Developmental expression and regulation of basic fibroblast growth factor and vascular endothelial growth factor in rat decidua and in a decidual cell line

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ABSTRACT

During pregnancy, the decidua is comprised of two separate tissues located either mesometrially or antimesometrially in the uterus. Trophoblast invasion takes place only in the mesometrial decidua, where extensive angiogenesis, essential for successful implantation, occurs. Both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been implicated in this phenomenon.

The aim of this study was to determine whether the expression of both growth factors is intrinsic to decidua and occurs in the absence of conceptuses, whether their genes are expressed specifically in the mesometrial decidua, the site of angiogenesis, and whether both growth factors are developmentally and hormonally regulated.

Decidual tissue was dissected from pseudopregnant rats and levels of both bFGF and VEGF mRNA were examined in mesometrial and antimesometrial decidua by semi-quantitative RT-PCR at different stages of pseudopregnancy. Although induction of decidualization triggered the mRNA expression of bFGF, VEGF mRNA expression remained unchanged. VEGF mRNA level was similar in both antimesometrial and mesometrial decidua, and remained constant throughout pseudopregnancy. In sharp contrast, bFGF mRNA was highly expressed in the mesometrial decidua at a time when extensive angiogenesis takes place in this tissue. Very little signal was observed in the antimesometrial decidua.

To examine the regulation of these growth factors, we used a temperature-sensitive decidual cell line developed by transforming antimesometrial decidual cells with SV-40 tsA 209 mutant virus. These cells express both bFGF and VEGF mRNA. Because progesterone is necessary for decidualization and decidua secretes prolactin (PRL)-related hormones, we examined the role of these hormones on VEGF and bFGF mRNA expressions. Neither progesterone nor PRL had any effect on VEGF mRNA levels. However, bFGF mRNA expression was greatly stimulated by PRL.

In conclusion, results of this investigation have revealed that bFGF, but not VEGF, mRNA becomes highly expressed in the mesometrial decidua at a time when extensive angiogenesis takes place in this tissue. Very little signal was observed in the antimesometrial decidua.

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INTRODUCTION

Decidualization in pregnant or pseudopregnant rat is marked by extensive proliferation and differentiation of endometrial stromal cells in response to either blastocyst implantation or artificial stimuli. Morphological changes that occur during decidualization have been extensively studied (De Feo 1967, O'Shea et al. 1983). Decidualization in the rat gives rise to two different cell populations, located on opposite sides of the uterine lumen (Bell 1983, Gu & Gibori 1995). The cells that decidualize in the antimesometrial site are highly differentiated and secrete several hormones and growth factors (Jayatilak et al. 1985, Gu et al. 1994, 1995, Gu & Gibori 1995), whereas the mesometrial decidua where blood
vessels gain access is the site of extensive angiogenesis. Angiogenesis is a fundamental process by which new blood vessels are formed (Folkman & Shing 1992) and it is essential in reproduction, development and wound repair. Decidualization is accompanied by extensive angiogenesis, and these two processes are interdependent for successful fetal survival. Angiogenesis, involving proliferation, maturation and migration of endothelial cells is not only regulated by ovarian steroids but also by angiogenic growth factors. The cells comprising the mesometrial region secrete α₂-macroglobulin (Gu et al. 1992), which is a potent protease inhibitor and, therefore, limits the invasion of trophoblast. α₂-Macroglobulin is also known to bind these growth factors (Dennis et al. 1989, Soker et al. 1993). The fibroblast growth factors (FGFs) are a distinct class of potent mitogens (Folkman & Klagsbrun 1987, Thomas 1987). These factors bind to heparin sulfate proteoglycans (Vlodavsky et al. 1987) and stimulate endothelial cells to migrate and form tubes (Montesano et al. 1986). Vascular endothelial growth factor (VEGF) also acts as mitogen specific to endothelial cells and it increases the vascular permeability in these cells (Ferrara & Henzel 1989, Gospodarowicz et al. 1989). VEGF mRNA expression is observed in the mouse uterus at the site of implantation (Chakraborty et al. 1995), and in artificially induced mouse decidual cells (Shweiki et al. 1993), whereas non-decidualized stromal cells fail to express this gene.

In this investigation, we examined the mRNA expression of these two potent angiogenic factors (bFGF and VEGF) in both the antimesometrial and mesometrial decidua of rats, throughout the course of their development. Because progesterone is also implicated in regulating the process of angiogenesis and the expression of these angiogenic factors in vivo (Cullinan-Bove & Koos 1993, Koos & Olson 1989, Rider & Psychoyos 1994), we examined the role of progesterone on the mRNA expression of bFGF and VEGF in a decidual cell line. Because decidual cells secrete decidual prolactin (PRL)-like hormones and possess the receptors for PRL (Gu et al. 1996) we also investigated the role of PRL in the expression of bFGF and VEGF mRNA.

MATERIALS AND METHODS

Materials

Cell culture supplies were purchased form Mediatech (Washington, DC, USA) unless otherwise indicated. Trypan blue and Hanks’ balanced salt solution (HBSS; without Ca²⁺ and Mg²⁺) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Taq DNA polymerase was procured from Perkin-Elmer Corp. (Foster City, CA, USA). Collagenase (type I) was obtained from Worthington Biochemical Corp. (Freehold, NJ, USA) and dispase (type II) and deoxyribonuclease were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA), and [α³²P]deoxy CTP (dCTP) obtained from Amersham (Arlington Heights, IL, USA).

Decidualization of animals

All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and approved by the institutional care and use committee. Pseudopregnancy was induced in Sprague–Dawley female rats by mating them with vasectomized male rats at the Harlan facilities (Madison, WI, USA). Rats were housed in a controlled temperature (20–22 °C) and photoperiod (14 h light: 10 h darkness). Purina rat chow (Ralston Purina, St Louis, MO, USA) and water were available ad libitum. Decidualization of the uterine endometrium was induced by scratching the antimesometrial surface of both uterine horns with a hooked needle on day 5 of pseudopregnancy under ether anesthesia. Rats were killed on days 6–14 of pseudopregnancy by an overdose of ether. The uterine horns were dissected out, freed from adjoining tissues and washed repeatedly in ice-cold PBS to remove excess blood. The antimesometrial and mesometrial decidua was dissected out as described previously (Gu & Gibori 1995).

Cell culture

Decidual cells (antimesometrial and mesometrial) were isolated and cultured as described earlier (Gu & Gibori 1995). The regulation of bFGF and VEGF mRNA was studied in an SV-40-transformed decidual cell line, termed GG-AD cells. The development and characterization of GG-AD cells has been described earlier (Srivastava et al. 1995). These cells were cultured at the permissive temperature (33 °C) in RPMI-1640 medium supplemented with 10% FBS, 2 × antibiotic–antimycotic solution, 1 mM sodium pyruvate, 1 × L-glutamine, 1 × non-essential amino acids and 0.5% d-glucose until they were 75% confluent. Cells were then transferred to non-permissive temperature (39 °C) for 3 days. All the experiments were conducted at 39 °C.
RNA extraction and reverse-transcription (RT)-PCR assay

Total RNA from dissected antimesometrial and mesometrial tissues were purified by homogenization in guanidinium thiocyanate and centrifugation through a cesium chloride cushion (Chirgwin et al. 1979), whereas total RNA from cultured GG-AD cells were isolated by a one-step guanidinium thiocyanate–phenol–chloroform extraction procedure (Chomczynski & Sacchi 1987).

For detecting bFGF mRNA, total RNA (1–5 µg) was reverse transcribed at 42 °C in the presence of random hexamer primers (100 pmol) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) in a 20-µl reaction mixture. Oligonucleotide primers specific to rat bFGF (5’GGA TTC CTG GCG ATC CA-3’ and 5’ AGA AGG TTA CAG ACG ATT CTC G-3’) were used for PCR amplification. A pair of oligonucleotide primers specific for a rat ribosomal protein L19 (5’-CGG AAT GTG-3’ and 5’-CTC TAG TAG TTC TGA GAC AGG-3’) was also included in each reaction as an internal control. A mixture containing the oligonucleotide primers (500 ng each), [α-32P]dCTP (2 µCi; 3000 Ci/mmol), dNTP (2 mM), MgCl2 (1·5 mM) and Taq DNA polymerase (2·5 U) was added to each reaction to a total volume of 90 µl. Amplification was carried out for 30 cycles using a 60 °C annealing temperature in a Perkin–Elmer/Cetus thermal cycler (Norwalk, CT, USA).

The conditions were such that amplification of the product was in the exponential phase and the assay was linear with respect to the amount of input RNA. For detecting VEGF mRNA, total RNA (1–5 µg) was reverse transcribed at 42 °C using Moloney murine leukemia virus reverse transcriptase and 100 pmol random hexamers in 20 µl reaction mixture. Primer pair specific for the rat VEGF sequence (5’-TCA CAT-3’ and 5’-AGA AGG TTA CAG ACG ATT CTC G-3’) were used for PCR amplification. A pair of L19 primers was also included in the reaction and the amplification was performed under conditions similar to those described for bFGF. Amplification was carried out for 30 cycles. The samples were separated on an 8% polyacrylamide non-denaturing gel. After autoradiography, the gel was dried and analyzed by a phosphorImager using Image Quant version software (Molecular Dynamics, Sunnyvale, CA, USA). The intensity of signals was normalized by using L19 as internal standard. The VEGF-specific primers used for PCR amplification gave three different PCR products (434, 564 and 631 bp), whereas L19 appeared at 194 bp. Two kinds of negative controls were prepared: 1) the reverse transcription was performed with total RNA without reverse transcriptase to detect the possible contamination in RNA samples by genomic DNA; 2) total RNA was omitted in reverse transcription. These controls confirmed that no contamination occurred during the course of the RT-PCR procedure.

Immunocytochemistry

Isolated antimesometrial and mesometrial cells were cultured on 12-mm glass coverslips that were coated with FBS and then washed three times with ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 1 h. Fixed cells were again washed three times in PBS, then treated for 15 min with 0·1 M glycine in PBS. The cells were permeabilized with 1% Triton X-100 in PBS for 4 min and washed three times with PBS. Non-specific antigenic sites were blocked with 1% BSA in PBS for 1 h at room temperature. Cells were then incubated with bFGF anti-rabbit IgG (Santacruz Biotechnology Inc., Santacruz, CA, USA) at 1 : 50 dilution and then washed with PBS. Cells were incubated with secondary antibody (anti-rabbit IgG-FITC) at 1 : 50 dilution from Santacruz Biotechnology Inc. for 1 h at room temperature and then washed to remove any non-specific bindings. Cells were mounted and immunostaining was visualized by fluorescence microscopy. For negative control, all the conditions remained the same, except that cells were not incubated with primary antibody.

RESULTS

We first examined the expression of VEGF mRNA by RT-PCR in the antimesometrial and mesometrial decidual tissues of rats from days 8 to 14 of pseudopregnancy, using VEGF-specific primers. Results revealed one major PCR product corresponding to 564 bp (Fig. 1B). In sharp contrast, bFGF mRNA levels were greater in the mesometrial decidua and its greatest expression was observed on day 11 of pseudopregnancy. bFGF mRNA declined thereafter, and totally disappeared by day 15 of pseudopregnancy (Fig. 1A).

To elucidate whether decidualization of the endometrial stroma induces a rapid expression of bFGF and VEGF mRNA, total RNA was obtained from uteri of pseudopregnant rats in which decidualization was induced on day 5 in one uterine horn, the other uterine horn serving as control. As shown in Fig. 2A, bFGF mRNA was barely detectable in the non-decidualized uteri on day 7, but in the decidualized uteri, there was a rapid
induction of bFGF mRNA. Nevertheless, VEGF mRNA was detectable equally both in the non-decidualized and the decidualized uteri (Fig. 2B).

To determine the expression of bFGF protein in the two populations of decidual cells, antimesometrial and mesometrial decidual cells were separated by elutriation and subjected to immunocytochemistry using the specific antibody to bFGF. Although bFGF was localized both in antimesometrial and in mesometrial cells, the signal was more intense in the mesometrial cells (Fig. 3). This observation corroborates the findings concerning bFGF mRNA expression, which was also more abundant in mesometrial decidua.

We recently developed an SV-40-transformed temperature-sensitive rat decidual cell line (Srivastava et al. 1995). To determine that this cell line indeed expresses bFGF and VEGF mRNA, we cultured the cells until confluence at permissive temperature (33°C) and total RNA was isolated. Meanwhile, another group of cells were cultured at 33°C until 75% confluence and then at a non-permissive temperature (39°C). Total RNA was obtained from these cells after they were cultured at 39°C for 1, 2, 3 and 4 days and subjected to RT-PCR using bFGF and VEGF-specific primers.
L19 was used as internal control. bFGF mRNA was detected in these cells cultured at both 33 °C and 39 °C, without any appreciable change (Fig. 4A). RT-PCR amplification of the total RNA obtained from the decidual cell line cultured at 33 °C and 39 °C for several days using VEGF-specific primers revealed three PCR products corresponding to 635, 564 and 434 bp respectively (Fig. 4B). The VEGF gene is alternatively spliced and gives rise to three transcripts encoding for protein of 188, 164 and 120 amino acids respectively that in turn form the active dimeric factors. The mRNAs for the VEGF164 and VEGF120 subunits are the dominant forms, whereas mRNA for the VEGF188 is inactive (Cullinan-Bove & Koos 1993).

To determine the progesterone regulation of expression of bFGF and VEGF mRNA, we cultured the SV-40-transformed decidual cells at 33 °C for several days until they were 75% confluent and then at 39 °C for 3 days. We have previously shown that these cells, when cultured at 39 °C, express progesterone receptor mRNA (Srivastava et al. 1995). Cells were then treated with progesterone (0, 1, 10, and 100 ng/ml) for 48 h. Total RNA was isolated from these cells and subjected to RT-PCR for detecting bFGF and VEGF mRNAs. L19 was used as an internal control. A single transcript of size 354 bp was discernible for bFGF mRNA, whereas three transcripts of sizes 631, 564 and 434 bp were detected for VEGF mRNA.

FIGURE 4. Expression of bFGF and VEGF mRNA in an SV-40-transformed decidual cell line at 33 °C (permissive) and 39 °C (non-permissive) temperatures.

SV-40-transformed decidual cells were cultured at 33 °C (permissive) until 75% confluent and total RNA was isolated from these cells. RNA was subjected to RT-PCR using (A) bFGF and (B) VEGF-specific primer. A group of cells that were confluent at 33 °C were cultured at 39 °C (non-permissive) for 4 days. Total RNA was prepared from these cells and subjected to RT-PCR for detecting bFGF and VEGF mRNAs. L19 was used as an internal control. A single transcript of size 354 bp was discernible for bFGF mRNA, whereas three transcripts of sizes 631, 564 and 434 bp were detected for VEGF mRNA.

decidual cell line at 33 °C until they were 75% confluent and then at 39 °C for 3 days. These cells were incubated with ovine PRL (0, 0.01, 0.1, 1 and 10 µg/ml) for 48 h. Total RNA was isolated from these cells and subjected to RT-PCR using (A) bFGF- and (B) VEGF-specific primers (B). L19 was used as an internal control for both reactions. Lower panel represents the quantification of the data (n=3). Gels were exposed to films for 24 h.

FIGURE 5. Effect of different concentrations of progesterone (P₄) on the expression of bFGF and VEGF mRNA in SV-40-transformed decidual cells.

Decidual cells were cultured at 33 °C for several days until they were 75% confluent and then at 39 °C for 72 h. Cells were treated with progesterone (0, 1, 10 and 100 ng/ml) for 48 h. Total RNA was isolated from these cells and subjected to RT-PCR using (A) bFGF- and (B) VEGF-specific primers (B). L19 was used as an internal control for both reactions. Lower panel represents the quantification of the data (n=3). Gels were exposed to films for 24 h.

DISCUSSION

The results of this investigation clearly demonstrate that the rat decidua expresses the genes for both bFGF and VEGF. Developmental studies revealed that VEGF mRNA remained expressed at similar levels throughout decidual development. In sharp contrast, bFGF mRNA expression was found to be
FGFs comprise a family of related proteins from at least nine distinct genes (Basillico & Moscatelli 1992, Miyamoto et al. 1993). Members of this growth factor family have been shown to have direct effects on endothelial cell growth, migration and protease synthesis. They are able to induce the formation of new blood vessels and, therefore, they have a direct role in angiogenesis (Gospodarowicz et al. 1987). bFGFs are not secretory proteins; they are, however, stored in the extracellular matrix and can be mobilized in a biologically active form by heparin or heparin sulfate (Vlodavsky et al. 1987, Folkman et al. 1988). The binding of FGF to heparin sulfate is a prerequisite for the binding of FGF to its high-affinity receptors on the cell surface (Rapraeger et al. 1991, Yayon et al. 1991). Cell-associated heparin sulfate proteoglycans appear to provide a sustained release reservoir that can mediate long-term responses to short exposures of bFGF (Moscatelli et al. 1991). bFGF is both chemotactic and mitogenic for endothelial cells (Friesel & Maciag 1988) and it induces a pathway leading to the phosphorylation and activation of mitogen-activated protein kinase (Johnson & Williams 1993). The expression of mRNA of this highly potent angiogenic factor in the decidua demonstrates that bFGF is instrumental in regulating the highly directional and stereospecific growth patterns of proliferating vessels during the decidual response. bFGF mRNA is expressed in decidua throughout the course of pseudopregnancy, and a striking feature of it is that it is expressed in abundance in the mesometrial decidua, where blood vessels gain access.

VEGF is a mitogen for endothelial cells in vitro and a potent angiogenic factor in vivo (Ferrara & Henzel 1989, Gospodarowicz et al. 1989, Ferrara et al. 1992). The presence of VEGF mRNA in the decidua suggests a role for it in angiogenesis. Such angiogenic activity of VEGF is probably due, not only to its mitogenic activity, but also to its ability to increase vascular permeability, which is believed to produce serum factors essential for new blood vessel growth (Connolly 1991). The mitogenic activity of VEGF is mediated by specific VEGF receptors, which are found on the cell surface of various endothelial cell types (Plouet & Moukadir 1990, Vaisman et al. 1990) and binding of VEGF to its cell surface receptor is dependent upon heparin-like molecules (Gitay-Goren et al. 1992). Recently, it has been shown that VEGF at approximately 2·2 nM concentration is capable of inducing the cells to invade the underlying matrix and form capillary-like tubules (Pepper et al. 1991), whereas bFGF is capable of inducing this response in concentrations about half those required with VEGF. Interestingly, these two growth factors have a potent

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**Figure 6.** Effect of ovine prolactin (PRL) on the expression of bFGF and VEGF mRNA in SV-40-transformed rat decidual cells. SV-40-transformed decidual cells were cultured at 33 °C until they were 75% confluent and then at 39 °C for 72 h. Cells were treated with different concentrations of PRL (0, 0·01, 0·1, 1 and 10 µg/ml) for 48 h. Total RNA isolated from these cells was subjected to RT-PCR using (A) bFGF- and (B) VEGF-specific primers. L19 was used as an internal control. Right panel represents the quantification of the data (n=3). Gels were exposed to films for 24 h.
synergistic effect on the proliferation of endothelial cells and the induction of angiogenesis (Pepper et al. 1991, Goto et al. 1993). Therefore, it is plausible that this synergism between bFGF and VEGF might be playing an important part in the regulation of angiogenesis in the rat decidua.

In order to study the hormonal regulation of these growth factors in decidua, we used an SV-40-transformed decidual cell line (Srivastava et al. 1995). These cells express bFGF, VEGF and progesterone receptor mRNA and, as progesterone is essential for the process of decidualization, we studied the effect of progesterone in vitro on the mRNA expression of bFGF and VEGF, using this cell line. However, progesterone was not able to alter the mRNA expression of either bFGF or VEGF. Progesterone is essential for stromal cell proliferation (Galassi 1968, Sakamoto et al. 1983) and, when progesterone action is blocked using a progesterone receptor antagonist, RU 486, stromal cell bFGF expression is lost, these cells stop dividing, and implantation does not occur (Rider & Psychoyos 1994). Our failure to detect any alteration in the bFGF mRNA expression despite the fact that these cells do possess progesterone receptors is probably due to the fact that the cells are derived from highly differentiated decidual cells and not from the stromal cells.

Decidual tissue is the source of PRL-like hormones or decidual luteotropins and it also possesses the receptors for PRL (Jayatilak et al. 1985, Gu et al. 1996). Similarly, placental tissue also secretes proteins of the prolactin and growth factor family (Soares et al. 1991). In mouse placenta, two such proteins have been identified – proliferin and proliferin-related proteins – and a direct role for such proteins have been identified – proliferin and its receptor mRNA in the mouse uterus around the time of implantation. Journal of Endocrinology 147 339–352.


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REFERENCES


Rider V & Psychoyos A 1994 Inhibition of progesterone receptor function results in loss of basic fibroblast growth factor expression and stromal cell proliferation during uterine remodeling in the pregnant rat. *Journal of Endocrinology* 140 239–249.


Soker S, Svaln CM & Neufeld G 1993 Vascular endothelial growth factor is inactivated by binding to α2-macroglobulin and the binding is inhibited by heparin. *Journal of Biological Chemistry* 268 7685–7691.


Yayıon A, Klagesbrun M, Eko JD, Leder P & Ormitz DM 1991 Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64 841–848.

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