSC/50% formamide/10 mM dithiothreitol at 50°C; 4X SSC/10 mM Tris/1 mM EDTA with 20 mcg/ml ribonuclease at 37°C; and 2X SSC/50% formamide/10 mM dithiothreitol at 65°C and 2X SSC. Slides were then dehydrated through graded alcohols containing 0.3 M ammonium acetate, dried, coated with Kodak NTB 2 emulsion (Rochester, New York), and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer, and the slides were counterstained with hematoxylin. Antisense single-stranded ^{35}S-labeled VPF/VEGF RNA probe and its sense control

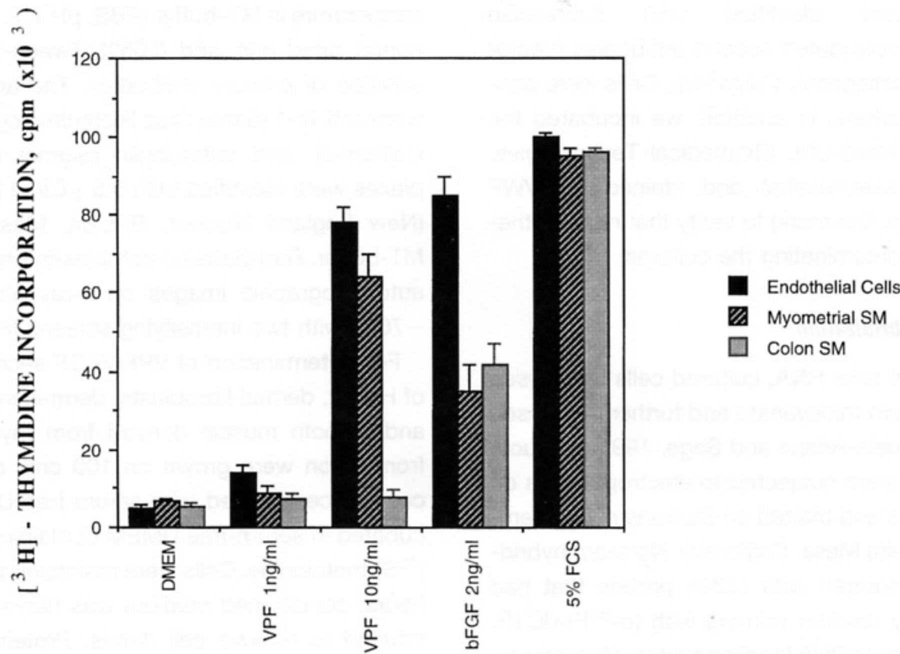

Figure 7.
Thymidine incorporation in response to VEGF/VPF (1 and 10 ng/ml), bFGF (2 ng/ml), 5% FCS, and DMEM alone was evaluated in cultures of dermal endothelial cells and smooth muscle derived from myometrium and colon. Subconfluent cultures in 48-well COSTAR dishes were incubated for 48 hours with 1 μCi/ml of [^{3}H]-thymidine, and incorporated cpm were measured by scintillation counting. Values are given as the mean ± SEM The mean represents the average of quadruplicate samples.

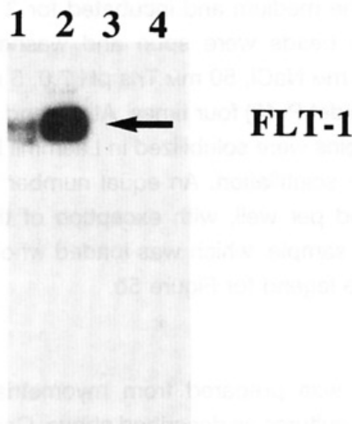

Figure 8.
Myometrial- and colon-derived smooth muscle cells were incubated in the presence or absence of 20 ng/ml VPF. Immune precipitates of anti-flt-1 were transferred and incubated with anti-phosphotyrosine antibodies. Lanes: 1, myometrial smooth muscle cells − VPF; 2, myometrial smooth muscle cells + VPF; 3, colon smooth muscle cells − VPF; 4, colon smooth muscle cells + VPF. Migration of phosphorylated flt-1 is indicated by *arrow.*

have been described previously (Brown et al, 1992a). The antisense probe hybridizes specifically with a region of VPF/VEGF mRNA common to all known VPF/VEGF-splicing variants. ^{35}S-labeled single-stranded antisense and sense RNA probes for the VPF/VEGF receptors flt-1 and KDR were designed by Robert W. Jackman (Assistant Professor, Department of Pathology, Beth Israel Hospital, Boston, Massachusetts) and have been described previously (Brown et al, 1993b).

Cell Isolation and Culture

Smooth muscle cells from colon or myometrium were obtained by enzymatic digestion with collagenase (Sigma, St. Louis, Missouri) (50 μg/ml) at 37°C for 2 hours. After digestion, the suspension was filtered through a 70-μm nylon mesh to remove undigested fragments. Cells were spun and washed four times in DMEM containing a 5-fold excess of antibiotics (500U/ml penicillin, 500 μg/ml streptomycin, and gentamycin) by repeated cycles of centrifugation and resuspension. Isolated cells were plated on tissue culture dishes coated previously with 50 μg/ml of fibronectin (Telios, San Diego, California). Cells were allowed to attach for 1 to 2 hours; these cells were mainly fibroblasts, and endothelial cells. Supernatants were removed and plated on flasks coated with Vitrogen (50 μg/ml) (Collagen Corporation, Palo Alto, California). After confluence, endothelial cells were removed by affinity-binding on E-selectin–coated magnetic beads after treatment with TNF for 4 hours. Remaining cells were characterized for the presence of smooth muscle actin (Sigma) and calponin (Sigma) by immunocytochemistry after fixation in 4% paraformaldehyde containing 0.2% Triton X-100. Immune

complexes were identified with fluorescein isothiocyanate-conjugated second antibodies (Vector Laboratories, Burlingame, California). Cells were positive to both markers. In addition, we incubated the cells with acetylated-LDL (Biomedical Technologies, Stoughton, Massachusetts) and stained for VWF (DAKO, Glostrup, Denmark) to verify that no endothelial cells were contaminating the cultures.

Northern Blot Hybridization

For extraction of total RNA, cultured cells were lysed in 4 M guanidinium thiocyanate and further processed as described (Iruela-Arispe and Sage, 1993). Aliquots of RNA (10 μg) were subjected to electrophoresis on 1% agarose gels and blotted on Biotrans nylon membranes (ICN, Costa Mesa, California). Northern hybridization was performed with cDNA probes that had been labeled by random priming with (α-^{32}P)-dCTP, using the Multiprime DNA labeling system (Amersham, Arlington Heights, Illinois), according to the manufacturer's instructions. The 546-bp human VPF/VEGF cDNA has been previously described (Detmar et al, 1994). The cDNA probes for flt-1 and KDR were amplified by PCR of human placental cDNA (Quick-Clone, Clontech, Palo Alto, California), using primers that surround a 2.446-kb fragment from position 245 to 2691 of the human flt-1 cDNA sequence and a 0.83-kb fragment from position 1650 to 2480 of the human KDR cDNA sequence. Purified products were cloned into pT7 (Novagen, Madison, Wisconsin) by means of single A overhangs. Clones were verified by restriction endonuclease mapping and by electrophoretic analysis of PCR products from two pairs of internal primers for each of the receptor fragments.

Western Blot Analysis and Immunoprecipitation

To determine the presence or absence of flt-1 on smooth muscle cells, cultures of HaCat cells, dermal microvascular endothelial cells, colon smooth muscle cells, myometrial smooth muscle cells, and dermal fibroblasts, were solubilized in Laemmli buffer (Laemmli, 1970) in the absence of dye. Total proteins were quantified utilizing a BCA kit (Pierce, Rockford, Illinois), and equal amounts of protein were loaded on a 7% SDS-PAGE gel. In independent experiments, the amounts of samples for SDS-PAGE were normalized according to total protein levels or cell number. Because similar results were obtained despite these different criteria, we have presented data from experiments in which the amount of each sample was normalized to cell number. Gels were then transferred to nitrocellulose and incubated for 16 hours at room temperature in MT-buffer (PBS, pH 7.7, containing 1% nonfat dried milk and 0.05% Tween-20) before the addition of primary antibodies. The antibodies used were anti-flt-1 (Santa Cruz Biotechnology, Santa Cruz, California), and anti-tubulin (Sigma) Immune complexes were identified with 0.5 μCi/ml [^{125}I]-protein A (New England Nuclear, Boston, Massachusetts) in MT-buffer. Radiolabeled complexes were detected as autoradiographic images on x-ray film exposed at −70°C with two intensifying screens (Kodak).

For determination of VPF/VEGF secretion, cultures of HaCat, dermal fibroblasts, dermal endothelial cells, and smooth muscle derived from myometrium and from colon were grown on 100 cm^2 dishes to near confluence, washed with serum-free DMEM, and incubated in serum-free DMEM containing 50 μCi/ml of [^{35}S]-methionine. Cells were maintained at 37°C for 48 hours; conditioned medium was harvested and centrifuged to remove cell debris. Proteinase inhibitors were added to produce the following final concentrations: 6.3 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 25 mM EDTA, and 5 μg/ml pepstatin A. Medium proteins were incubated end-over-end overnight with anti-VPF antibodies at 4°C. On the next day, protein A sepharose linked to anti-rabbit IgG was added to the medium and incubated for 2 additional hours. The beads were spun and washed in NET buffer (150 mM NaCl, 50 mM Tris pH 7.0, 5 mM EDTA, 0.05% Nonidet P-40) four times. At the end of the last wash, proteins were solubilized in Laemmli buffer and counted by scintillation. An equal number of counts were loaded per well, with exception of the dermal endothelial sample, which was loaded whole as indicated in the legend for Figure 5b.

RT-PCR

Total RNA was prepared from myometrial smooth muscle cell cultures as described above. Complementary DNA was synthesized using murine leukemia virus RT and oligo(dT) primers (GeneAmp Kit; Perkin-Elmer Cetus, Norwalk, Connecticut), according to the manufacturer's instructions. Amplification was performed through 35 cycles (30 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C), using the two VPF/VEGF specific primers: 5′ GACCACCTGCCTCCTGA-CACTTC 3′ (bases 433 to 455 in exon 3), and 5′ GAGTGTGTGCCCACTGAGGAGTC 3′ (bases 785 to 807 in exon 8) (Keck et al, 1989). The primers were chosen because they amplified exons 3 to 8 and allowed for distinguishing between the different VPF/VEGF splicing variants. PCR products of 171-bp, 303-bp, and 375-bp corresponded with VPF/VEGF$_{121}$, VPF/VEGF165, and VPF/VEGF$_{189}$, respectively.

Proliferation Assays

To determine the effect of VPF/VEGF on the proliferation of colon- and myometrial-derived smooth muscle cells, we cultured 10,000 cells per well of a 48-well COSTAR plate (COSTAR, Pleasanton, California) overnight at 37°C in complete DMEM. The medium was removed and fresh serum-free medium containing [^{3}H]-thymidine 1 μCi/ml and 0 to10 ng/ml VPF/VEGF; 2ng/ml bFGF; or 5% FCS was added to four replicate wells. Cells were cultured for 48 hours, washed, and fixed in 10% TCA. Incorporation of [3H]-thymidine was determined by scintillation counting as previously described (Iruela-Arispe and Sage, 1993). Experiments were performed in quadruplicate and were repeated in three independent isolates of cells from different individuals.

Phosphorylation Studies

The ability of endogenous VPF/VEGF to induce phosphorylation of flt-1 in an autocrine manner was examined by immunoprecipitation of flt-1 followed by Western blotting with anti-phosphotyrosine antibodies. Cultures of smooth muscle cells from myometrium and from colon were cultured for 3 days and washed; cells were solubilized in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS). flt-1 was immunoprecipitated with an antibody to the human protein (Santa Cruz Biotechnology) and protein A Sepharose beads (Pharmacia, Piscataway, New Jersey) linked to rabbit anti-mouse IgG. After several washes with NET buffer, beads were boiled and proteins were resolved on an SDS-PAGE gel. The gel was subsequently transferred to a nitrocellulose membrane, blocked, and probed with an anti-phosphotyrosine antibody (Signal Transduction Laboratories, Lexington, Kentucky). Immune complexes were detected by chemoluminescence (Amersham, Arlington Heights, Illinois).

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