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Abstract. FGF-2 and VEGF are potent angiogenesis inducers in vivo and in vitro. Here we show that FGF-2 induces VEGF expression in vascular endothelial cells through autocrine and paracrine mechanisms. Addition of recombinant FGF-2 to cultured endothelial cells or upregulation of endogenous FGF-2 results in increased VEGF expression. Neutralizing monoclonal antibody to VEGF inhibits FGF-2–induced endothelial cell proliferation. Endogenous 18-kD FGF-2 production up-regulates VEGF expression through extracellular interaction with cell membrane receptors; high-Mr FGF-2 (22–24-kD) acts via intracellular mechanism(s). During angiogenesis induced by FGF-2 in the mouse cornea, the endothelial cells of forming capillaries express VEGF mRNA and protein. Systemic administration of neutralizing VEGF antibody dramatically reduces FGF-2–induced angiogenesis. Because occasional fibroblasts or other cell types present in the corneal stroma show no significant expression of VEGF mRNA, these findings demonstrate that endothelial cell-derived VEGF is an important autocrine mediator of FGF-2–induced angiogenesis. Thus, angiogenesis in vivo can be modulated by a novel mechanism that involves the autocrine action of vascular endothelial cell-derived FGF-2 and VEGF.
apparent defects related to impaired angiogenesis (Ortega et al., 1991; Zhou et al., 1998).

FGF-2 exists in four different molecular weight isoforms: 18-kD or low molecular weight (LMW) FGF-2, and 22-, 22.5- and 24-kD or high molecular weight (HMW) FGF-2. The HMW forms derive from alternative translation initiation (CUG) codons and contain the complete LMW sequence in addition to an NH2-terminal extension of varying length (Moscatelli et al., 1987; Sommer et al., 1987; Florkiewicz and Sommer, 1989; Prats et al., 1989). The different forms of FGF-2 have been associated with different cell functions and cellular compartmentalization. LMW FGF-2 is released by the cells and stimulates cell migration, proliferation, and FGF receptor downregulation through binding to surface receptors; HMW FGF-2 forms primarily localize to the nucleus and modulate cell proliferation (Bugler et al., 1991; Mignatti et al., 1991; Florkiewicz et al., 1991; Quarto et al., 1991a,b; Renko et al., 1991; Bikfalvi et al., 1995). The biological activity of FGF-2 is mediated through a dual receptor system consisting of four high-affinity, tyrosine kinase receptors and low-affinity, heparan sulfate proteoglycans located at the cell surface (Moscatelli et al., 1987; Flau menhaft et al., 1989; Lee et al., 1989; Dionne et al., 1990; Keegan et al., 1991; Partanen et al., 1991). However, FGF-2 lacks a signal peptide that directs secretion through the classical secretory pathway (Abraham et al., 1986). Although the mechanism(s) by which FGF-2 is released from cells is/are still unknown (Mignatti and Rifkin, 1991; Mignatti et al., 1991, 1992), FGF-2 is found extracellularly and modulates several cell functions in an autocrine manner (Sato and Rifkin, 1988; Mignatti et al., 1991; Sato et al., 1991; Peverali et al., 1994; Bikfalvi et al., 1995; Klein et al., 1996). Because of its high affinity for heparin and heparan sulfate glycosaminoglycans, significant amounts of FGF-2 are associated with the extracellular matrix of in vitro cell cultures (Vlodavski et al., 1987; Bashkin et al., 1989; Flau menhaft et al., 1989; Rojeli et al., 1989). In vivo, FGF-2 has been detected in the basal lamina of blood capillaries, primarily at sites of vessel branching, and in the endothelium of the capillaries of some tumors (Folkman et al., 1988; Di Mario et al., 1989; Cordon et al. 1990; Schulze-Osthoff et al., 1990), suggesting that endothelial cell-derived FGF-2 may mediate angiogenesis with an autocrine mode of action. This hypothesis is supported by the observation that FGF-2 has an autocrine effect on several cell functions required for angiogenesis, including proliferation, migration, proteinase production, and integrin expression (Sato and Rifkin, 1988; Mignatti et al., 1991; Sato et al., 1991; Peverali et al., 1994; Bikfalvi et al., 1995; Klein et al., 1996). VEGF, the prototype member of a family of four structurally related growth factors, is a potent mitogen for micro- and macrovascular endothelial cells but lacks appreciable mitogenic activity for other cell types (Ferrara and Davis-Smyth, 1997; Senger et al., 1993). VEGF exists in five molecular species of 121, 145, 165, 189, and 206 amino acids (VEGF121, VEGF145, VEGF165, VEGF189, VEGF206) that derive from exon splicing of a single gene (Houck et al., 1991; Keck et al., 1989; Leung et al., 1991; Tisher et al., 1991; Politorak et al., 1997). VEGF165, a 46-kD homodimeric glycoprotein, is the predominant isoform produced by a variety of normal and transformed cells. VEGF121 is a freely soluble protein. The other VEGF isoforms show increased affinity for heparan sulfate proteoglycans: VEGF145 is soluble although a fraction can remain bound to the extracellular matrix; VEGF189 and VEGF206 are almost exclusively sequestered in the extracellular matrix (Ferrara et al., 1992; Houck et al., 1992; Park et al., 1993).

The promoter region of VEGF contains hypoxia-responsive elements in addition to several potential Ap-1, Ap-2 and Sp-1 binding sites, indicating that VEGF transcription can be enhanced in response to multiple stimuli (Tisher et al., 1991; Levy et al., 1995; Liu et al., 1995). Oxygen tension plays a major role in the regulation of VEGF expression in a variety of cell types, including endothelial cells. Several findings have implicated VEGF as the major mediator of the angiogenic effect of hypoxia (Sheweiki et al., 1992; Goldberg and Schneider, 1994; Minchencko et al., 1994; Liu et al., 1995; Shima et al., 1995; Forsythe et al., 1996). In addition, several cytokines and growth factors, as well as tumor promoters upregulate VEGF expression in many cell types (Garrido et al., 1993; Goldman et al., 1993; Pertovaara et al., 1994; Li et al., 1995; Tsai et al., 1995; Ryuto et al., 1996). It has been proposed that VEGF may act as a paracrine mediator for indirect-acting angiogenic factors, such as transforming growth factor beta (Brogi et al., 1994). However, the effect of cytokines and growth factors on VEGF expression in vascular endothelial cells has not been investigated.

Numerous lines of evidence implicate VEGF as a pivotal factor in the regulation of normal and pathological vasculogenesis and angiogenesis. VEGF promotes angiogenesis in different experimental models in vitro and in vivo (Leung et al., 1989; Plouet et al., 1989; Pepper et al., 1992; Nicosia et al., 1994; Phillips et al., 1995). The loss of even a single VEGF allele results in embryonic lethality, showing the irreplaceable role of this factor in the development of the vascular system (Carmeliet et al., 1996; Ferrara et al., 1996). In addition, VEGF has been shown to have therapeutic effects on coronary and limb ischemia (Takeshita et al., 1994; Pearlman et al., 1995; Harada et al., 1996; Isner et al., 1996). VEGF mRNA is markedly upregulated in the majority of human tumors so far examined (for review see Ferrara and Davis-Smyth, 1997). Its expression in several tumors has been correlated with high vascularity, lymph node metastasis, and liver metastasis, and a poorer prognosis than VEGF-negative tumors (Toi et al., 1994; Maeda et al., 1996). Antibodies to VEGF or expression of a dominant-negative VEGF receptor inhibit tumor growth in vivo without affecting tumor cell proliferation in vitro. In animals treated with anti-VEGF antibodies the density of blood vessels in tumor sections is lower than in the tumors of control animals, showing that the inhibitory effect of the antibody on tumor growth is mediated by a blockade of the angiogenic activity of VEGF (Kim et al., 1993; Mullauer et al., 1994; Warren et al., 1995; Borgstrom et al., 1996). These findings implicate VEGF as the major tumor angiogenesis factor so far identified.

In addition to promoting vasculogenesis and angiogenesis, VEGF enhances vascular permeability and induces fenestrations in the endothelium of small capillaries and venules (Senger et al., 1983; Roberts and Palade, 1995). An increase in vascular permeability may be required for
angiogenesis during tumor growth and wound healing (Dvorak, 1986). The observation of VEGF expression around microvessels whose endothelium is normally quiescent has generated the hypothesis that VEGF is also required for the maintenance of the differentiated state of blood vessels (Ferrara et al., 1992; Alon et al., 1995).

The findings that FGF-2 can modulate angiogenesis with an autocrine mechanism (Sato and Rifkin, 1988; Mignatti et al., 1991; Sato et al., 1991; Peverali et al., 1994; Bickf alvi et al., 1995; Klein et al., 1996) and that FGF-2 and VEGF have synergistic effects on angiogenesis (Pepper et al., 1992) prompted us to investigate potential interactions between these two potent angiogenic factors in vascular endothelial cells. Here we report that FGF-2 modulates endothelial cell expression of VEGF through both autocrine and paracrine mechanisms of action. In vivo the endothelial cells of quiescent vessels do not express VEGF; upon stimulation with FGF-2 the endothelium of newly forming capillaries produces VEGF. The systemic administration of VEGF antibody to mice that received corneal implants of FGF-2 pellets results in a dramatic decrease in angiogenesis, implicating endothelial cell VEGF as a major mediator of the angiogenic activity of FGF-2.

Materials and Methods

Materials

Human recombinant VEGF{sub}_{165} and mouse recombinant VEGF{sub}_{165} were purchased from R & D Systems, Inc. (Minneapolis, MN); anti-human VEGF antibody (αVEGF sc507) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the neutralizing monoclonal antibody to human VEGF, mAb #577B11, was a gift of Texas Biotechnology, Inc. (Houston, TX); mouse and rabbit nonimmune (n.i.) IgG were purchased from Sigma Chemical Co. (St. Louis, MO); human recombinant FGF-2 was obtained from Scios Nova and Synergen, Inc. (Boulder, CO); anti–FGF-2 antibody has been described (RS8; Pintucci et al., 1996); anti–von Willebrand factor antibody (vWF A0082) was purchased from Dako Corp. (Carpinteria, CA), and doxycycline from Sigma Chemical Co. Protein concentrations were measured by the Bio-Rad DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.

Cells and Media

Bovine aortic endothelial cells (BAE) and bovine capillary endothelial cells (BCE) were isolated as described (Folkman et al., 1979) and grown in DME supplemented with 10% FBS, 2 mM l-glutamine, and 500 μg/ml geneticin (G418; Sigma Chemical Co.). The blots were probed with an antibody to human VEGF (αVEGF sc507; Santa Cruz Biotechnology, Inc.) or to human rFGF-2 (R58; Pintucci et al., 1996) for 1 h at room temperature. After incubation with anti–FGF-2 antibody or mouse n.i. IgG. Control cultures received equivalent volumes of medium with no addition. The medium was replaced every third day with fresh medium with or without addition of FGF-2, VEGF, and the IgGs. Triplicate samples were trypsinized every 24 h and counted with a hemocytometer.

Transfection of NIH 3T3 Cells with FGF-2 cDNA under Control by the Tetracycline-dependent Transactivator

EcoRI–EcoRI cDNAs for LMW and HMW FGF-2 were cloned into the EcoRI site of the plasmid pUHD10-3 (provided by H. Bujard, Zentrum für Molekuleare Biologie der Universität, Heidelberg, Germany), which contains the binding sequence for the tetracycline-controlled transactivator (rtTA) in front of a multiple cloning site (Gossen et al., 1995). NIH 3T3 cells were transfected with the plasmid pUHD172-Neo (also provided by H. Bujard), which contains a mutated form of rtTA spliced to a nuclear translocation sequence and the neomycin resistance gene (Gossen et al., 1995). Transfected cells were selected in medium containing 500 μg/ml of G418. One clone of G418-resistant cells that constitutively expressed rtTA was cotransfected with the plasmids pUHD–LMW FGF-2 or pUHD–HMW FGF-2 and the plasmid pCEP4 containing the hygromycin resistance gene (Invitrogen, Carlsbad, CA). Stable cotransfectants were selected in medium containing 500 μg/ml of both G418 and hygromycin (Calbiochem-Novabiochem, San Diego, CA). Hygromycin/G418-resistant cells were characterized for FGF-2 expression by Western blotting of extracts of cells grown in serum-free medium with or without 1 μg/ml of doxycycline for 24 h.

Preparation of SK-Hep1 Cell-conditioned Medium

Confluent cultures of SK-Hep1 cells in 150 cm{sup 2} culture flasks were washed twice with PBS and incubated for 17 h in the presence of 15 ml of serum-free DMEM. The culture supernatant was centrifuged at 1,000 g for 15 min and either used immediately or stored at −20°C.

Northern Blotting

Total RNA was extracted from cells with the Trizol reagent (GIBCO BRL) according to the manufacturer’s instructions. The RNA (20 μg) was run in a 1% formaldehyde–agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim Biochemicals, Indianapolis, IN). The blot was prehybridized in digoxigenin (DIG) Easy Hybridization Buffer (Boehringer Mannheim Biochemicals) for 2 h at 42°C and then hybridized overnight at 42°C with a 403-bp DIG-labeled cDNA probe to human VEGF{sub}_{165}, obtained by reverse transcriptase–PCR using the primers described in Houk et al. (1991). The cDNA was labeled by running the PCR reaction in the presence of DIG-labeled nucleotides (Boehringer Mannheim Biochemicals). After hybridization, the membrane was washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 1× SSC, 0.1% SDS at 50°C for 15 min. The detection of the probe was performed using the Genius 7 kit (Boehringer Mannheim Biochemicals) according to the manufacturer’s instructions. The membranes were exposed to autoradiographic films (Hyperfilm MP; Amersham Life Technologies, Arlington Heights, IL) for 1 min.

Western Blotting

Confluent endothelial cells or NIH 3T3 cells in 100-mm dishes were washed with PBS and incubated overnight in serum-free medium. The conditioned medium was concentrated 80-fold by ultrafiltration in Centricon tubes (Amicon, Inc., Beverly, MA). The cells were lysed in 100 mM Tris–HCl, pH 8.1, containing 0.5% Triton X-100, 10 μg/ml leupeptin (Sigma Chemical Co.), and 400 μM Pefablock (Boehringer Mannheim Biochemicals). Cell extract protein (200 μg) from endothelial cells, 50 μg from NIH 3T3 cells) or conditioned medium medium was electrophoresed in SDS–12% polyacrylamide gel under reducing conditions and then blotted to a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Waters Chromatography, Bedford, MA) for 4 h at 45 V. The membrane was incubated with 5% skim milk (Carnation; Nestlé Food Co., Glendale, CA) in 20 mM Tris buffer, pH 7.4, overnight at 4°C to block nonspecific binding. VEGF or FGF-2 were detected by incubating the membrane with 0.2 μg/ml of antibody to human VEGF (αVEGF sc507; Santa Cruz Biotechnology, Inc.) or to human rFGF-2 (R58; Pintucci et al., 1996) for 1 h at room temperature. After incubation with

Cell Proliferation Assay

HUVE or HAE cells were seeded into 96-well plates (2.5 × 10{sup 3} cells/well) in 50 μl of endothelial cell basal medium supplemented with heparin and 1% FCS. After overnight incubation, FGF-2 or VEGF were added to a final concentration of 10 or 30 ng/ml, respectively, in the presence or absence of 10 μg/ml of either anti-human VEGF monoclonal antibody or 100 μl of endothelial cell basal medium supplemented with heparin and 1% FCS. After overnight incubation, FGF-2 or VEGF were added to a final concentration of 10 or 30 ng/ml, respectively, in the presence or absence of 10 μg/ml of either anti-human VEGF monoclonal antibody or
horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5,000) for 1 h (Amersham Life Technologies), immune complexes were detected with the enhanced chemiluminescence ECL™ detection system (Amersham Life Technologies). The membranes were exposed to autoradiographic films (Hyperfilm MP; Amersham Life Technologies) for 10 s–1 min.

**Metabolic Labeling and Immunoprecipitation**

Confluent endothelial cells were incubated for 2 h with SK-Hepl cell-conditioned medium or with control medium. After incubation with methionine/cysteine-free DME for 1 h, the medium was replaced with methionine/cysteine-free DME containing 150 μCi/ml of [35S]methionine/cysteine (ICN Biomedicals, Inc., Costa Mesa, CA) and the incubation was continued for 4 h. The medium was collected and centrifuged at 1,000 × g (Amersham Life Technologies) for 7 d at 80°C. The dried gels were exposed to autoradiographic films (Hyperfilm MP; Amersham Life Technologies) for 7 d at 80°C.

**Densitometry**

Northern blot and Western blot bands were analyzed with a Shimadzu scanning densitometer (model C6-93-01PC; Shimadzu Scientific Instruments, Inc., Columbia, MD) using dedicated software.

**In Vivo Angiogenesis Assay**

Angiogenesis assays in the mouse cornea were performed as described (Chen et al., 1995). Briefly, a corneal pocket was created with a modified von Graefe cataract knife in both eyes of 4–6-wk-old C57B mice (The Jackson Laboratory, Bar Harbor, ME). One 0.34 × 0.34-mm sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) pellet coated with hydron polymer type NCC (IFN Sciences, New Brunswick, NJ), containing 50 ng of rFGF-2 was implanted into each pocket. Pellets without rFGF-2 were used as negative controls. The corneas were routinely examined by slit-lamp biomicroscopy on postoperative days 5–7. After examination, the mice were killed and the eyes excised, embedded in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC), and then frozen in a mixture of dry ice and 2-methylbutane (Fisher Scientific Co.). The slides were counterstained with Gil's hematoxylin 1 (Sigma Chemical Co.).

**Immunohistochemistry**

30-µm frozen sagittal sections of mouse eyes were fixed in 4% paraformaldehyde in PBS for 15 min and then incubated with 1 µg/ml goat n.i. IgG for 1 h to block nonspecific binding. To detect VEGF or von Willebrand factor (vWF), the specimens were incubated overnight at 4°C with 2 µg/ml of rabbit antibody to murine VEGF (αVEGF sc507) or with 2 µg/ml of rabbit antibody to human vWF that cross-reacts with mouse vWF. Rabbit n.i. IgG was used as a negative control. The sections were washed in 20 mM Tris buffer, pH 7.4, incubated with biotinylated goat anti–rabbit antibody serum (Vectorstain ABC Kit; Burlingame, CA) for 45 min and subsequently in 1% Triton X-100 (Sigma Chemical Co.) for 30 min. After washing in 20 mM Tris buffer, pH 7.4, avidin–biotin amplification was performed according to the manufacturer’s instructions (Vectorstain ABC Kit). Anti–genotype antibodies complexes were detected by incubation with 3,3-diaminobenzamine (Sigma Chemical Co.) at room temperature for 10 min. The slides were counterstained with Gill’s hematoxylin 1 (Sigma Chemical Co.).

**In Situ Hybridization**

Single-stranded, sense (S) and antisense (AS) RNA probes were generated by run-off in vitro transcription in the presence of DIG-labeled nucleotides (Transcription kit; Boehringer Mannheim Biochemicals) of a linearized pCR®II plasmid (TA cloning kit; Invitrogen) containing the 403-bp VEGF cDNA insert used for Northern blotting. 30-µm frozen sections of mouse eyes were fixed in 4% paraformaldehyde in PBS for 15 min, digested with proteinase K (1 µg/ml; Boehringer Mannheim Biochemicals) at room temperature for 10 min and then acetylated with 0.25% acetic anhydride in 100 mM triethanolamine. Hybridization with 0.2 µg/ml of the DIG-labeled S or AS VEGF riboprobe was performed in deionized 40% formamide, 10% dextran sulphate, 1% Denhardt’s solution, 4× SSC, 10 mM DTT, and 1 mg/ml sheared salmon sperm DNA overnight at 42°C in a moist chamber. The slides were washed three times in 0.1× SSC for 20 min at 55°C and digested with RNase A (20 µg/ml; Boehringer Mannheim Biochemicals). The immunological detection was performed using sheep anti-DIG IgG conjugated with alkaline phosphatase 0.5 U/ml (anti-DIG-AP Fab fragment; Boehringer Mannheim Biochemicals) according to the manufacturer’s instructions. The chromogenic reaction with NBT/BCIP (Boehringer Mannheim Biochemicals) was developed in 100 mM Tris buffer, 50 mM NaCl, 50 mM MgCl2, and 1 mM levamisole, pH 9.5, for 4 h at room temperature.

**Results**

FGF-2 Stimulates Endothelial Cell Expression of VEGF through Both Autocrine and Paracrine Mechanisms

To test the effect of FGF-2 on VEGF expression by endothelial cells, confluent BCE or BAE cells were incubated for 4 h with serum-free medium or with medium containing 10 ng/ml of human recombinant FGF-2 (rFGF-2). Northern blotting with a cDNA probe to human VEGF showed a band of 3.9 kb, consistent with the expected size of VEGF mRNA (Tischer et al., 1991; Shima et al., 1995) (Fig. 1 A). The intensity of this band was ten- and fivefold higher in rFGF-2–treated BCE and BAE cells, respectively, than in control cells. Western blotting under nonreducing conditions with antibody to human VEGF showed a band of 46 kD that comigrated with human recombinant VEGF165 (R & D Systems) in medium conditioned by FGF-2–treated endothelial cells. This band was very faint or absent in the conditioned medium or extracts of control BCE or BAE cells (Fig. 1 B). Reverse transcriptase–PCR with primers for VEGF (Houk et al., 1991) showed two transcripts whose sequences corresponded to VEGF165 and VEGF121 and a minor transcript corresponding to VEGF189 (data not shown). However, Western blotting of cell-conditioned medium or extract showed no immunoreactive proteins other than VEGF165. These data showed that exogenous FGF-2 upregulates the expression of VEGF165 in cultured endothelial cells.

The endothelial cells we used had been selected in our laboratory for low expression of endogenous FGF-2, relative to other strains of BCE or BAE cells. The cells expressed 18, 22, and 24 kD FGF-2, as assessed by Western blotting with antibody to human rFGF-2 (Fig. 2 A). Because in the absence of exogenous rFGF-2 BCE or BAE cells did not express VEGF (Fig. 1), this finding indicated that either VEGF expression is regulated only by exogenous FGF-2 or that the levels of endogenous FGF-2 in our endothelial cells were too low to induce VEGF expression. To test these hypotheses, BCE cells were incubated with SK-Hepl hepatoma cell-conditioned medium, which up-regulates endothelial cell expression of FGF-2 mRNA (Peverali et al., 1994). Consistent with previous findings (Moscatelli et al., 1986b; Peverali et al., 1994), Western blotting of concentrated SK-Hepl cell-conditioned medium showed no detectable FGF-2 (<0.25 ng/ml) (Fig. 2 A). Incubation with SK-Hepl cell-conditioned medium resulted in a five- and twofold increase in LMW FGF-2 and HMW FGF-2, respectively (Fig. 2 A), and in a fourfold increase in VEGF mRNA (Fig. 2 B). Addition of neutralizing rFGF-2 IgG (10 µg/ml) decreased VEGF mRNA expression to control levels. In contrast, n.i. IgG was ineffective.
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Seghezzi et al.
HMW FGF-2 (Fig. 2
Our endothelial cells expressed both LMW FGF-2 and
Mechanisms of Action
FGF-2 Modulate VEGF Expression through Different
Endogenous LMW FGF-2 and HMW
VEGF Mediates FGF-2--induced Proliferation of
Endothelial Cells
To test the functional significance of VEGF produced by
endothelial cells stimulated with FGF-2, we characterized
the effect of neutralizing monoclonal antibody to VEGF
on the proliferation of endothelial cells treated with FGF-2.
Because the monoclonal antibody we used was raised
against human VEGF, HUVE or HAE cells were used for
these experiments. As shown in Fig. 3 (A and C), addition of
VEGF antibody to FGF-2--treated HUVE or HAE cells
inhibited cell proliferation by 100 and 50%, respectively.
In control cultures, the VEGF antibody completely neu-
tralized the activity of recombinant VEGF on HUVE or
HAE cell proliferation (Fig. 3, B and D). Thus, endothe-
rial cell-derived VEGF is an important downstream medi-
ator of the mitogenic activity of FGF-2.
Endogenous LMW FGF-2 and HMW
VEGF165 expression was increased in FGF transfectant cell
mRNA is shown as a control. This experiment was repeated three
times with similar results.

VEGF Mediates FGF-2--induced Proliferation of
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Endogenous LMW FGF-2 and HMW
FGF-2 Modulate VEGF Expression through Different
Mechanisms of Action
Our endothelial cells expressed both LMW FGF-2 and
HMW FGF-2 (Fig. 2 A). To characterize possible differen-
tial roles of the FGF-2 isoforms in VEGF upregulation we
absence or in the presence of doxycycline, a tetracycline an-
alogue (data not shown). However, HMW FGF-2 transfec-
tants had a higher growth rate than LMW FGF-2 transfec-
tants. Addition of doxycycline to the culture medium resulted in a dramatic upregulation of both FGF-2 and VEGF165 expression by the LMW FGF-2 and the HMW FGF-2 trans-
fectants (Fig. 5) but did not affect FGF-2 or VEGF expres-
sion in control, vector-transfected cells (data not shown).

To confirm that VEGF expression in NIH 3T3 cells was mediated by FGF-2, HMW FGF-2 and LMW FGF-2 transfectants were grown for 9 h in the presence of neu-
tralizing antibody to FGF-2. VEGF expression was char-
acterized by Northern blotting (data not shown) and by Western blotting (Fig. 6). Addition of the antibody abol-
ished VEGF165 expression in a dose-dependent manner in LMW FGF-2 transfectants but had no effect on HMW FGF-2 transfec-tants. This result is consistent with previous findings of different cell compartmentalization and mecha-
nisms of action of LMW FGF-2 and HMW FGF-2 (Quarto et al., 1991b; Bikfalvi et al., 1995; Klein et al., 1996).

These data showed that VEGF expression is modulated by endogenous FGF-2 as well as by exogenous FGF-2. VEGF upregulation by endogenous LMW FGF-2 requires release of FGF-2 from the cells and interaction with cell membrane receptors. In contrast, endogenous HMW FGF-2 acts through an intracellular mechanism.

**FGF-2 Induces VEGF Expression in the Endothelial Cells of Growing Capillaries**

To test the hypothesis that FGF-2 can upregulate endothelial cell expression of VEGF in vivo, we used the angio-
genesis assay in the mouse cornea. Hydron pellets contain-
ing either 50 ng of rFGF-2 or no rFGF-2 were implanted in corneal pockets of C57B mice 1 mm away from the limbic vessels. 6 d after implantation, when maximum capillary formation was observed in eyes with the rFGF-2 pellets, 30-
m agar sagittal sections of the entire eye were prepared and analyzed both by in situ hybridization and by immu-
nohistochemistry.

In situs hybridization with a DIG-labeled antisense VEGF riboprobe of sections of eyes that received sham pellets showed that the endothelial cells of the limbic ves-
sels expressed no detectable VEGF mRNA (Fig. 7 A). In sections of eyes that received rFGF-2 pellets, the VEGF riboprobe hybridized to VEGF mRNA present in the endo-
thelial cells of the newly formed capillaries in the limbic region (Fig. 7 B). Photomicrographs taken in a through-focus
rFGF-2 results in VEGF expression by the endothelial vascular endothelium, induction of angiogenesis with 10). Thus, although no VEGF is expressed by quiescent producing cells were indeed endothelial cells (Figs. 9 and 10). Western blotting analysis of medium conditioned by LMW FGF-2 or HMW FGF-2 transfectants (clones I and 3 shown in Fig. 4) in the absence or in the presence of the indicated concentrations of anti-FGF-2 antibody (aFGF-2) or n.i. (n.) IgG (50 μg/ml). (A) LMW FGF-2 transfectants (clone 3). (B) HMW FGF-2 transfectants (clone I). FGF-2 expression by these clones is shown in Fig. 4 A. The cells were preincubated for 3 h in serum-free medium with or without the indicated concentrations of anti-FGF-2 antibody or n.i. IgG; the medium was replaced with fresh, serum-free medium with or without anti-FGF-2 antibody or n.i. IgG and the incubation was continued for 9 h. The conditioned medium was analyzed by Western blotting under reducing conditions with anti-VEGF antibody as described in Materials and Methods. Recombinant VEGF<sub>165</sub> (10 ng) was run as a control in the leftmost lane of the gels shown in A and B. This experiment was repeated three times with similar results.

Endothelial Cell VEGF Mediates FGF-2–induced Angiogenesis

To investigate the functional significance of endothelial cell expression of VEGF during FGF-2–induced angiogenesis, 18 mice that received corneal implants of pellets containing rFGF-2 (50 ng) in both eyes were randomized into three groups of six animals and given i.v. injections of either PBS, PBS containing n.i. IgG (100 μg), or neutralizing anti-human VEGF monoclonal antibody (100 μg). This antibody neutralizes mouse VEGF in in vitro proliferation assay almost as efficiently as human VEGF (Fig. 11; see Fig. 3 for comparison). The mice were injected 1 d before pellet implantation and on postoperative days 1 and 3. The corneas were photographed on day 5.

The animals injected with anti-VEGF antibody consistently showed markedly reduced angiogenesis relative to control mice injected with PBS or n.i. IgG. As shown in Fig. 12, the length of the vessels was the same in the three groups. However, the capillaries of mice treated with anti-VEGF antibody appeared to be consistently reduced in number, and were thinner and less branched than in control animals. Similar results were obtained when the VEGF antibody (1 μg) or n.i. IgG were added in the same pellets containing rFGF-2 (Fig. 13).

To quantitate these effects, the number of vessels was measured on enlarged photographs of all the eyes. Because vessel branching also appeared to be reduced in antibody-treated animals, the vessels were counted both proximally to and distally from the limbus (i.e., close to the pellet). As shown in Table I, in control mice the number of vessels distal from the limbus was 2.0- to 2.5-fold higher than in the paralimbic region. In mice receiving VEGF antibody either by intravenous injection or by corneal implant the number of vessels proximal to the limbic region showed only 12 and 26% decrease, respectively. However, distally from the limbus, the systemic and the topical antibody treatments resulted in
Figure 7. Expression of VEGF mRNA by vascular endothelium in vivo. In situ hybridization with DIG-labeled sense or antisense VEGF riboprobes of adjacent 30-μm sections of mouse corneas that received either (A) sham pellets or (B) pellets containing 50 ng of rFGF-2. Implantation of pellets in the cornea, preparation of the probes, and in situ hybridization were carried out as described in Materials and Methods. Contrast was enhanced by computer to increase the appearance of the reaction product. Arrowheads, vessel’s wall. (A) Sections of limbic vessels show no hybridization with the probes. (B) Sections of newly forming capillaries in the stroma of the cornea show hybridization of the endothelium with the antisense but not with the sense probe. Hybridization signals (brown-black staining) are present only in the endothelium of newly formed vessels in FGF-2–treated eyes.

Discussion

Experimental evidence has shown that angiogenesis is regulated by a variety of growth factors and cytokines with a paracrine mode of action. FGF-2 and VEGF are the most potent angiogenesis inducers and have a synergistic effect on angiogenesis (Pepper et al., 1992; Asahara et al., 1995). In this work we provide evidence that vascular endothelial cell-derived FGF-2 and VEGF are involved in a cascade of growth factor activities that modulate angiogenesis with an autocrine mechanism of action. In this cascade, endogenous or exogenous FGF-2 regulates endothelial cell expression of VEGF, which is a major autocrine mediator of FGF-2-induced angiogenesis. These conclusions are based on the following observations: (a) although quiescent endothelial cells in vitro do not express VEGF, addition of exogenous FGF-2 or upregulation of endogenous FGF-2 expression result in increased VEGF synthesis; (b) antibody to VEGF inhibits FGF-2-induced proliferation of endothelial cells; (c) in cultured cells endogenous FGF-2 upregulates VEGF expression through both extracellular and intracellular mechanisms: VEGF upregulation by LMW FGF-2 requires FGF-2 release from the cells and interaction with cell membrane receptors; HMW FGF-2 acts through an intracellular mechanism; (d) although the quiescent endothelial cells of the limbic vessels of mouse cornea express no VEGF, during angiogenesis induced by implantation of FGF-2 pellets the endothelial cells of newly formed capillaries express both VEGF mRNA and protein; VEGF mRNA is located primarily to the endothelium of branching vessels; and (e) systemic administration of neutralizing VEGF antibody inhibits angiogenesis induced by FGF-2 in the mouse cornea.

Our cultured endothelial cells expressed significant amounts of FGF-2 but no VEGF. Upregulation of VEGF mRNA and protein required addition of exogenous FGF-2 or upregulation of endogenous FGF-2, indicating that the endogenous levels of FGF-2 were not sufficient to induce VEGF expression. This conclusion is also supported by our experiments with NIH 3T3 fibroblasts transfected with FGF-2 cDNA and the tetracycline resistance transactivator. Because the tetracycline-controlled transactivator was leaky, in the absence of doxycycline LMW FGF-2 and HMW FGF-2 transfectants expressed detectable amounts of FGF-2. However, the expression of VEGF was very low or below detection limit (refer to Fig. 5). Upregulation of FGF-2 synthesis...
by addition of doxycycline resulted in a dramatic increase in VEGF expression. Therefore, the level of endogenous FGF-2 or the presence of exogenous FGF-2 are important factors in the regulation of VEGF expression in endothelial cells.

Our characterization of NIH 3T3 cells transfected with LMW FGF-2 or HMW FGF-2 cDNA showed that both HMW FGF-2 and LMW FGF-2 upregulate VEGF expression. Transfected that constitutively express high levels of LMW FGF-2 or HMW FGF-2 also produce high levels of VEGF, whereas control, vector-transfected cells express no VEGF. LMW FGF-2 and HMW FGF-2 transfecteds produce comparable levels of VEGF. However, the high amounts of FGF-2 produced by these cells may mask differences in the efficiency of VEGF regulation between LMW FGF-2 and HMW FGF-2. Two observations suggest that LMW FGF-2 may upregulate VEGF expression more efficiently than HMW FGF-2. Treatment of endothelial cells with SK-Hep1 cell-conditioned medium resulted in five- and twofold increased synthesis of LMW FGF-2 and HMW FGF-2, respectively. Concomitantly, VEGF mRNA and VEGF<sub>165</sub> levels were also upregulated. Addition of neutralizing antibody to FGF-2 blocked the upregulation of VEGF mRNA and protein. Because HMW FGF-2 is primarily intracellular and acts through an intracrine mechanism(s) (Bugler et al., 1991; Florkiewicz et al., 1991; Quarto et al., 1991a,b; Renko et al., 1991; Bikfalvi et al., 1995), the antibody did not neutralize HMW FGF-2 activity. However, the upregulation of HMW FGF-2 did not result in increased VEGF levels. This observation is consistent with the results of our analysis of VEGF expression in clones of NIH 3T3 cells transfected with FGF-2 cDNA and the tetracycline resistance transactivator. In the absence of doxycycline, LMW FGF-2 transfecteds expressed low amounts of both LMW FGF-2 and VEGF. In contrast, the HMW FGF-2 transfecteds had higher levels of HMW FGF-2 but expressed no VEGF (refer to Fig. 5).

Neutralizing antibody to FGF-2 abolished VEGF expression in NIH 3T3 cells that produce LMW FGF-2 but had no effect on cells that express HMW FGF-2, showing that LMW FGF-2 and HMW FGF-2 modulate VEGF ex-

Figure 8. Expression of VEGF mRNA by the endothelial cells of branching capillaries. Photomicrographs taken in a through-focus series (1-μm steps) of a 30-μm-thick section of mouse cornea hybridized in situ with a DIG-labeled antisense ribo-probe to VEGF. A hydron pellet containing 50 ng of rFGF-2 was implanted in the cornea 5 d before sectioning. Implantation of the pellet in the cornea, preparation of the probes, and in situ hybridization were carried out as described in Materials and Methods. Contrast was enhanced by computer to increase the appearance of the reaction product. Arrowheads, the wall of a capillary branching out of a larger vessel (top left corner of each panel). A homogenous hybridization signal is associated with the endothelium of the branching capillary (arrowheads, panels 2–5) but not with the endothelium of the larger vessel. In panels 1 and 5 the capillary is below and above the focus plane, respectively.

Figure 9. Lack of VEGF expression in quiescent endothelium. Adjacent 30-μm sections of mouse corneas that received pellets with no FGF-2 were immunostained with antibody to mouse VEGF (VEGF IgG) or to vWF (vWF IgG) or with n.i. IgG (n.i. IgG) as described in Materials and Methods. The endothelium of the limbic vessels (arrowheads) stains positively for vWF but not for VEGF.
pression with different mechanisms of action. This finding is consistent with previous observations that HMW FGF-2 is exclusively found intracellularly and accumulates in the nucleus, whereas LMW FGF-2 is released by the cells and binds to surface receptors (Bugler et al., 1991; Mignatti et al., 1991; Florkiewicz et al., 1991; Quarto et al., 1991a,b; Renko et al., 1991; Bikfalvi et al., 1995). LMW FGF-2 and HMW FGF-2 modulate different cell functions, including migration, proliferation and FGF receptor downregulation (Bikfalvi et al., 1995). It has not been established whether these cell functions are also mediated through VEGF expression.

Many tumor or transformed cells express high levels of VEGF (Ferrara and Davis-Smyth, 1997). The upregulation of VEGF expression in endothelial cells or in FGF-2–transfected NIH 3T3 cells might result from the growth stimulatory effect of FGF-2 or from nonspecific effects derived from cell transformation. However, our analysis of NIH 3T3 cells transfected with FGF-2 cDNA and the tetracycline resistance transactivator rules out this hypothesis. Whereas expression of both FGF-2 and VEGF was dramatically upregulated in the presence of doxycycline, the cells had comparable growth rates in the presence or in the absence of the antibiotic (data not shown). In the presence of doxycycline, LMW FGF-2 transfectants had lower growth rates than HMW FGF-2 transfectants (data not shown) but expressed comparable amounts of VEGF.

VEGF expression is regulated by a variety of factors, including hypoxia, cytokines, and tumor promoters. FGF-2 upregulates VEGF levels in vascular smooth muscle cells (Stavri et al., 1995). However, only hypoxia and advanced glycation end products have been shown to regulate VEGF expression in vascular endothelial cells (Liu et al., 1995; Yamagishi et al., 1997). Our study is the first demonstration that VEGF expression in endothelial cells is modulated by FGF-2, a potent angiogenesis inducer. This finding has several important implications. First, VEGF is an important downstream mediator of the angiogenic activity of FGF-2, as shown by the inhibitory effect of VEGF antibody in vitro and in vivo. Second, although other cell types, including pericytes, smooth muscle cells, and inflammatory cells may participate in angiogenesis, capillary formation can be mediated by endothelial cell-derived growth factors through an autocrine mechanism independent of accessory cells. Third, FGF-2 and VEGF have synergistic effects on angiogenesis (Pepper et al., 1992; Asahara et al., 1995). Thus, a cascade of autocrine events in which one angiogenic factor upregulates the other appears to represent a very efficient mechanism for angiogenesis. Recently, FGF-2 antibodies have been shown to block VEGF-induced angiogenesis in vitro (Mandriota and Pepper, 1997). Although this effect is opposite to the one we report here, it is consistent with our observation of an autocrine mechanism of angiogenesis mediated by FGF-2 and points to complex interactions between FGF-2 and VEGF. It is possible that endogenous endothelial cell
FGF-2 is required for efficient angiogenesis and/or that the simultaneous presence of both FGF-2 and VEGF in vascular endothelial cells is required for angiogenesis in the adult organism. Mice genetically deficient in FGF-2 (FGF-2 knockout) have a normal vasculature and no apparent defects related to impaired angiogenesis (Ortega et al., 1998; Zhou et al., 1998). However, whereas VEGF expression during development may be modulated by factors other than FGF-2, the levels of VEGF in the endothelium of forming capillaries in adult FGF-2 knockout mice have not yet been characterized. More important, whether FGF-2 knockout mice have normal VEGF levels and angiogenesis in pathological settings such as tumor growth also remains to be investigated.

Our in vivo angiogenesis experiments with the mouse cornea show that FGF-2 also modulates endothelial cell expression of VEGF in vivo, and that endothelial cell VEGF is an important, autocrine effector of FGF-2-induced angiogenesis.

Consistent with previous findings, our in situ hybridization and immunohistochemistry experiments showed that the endothelium of the quiescent limbic vessels of the mouse cornea does not express VEGF. However, after stimulation with FGF-2, the endothelial cells of newly formed capillaries produce both VEGF mRNA and protein. The analysis of VEGF mRNA in several tumors has shown expression in the tumor cells but not in the endothelium of the tumor vasculature (Ferrara and Davis-Smyth, 1997). Conversely, immunohistochemical studies have shown VEGF associated with the endothelium of tumor capillaries, indicating that VEGF secreted by tumor cells is bound by its receptors present exclusively on endothelial cells (Plate et al., 1992, 1994; Brown et al., 1993; Qu-Hong et al., 1995). In these studies the relative abundance of VEGF transcript in the tumor cells might mask the VEGF mRNA of the endothelial cells. Our experimental model of angiogenesis in the mouse cornea obviates the problem of identifying the source of endothelium-associated VEGF, since only occasional fibroblasts are normally present in the stroma of the cornea. Inflammatory cells may be present in the cornea during angiogenesis. However, in our in situ hybridization experiments, the VEGF mRNA probe hybridized significantly only to vWF–positive cells of newly formed capillaries, indicating endothelial cells as the major source of VEGF.

Figure 12. Effect of anti-VEGF antibody on FGF-2-induced angiogenesis. Hydron pellets containing 50 ng of rFGF-2 were implanted in the cornea of both eyes of 18 Swiss Webster mice as described in Materials and Methods. The animals were randomized into three groups of six mice and given i.v. injections of either PBS or PBS containing n.i. IgG (100 μg) or neutralizing anti-human VEGF monoclonal antibody (VEGF IgG; 100 μg) 1 d before pellet implantation and on postoperative days 1 and 3. The corneas were photographed by slit-lamp biomicroscopy on day 5 after implantation of the pellet. The eyes of the animals injected with VEGF antibody have fewer and thinner corneal limbic capillaries than those of animals injected with PBS or n.i. IgG. An enlargement of the limbic area containing the newly formed vessels is shown below each photograph of the corresponding eye. This experiment was repeated twice with comparable results. In control mice that received corneal implants of pellets containing 200 ng of human recombinant VEGF, the same treatment with the VEGF antibody abolished the angiogenic response almost completely (data not shown).
On the basis of our data, we cannot exclude that in the cornea assay VEGF from cell types other than endothelial cells, including smooth muscle cells, macrophages, or T cells (Freeman et al., 1995; Stavri et al., 1995; McLaren et al., 1996), may contribute to early stages of angiogenesis, e.g., capillary sprouting from the limbic vessel. However, our finding that VEGF antibody did not significantly reduce the number of capillaries sprouting from the limbic vessel but did considerably decrease subsequent branching indicates that the contribution of cell types other than endothelial cells may be independent of VEGF. In contrast, VEGF derived from endothelial cells may play a role at later stages of angiogenesis. Whatever the contribution of other cell types to angiogenesis, our finding that in vitro FGF-2–stimulated endothelial cells rapidly (2–4 h) upregulate VEGF mRNA expression suggests that in vivo VEGF receptors can be more promptly saturated by the endothelial cell–derived ligand with an autocrine mechanism than with a paracrine mechanism by VEGF derived from other sources.

VEGF mRNA was located predominantly to sites of vessel branching. Interestingly, FGF-2 has also been found associated with the basal lamina of branching capillaries (Cordon et al., 1990), indicating that FGF-2 and VEGF may be primarily expressed by the endothelium of growing capillaries and play a role in vessel formation. This hypothesis is confirmed by our finding that neutralizing VEGF antibody dramatically inhibits FGF-2–induced angiogenesis in the mouse cornea. It is noteworthy that the antibody appears to predominantly reduce branching of the newly formed vessels, an observation that correlates with the location of FGF-2 and VEGF.

A number of experimental and clinical studies have implicated VEGF as a paracrine angiogenesis factor in different physiological and pathological settings. The levels of VEGF in several tumors have been shown to correlate with tumor vascularity, a negative prognostic indicator for a variety of malignancies (Toi et al., 1994; Maeda et al., 1996). However, other studies have failed to demonstrate such correlation (Dvorak et al., 1995). The autocrine or paracrine regulation of endothelial cell VEGF by FGF-2 may represent an important mechanism of angiogenesis under conditions in which insufficient or no VEGF is derived from other cell types. This autocrine mechanism may be triggered by normal or tumor cell–derived FGF-2 or by upregulation of endothelial cell FGF-2. Factors that regulate endothelial cell expression of FGF-2 are essentially unknown. Some tumor cell lines secrete a peptide(s) that upregulates endothelial cell expression of FGF-2 and induces in vitro angiogenesis (Peverali et al., 1994). FGF-2 upregulation results in increased expression of urokinase, a proteinase implicated in angiogenesis. Urokinase upreg-

**Table I. Number of Capillaries Proximal to and Distal from the Limbic Region of the Cornea of Mice Receiving mAb to VEGF by i.v. Injection or by Corneal Implant in the FGF-2–Containing Pellet**

<table>
<thead>
<tr>
<th>i.v. injection</th>
<th>Proximal</th>
<th>Distal</th>
<th>Pellet</th>
<th>Proximal</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vessel</td>
<td>vessel</td>
<td>vessel</td>
<td>vessel</td>
<td>vessel</td>
</tr>
<tr>
<td></td>
<td>(n)*</td>
<td>(%)</td>
<td>(n)*</td>
<td>(%)</td>
<td>(n)*</td>
</tr>
<tr>
<td>PBS</td>
<td>15.7 ± 1.1</td>
<td>100.0</td>
<td>41 ± 3.1</td>
<td>97.2</td>
<td>—</td>
</tr>
<tr>
<td>n.i. IgG</td>
<td>15.7 ± 1.1</td>
<td>100.0</td>
<td>42.3 ± 1.5</td>
<td>100.0</td>
<td>—</td>
</tr>
<tr>
<td>VEGF Ab</td>
<td>13.8 ± 1.5</td>
<td>87.9‡</td>
<td>25.2 ± 0.9</td>
<td>59.6‡</td>
<td>11.3 ± 0.9</td>
</tr>
</tbody>
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*Mean of 12 eyes per group ± SEM.
‡P < 0.01.
§P < 0.01.

Statistical analysis performed by analysis of variance.

Figure 13. Effect of anti-VEGF antibody on FGF-2–induced angiogenesis. Hydron pellets containing 50 ng of rFGF-2 with or without 1 μg of anti-VEGF monoclonal antibody were implanted in the cornea of both eyes of Swiss Webster mice as described in Materials and Methods. Each group consisted of six animals. The corneas were photographed by slit-lamp biomicroscopy on day 5 after implantation of the pellet. FGF-2 pellet: eyes that received corneal implantation of pellets containing FGF-2 alone. FGF-2/VEGF IgG pellet: eyes that received corneal implantation of pellets containing FGF-2 and antibody to VEGF. The eyes that received corneal implants of pellets containing FGF-2 and VEGF antibody have fewer and thinner corneal limbic capillaries than eyes that received corneal implants of pellets containing FGF-2 alone.
ulation and in vitro angiogenesis are mediated by endothelial cell FGF-2 with an autocrine mechanism of action (Peverali et al., 1994). The purification and characterization of this and other potential factors that upregulate endothelial cell FGF-2 will be important for our comprehension of the paracrine and autocrine mechanisms of angiogenesis.

In all the experiments we performed, i.e. or local administration of VEGF antibody consistently inhibited angiogenesis. However, our antibody did not totally abrogate FGF-2-induced capillary formation. This result can be explained by two hypotheses: (a) the monoclonal antibody we used was raised against human VEGF; although we found that in vitro it can completely neutralize mouse VEGF as efficiently as human VEGF, it is possible that in vivo this antibody does not completely neutralize or the doses we administered did not affect complete neutralization of mouse VEGF; and (b) FGF-2 may not only act by inducing endothelial cell expression of VEGF but also induce angiogenesis through VEGF-independent mechanisms.

Mouse embryos lacking a single VEGF allele have several abnormalities in their vasculature and die in utero (Carmeliet et al., 1996; Ferrara et al., 1996), showing that FGF-2 alone is not sufficient for normal vasculogenesis and angiogenesis during development. However, in the adult organism FGF-2 might at least in part complement the lack of VEGF. Although these hypotheses await confirmation, our experiments clearly show endothelial cell VEGF as a major downstream mediator of the angiogenic activity of FGF-2.

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Autocrine Control of Angiogenesis by FGF-2 and VEGF

Seghezzi et al.


