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Vascular Endothelial Growth Factor Confers a Growth Advantage *in Vitro* and *in Vivo* to Stromal Cells Cultured from Neonatal Hemangiomas

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Neonatal hemangioma is a common benign proliferation of unorganized structures containing stromal and capillary endothelial cells. We tested the hypothesis that such cell proliferation might result from the release by stromal cells of endothelial cell mitogens. Stromal cells cultured from biopsies of surgically removed life-threatening hemangiomas released an endothelial cell mitogen* in vitro *that was indistinguishable from vascular endothelial growth factor (VEGF) based on independent criteria such as affinity chromatography for heparin or anti-VEGF IgG and radioreceptor assay. A functional product of the* KDR *gene encoding a cognate VEGF receptor was also expressed by these stromal cells. Transient transfection with antisense oligonucleotides targeted on the translation initiation codon of* KDR *abolished its tyrosine phosphorylation and mitogenic response of neonatal hemangioma cells to VEGF, confirming the existence of an autocrine loop of proliferation. When grafted in nude mice, these stromal cells elicited an angiogenic response that was blocked by neutralizing anti-VEGF IgG. These results might provide a clue to the importance of stromal cells in the pathogeny of neonatal hemangiomas. (Am J Pathol 1997, 150:1315–1326)

Several physiological and pathological situations such as embryonic development, wound healing, tumor progression, and diabetes involve the proliferation of new vessels from pre-existing capillaries (reviewed in Ref. 1). The mechanisms that control such local angiogenesis are not yet understood and may be due to a local hyperproduction of growth factors for capillary endothelial cells. During the past decade, several angiogenic growth factors, including transforming growth factor-α and -β, angiogenin, acidic and basic fibroblast growth factors (aFGF and bFGF), and platelet-derived growth factor (PDGF) have been purified and their cDNAs cloned. Vasculotropin,[2] also called vascular endothelial growth factor (VEGF)[3,4] or vascular permeability factor,[5,6] is secreted and released in the vicinity of endothelial cells, which in turn are able to proliferate, migrate, and differentiate along this gradient of concentration. cDNA cloning showed an overall homology of 15 and 18% with the A and B chains of PDGF.[7,8] Alternative splicing of the pre-mRNA generated four different forms encoding 121-, 165-, 189-, and 210-amino-acid peptides.[9,10] Although all four forms are secreted, only the two smaller ones are efficiently released in the conditioned medium.[10] Several tumor cell lines[2,4–6] as well as some normal cultured cells such as folliculo-stellar cells of the anterior pituitary,[3] vascular smooth muscle cells,[11,12] monocytes, and mesangial cells[13] release VEGF in their conditioned medium. VEGF binds to cultured vascular endothelial cells[14–17] and retinal pigment epithelial cells[18] on two high-affinity binding sites and promotes their proliferation and their migration. However, other cells such as corneal endothelial cells,[19] osteoblasts,[20] or

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monocytes[21] bind VEGF on a single high-affinity binding site and migrate, but do not proliferate, upon its addition. So far, two genes, *flt*-1[22] and *KDR*[23] or its murine homologue *flk*-1,[24,25] encoding membrane-spanning tyrosine kinase type III receptors, have been identified as VEGF receptors.

Recent reports have shown that the knock-out of the VEGF gene, even in heterozygous animals, is lethal for embryos at day 10.5, thus demonstrating that VEGF is a major regulator of vasculogenesis.[26,27] Accordingly, knock-out of the *KDR* and *flt*-1 genes induces a vessel reduction or disorganization, respectively.[28,29] Although their expression has been reported as restricted to vascular endothelial cells *in vivo*, their temporal and spatial patterns are different. *KDR/flk*-1 is expressed mainly in the embryo,[24,25] whereas *flt*-1 is still present in adult tissues.[30]

An early pathological feature of neonatal hemangioma (NHA) is uncontrolled angiogenesis, as capillary proliferation occurs soon after birth, reaching a maximal size by 6 to 10 months, and then regresses over several years.[31] Although hemangiomas are frequent and can be diagnosed in up to 10% of babies, they do not usually require treatment due to their spontaneous involution. However, the size or location of certain hemangiomas may be life threatening and therefore require surgical removal. As it has been recently reported that VEGF is present in hemangiomas during the proliferating phase[32] and that stromal cells cultured from fragments of such hemangiomas release large amounts of mitogenic activity for microvessel-derived endothelial cells,[33] we decided to study the role of VEGF in hemangioma pathogeny. VEGF is located in the vessel wall and expressed in NHA cells cultured *in vitro*. It acts as an autocrine growth factor through the activation of KDR, a cognate VEGF receptor. The use of specific antibodies directed against VEGF abolishes the vascular endothelial growth-promoting activity *in vitro* and the angiogenesis developed by NHA cells grafted in nude mice.

Materials and Methods

Cell Cultures and Reagents

Three hemangiomas were obtained during the proliferative phase in the first year of evolution by diagnostic biopsy or therapeutic surgical removal. Hemangiomas were separated from the skin and digested in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg/ml collagenase and 50 μg/ml hyaluronidase for 3 hours at 37°C. The dissociated cells were cultured in DMEM supplemented with 20% fetal calf serum. Once at confluence, the cell monolayer was dissociated and the cells plated on dishes coated with *Ulex europaeus* agglutinin I lectin (Sigma Chemical Co., St. Louis, MO) to retain the endothelial cells.[29] After 5 minutes, nonadhering cells were washed out and further cultured in the same medium on uncoated dishes. These selected cells were named NHA cells and used from passages 3 to 7. Human endothelial cells were cultured from umbilical vein (HUVECs) in the same medium and used at passage 2.[34] Bovine vascular endothelial cells were cultured from aorta (FBAE) or adrenal cortex (ACE) as described.[35,36] Cell culture reagents were from Gibco and plasticware from Nunc (Roskilde, Denmark).

Recombinant human VEGF (165-amino-acid isoform) was produced in insect cells,[37] placenta growth factor (152-amino-acid isoform, PlGF) in mammalian cells[38] (generous gift of N. Ferrara), and FGF2 in *Escherichia coli*.[39] A specific agonist of the KDR receptor has been produced in rabbits by the anti-idiotypic strategy.[40] Neutralizing antibodies were elicited in rabbits by monthly injections in lymph nodes of 10 μg of recombinant human VEGF synthesized in insect cells. After 3 months, the IgGs were purified on protein-A-Sepharose and used as immunogens for a new set of rabbits (10 μg of anti-VEGF IgG per injection). The anti-idiotypic IgGs were purified by affinity chromatography on preimmune IgG (PI-IgG) to remove anti-allotypic antibodies followed by anti-VEGF IgG affinity chromatography. The VEGF and IgG affinity columns were prepared by mixing overnight at 4°C 1 mg of protein dialyzed against 50 mmol/L carbonate buffer, pH 8.5, and 1 ml of CNBr-activated Sepharose. The reaction was then blocked with 0.2 mol/L ethanolamine. The specific IgGs were eluted by 0.2 mol/L glycine, pH 2.5, immediately neutralized, and dialyzed against phosphate-buffered saline (PBS). One anti-idiotypic IgG fraction bound only to *KDR*- but not to *flt*-1-expressing COS cells and triggered endothelial cell proliferation, and this KDR agonist is referred as Anti-IdJ. For immunocytochemistry, recombinant human VEGF 121 synthesized in *E. coli* was injected in chicken breast and the antibodies purified from eggs on an anti-VEGF affinity column. Control IgGs were prepared by loading anti-VEGF IgG on VEGF affinity chromatography and recycling the flow-through until it was totally depleted of anti-VEGF IgG as checked by enzyme-linked immunosorbent assay. Neutralizing IgG anti-FGF2 was prepared and purified as already described.[41] Anti-flk-1 and anti-phosphotyrosine antibodies SC315 and PY69 were from Santa-

Table 1. *Antibodies Used for Immunocytochemistry*

Antibody 1	Origin	Dilution	Antibody 2	Dilution	Method
VEGF	This laboratory	1:50	Biotinylated-rabbit IgG/ chicken Ig	1:10	ABC
vWF	Dakopatts	1:800	Peroxidase-conjugated goat IgG/rabbit IgG	1:20	Two-step immunoperoxidase
S-endo-1	Ref.42	1:25	Biotinylated rabbit IgG/ mouse IgG	1:100	ABC
SMC α-actin	Dakopatts	1:25	TRITC-rabbit IgG/mouse IgG	1:200	Two-step immunofluorescent
UEA1	Dakopatts	1:100			ABC

SMC, smooth muscle cell; TRITC, tetraethylrhodamine isothiocyanate.

Cruz Biotechnology (Santa Cruz, CA). Biotinylated and peroxidase-conjugated goat anti-rabbit IgG, streptavidin peroxidase, and the ECL detection kit were from Amersham (Arlington Heights, IL). The Superscript amplification kit and reverse transcriptase were from Gibco. Other reagents were from Sigma.

Immunohistochemistry

We carried out an immunohistochemistry study to characterize the NHA cells by using three endothelial markers (von Willebrand factor (vWF), *Ulex europaeus* 1 (UEA1), and S-endo-1[42]) and one marker of the smooth muscle cell lineage (α-actin). NHA cells and HUVECs were cultured on Labtek chambers, fixed with 4% paraformaldehyde, washed with PBS and either permeabilized with methanol/acetone for 5 minutes at −20°C (vWF and smooth-muscle-cell-specific α-actin) or with pepsin for 3 minutes at 37°C (VEGF) or not permeabilized (S-endo-1 and UEA1). The slides were then incubated with antibodies listed in Table 1, diluted at the desired concentrations except that biotinylated UEA1 was incubated instead of antibody. The second antibodies (Dakopatts, Glostrup, Denmark) were conjugated as indicated with peroxidase, biotin, or tetramethylrhodamine isothiocyanate and processed with the avidin-biotin complex or two-step immunofluorescence.

mRNA Analysis

Confluent NHA cells were lysed in guanidinium thiocyanate, and total RNA was purified by the phenol chloroform method.[43] For Northern blot analysis, total RNA (20 μg) was run on a 1.2% formaldehyde agarose gel, blotted on to a Hybond-N nylon membrane (Amersham) by capillary transfer and ultraviolet cross-linked. Hybridization was performed with a 605-bp *Bam*HI fragment of human VEGF cDNA kindly provided by J. Abraham. This probe was ^{32}P labeled by using a multiprime DNA labeling system (Promega, Madison, WI). The control for equal lane loading and transfer was assessed by ethidium bromide staining. Total RNA (2 μg) was used for the reverse transcriptase reaction using the Superscript kit and random hexamers as primers. The polymerase chain reaction was performed on 1/10th of the cDNAs in a 50-μl volume using 2.5 U of *Taq* polymerase and 50 pmol of each oligonucleotide flanking the 5′ and 3′ ends of the VEGF coding sequence (CAAGTACCAAAGCCTCC and ACTGTTCGGCTCCGCCACT). Amplification was performed for 30 cycles (94°C for 1 minute, 57°C for 1 minute, and 72°C for 1.5 minutes). In another set of experiments, *KDR* cDNA was amplified as described previously[18] with primers aligned on the sequence 42 to 59 (direct GACCCGGGTGGGACATACACAAC) and 1240 to 1262 (reverse GCACCTTGGTTGTGGCTGAC). The amplified polymerase chain reaction (PCR) products (6 μl) were separated by electrophoresis on a 1.5% agarose gel. Negative controls were performed in the absence of cDNA. The specificity of the amplification was ascertained by enzymatic restriction on a unique site. One site for *Nco*I is present in position 151, and cleavage of a VEGF cDNA with *Nco*I should therefore yield to a shift of 86 bp of the size of the fragment.

Purification of the Conditioned Medium

NHA cells were grown in 560-cm^2 plates until confluency (8×10^7 cells per plate). The plates were then rinsed twice with serum-free medium and incubated in 120 ml of serum-free medium supplemented with antibiotics, 10 μg/ml transferrin, and 5 μg/ml insulin as previously described.[2] After 48 hours, the culture medium (1.4 L) was collected, centrifuged, concentrated 100-fold by ammonium sulfate precipitation (500 g/L), further dialyzed against 10 mmol/L Tris, pH 7.4, and 50 mmol/L NaCl, and then loaded on a heparin-Sepharose affinity col-

umn (1 × 0.2 cm). The retained material was subsequently eluted by a NaCl gradient. The 0.25-ml fractions were collected and processed for bioassays.

In parallel experiments, 30 ml of NHA-conditioned medium was loaded on an anti-VEGF IgG immunoaffinity column (1 × 0.2 cm), eluted by 0.2 mol/L glycine, pH 2.5, and immediately neutralized with 1 mol/L K_2HPO_4.

Bioassays for VEGF

Proliferation assays were performed on ACE or NHA cells seeded at low density (5000 cells per 12-well cluster plate) in 1 ml of DMEM containing 10% newborn or fetal calf serum. The modulators were inoculated every other day and the cells trypsinized and counted after 4 days in a Coulter counter. In some experiments, growth-arrested ACE or NHA cells seeded in 24-well cluster plates were incubated in serum-free medium with the modulators and 1 μCi of [^{3}H]thymidine (46 Ci/mmol) for 24 hours. The insoluble material was precipitated 10 minutes with 10% trichloracetic acid, neutralized, and dissolved in 0.2 mol/L NaOH, and the radioactivity was counted in a scintillation counter. VEGF content was measured with a radioreceptor assay using FBAE as target cells as already described.[14] Briefly 1 ng of iodinated VEGF and 10 μl of the chromatography fractions were incubated with subconfluent FBAE cells for 3 hours at 4°C. After three washes with PBS, the cells were lysed with 0.2 mol/L NaOH and the radioactivity counted in a gamma counter. Results were expressed in comparison with a standard curve of recombinant VEGF.

Binding Assays

NHA cells were seeded at 250,000 cells per 35-mm dish in DMEM supplemented with 20% calf serum and antibiotics. After 2 days, subconfluent cells (5 × 10^5 to 6 × 10^5 cells) were transferred at 4°C, and all subsequent operations were done in the cold.[14] The cells were washed twice for 10 minutes with binding buffer (DMEM containing 20 mmol/L Hepes and 2 mg/ml gelatin), adjusted to pH 7.4, and then incubated with the desired concentrations of iodinated VEGF in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of an excess of purified growth factor (500 ng). Both total and nonspecific binding were determined in duplicate. The dishes were shaken on an oscillating platform rotating at 1 cycle/second. After 2 hours, the cells were washed three times with cold binding buffer, washed once more with PBS containing 2 mol/L NaCl for 2 minutes, and then lysed and processed as above. The nonspecific binding was less than 20% at saturating concentrations. Values were analyzed according to the Scatchard's procedure[44] using the LIGAND fitting program version 2.3.11.[45]

Oligonucleotide Transfection

The 15-mer oligonucleotides (3′-ACGTCCTACCTGTCG-5′antisense and 3′GCTCTCCATCCTGCA-5′ sense) targeted on the translation initiation codon were synthesized to study *KDR* gene expression. Oligonucleotides were mixed with lipofectin diluted 200-fold in DMEM. After 30 minutes at room temperature, the mixture was added dropwise to subconfluent NHA cells (0.1 ml/cm^2). After 8 hours, the cells were rinsed and incubated for 16 more hours in antibiotic-free DMEM containing 5% fetal calf serum. Preliminary experiments established that maximal effect was obtained with a 2 μmol/L final concentration of oligonucleotides. *KDR* expression was monitored by VEGF Western blot and binding and proliferation assays 24 hours after transfection. NHA cells (3 × 10^6) were incubated 5 minutes at 37°C in the presence or absence of 2 nmol/L VEGF and then lysed with RIPA (0.1% sodium dodecyl sulfate (SDS), 1% cacodylate, 1 mmol/L EDTA in 10 mmol/L phosphate buffer, pH 7.4, containing 40 mmol/L sodium pyrophosphate and 1 mmol/L orthovanadate). Lysates were incubated 2 hours with 2 μg of anti-phosphotyrosine PY69 monoclonal antibody. The immune complexes adsorbed on protein-A-Sepharose beads at 4°C for 2 hours were then washed five times with lysis buffer and solubilized in SDS-polyacrylamide gel electrophoresis sample buffer. The immunoprecipitates were reduced and separated on a 6% polyacrylamide gel followed by transfer to nitrocellulose. The filters were stained with Ponceau red, treated with 5% nonfat milk in Tris-buffered saline with Tween 20 (10 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4) and incubated with 1 μg/ml of anti-flk-1 antibody. Immunoreactive proteins were detected by incubation with a 1:20,000 dilution of peroxidase-conjugated anti-rabbit IgG, followed by development with luminol substrate. In a second set of experiments, NHA cell proliferation was monitored on parallel dishes. At 24 hours after transfection, the cells were incubated with 2 ng/ml VEGF or FGF2 in the presence or absence of anti-VEGF IgG, and 20 hours later, the cells received 1 μCi of [^{3}H]thymidine (46 Ci/mmol) for 4 more hours and were then processed as above.

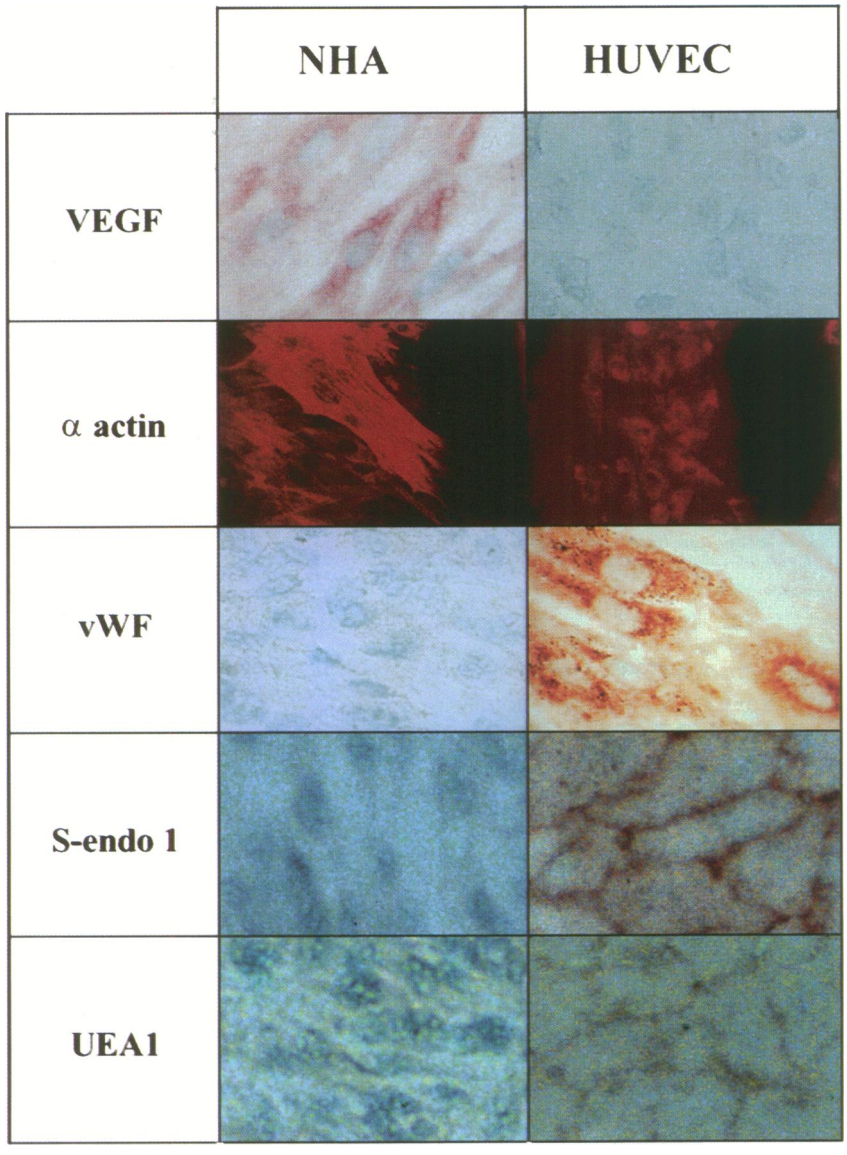

Figure 1. *Immunotyping of cultured NHA and HUVECs. NHA and HUVECs were fixed and incubated with anti-VEGF, anti-α-actin, anti-vWF, and S-endo-1 IgG and UEA1 and processed as described in the text. Magnification, ×400. Cells were counterstained with toluidine blue and photographed; magnification, ×1000.*

NHA Graft in Nude Mice

Cells were mechanically dissociated, washed once in serum-containing medium and twice in PBS, and then resuspended to 10^7 cells/ml. A total of 2×10^6 cells were injected subcutaneously in the dorsal areas of 8- to 10-week-old female athymic mice. Three days later, each group of five received an intraperitoneal injection of 200 μg of either anti-VEGF or preimmune IgG (PI-IgG) twice weekly. After 2 weeks, the mice were killed and the injection sites were dissected and photographed or fixed in Bouin's reagent. Tissues were processed for paraffin embedding, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) and saffron.

Results

Immunocharacterization of NHA Cells

NHA tissues were cultured *in vitro* and depleted of the endothelial-like cells by adsorption on *Ulex europaeus*. The NHA cells appeared as fusiform, fibroblastic-like cells. The presence of five distinct markers was tested on NHA cells and HUVECs. NHA cells

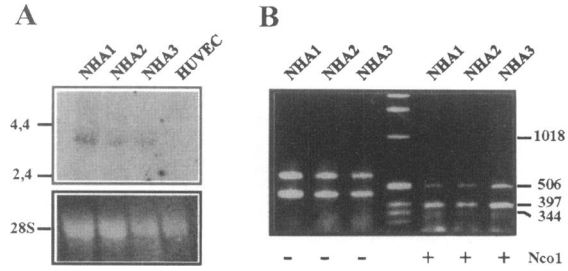

Figure 2. *Analysis of VEGF mRNAs in NHA and HUVECs.* A: *Total RNA (20 μg per lane) from three different samples of NHA cells (patients 1 to 3) and HUVECs was electrophoresed and hybridized with a ^{32}P-labeled human VEGF probe. Autoradiography was obtained by exposure to X-Omat AR film at − 70°C for 24 hours. The size of VEGF mRNA was estimated by the migration of calibrated RNA (RNA ladder, Gibco). An equal amount of loading is indicated in the lower panel by ethidium bromide staining of the 28S ribosomal RNA.* B: *Total RNA (2 μg) was used for cDNA synthesis by reverse transcriptase using random hexamers. The product of the reaction (1/10th) was used for PCR amplification with primers in a final volume of 50 μl. Products from each of the reactions (6 μl) were resolved on a 1.5% agarose gel. cDNAs as templates were run before* (left) *or after* (right) *NcoI digestion. The 1-kb marker was run in the middle (344, 397, 506, 1018, 1636, and 2036 bp).*

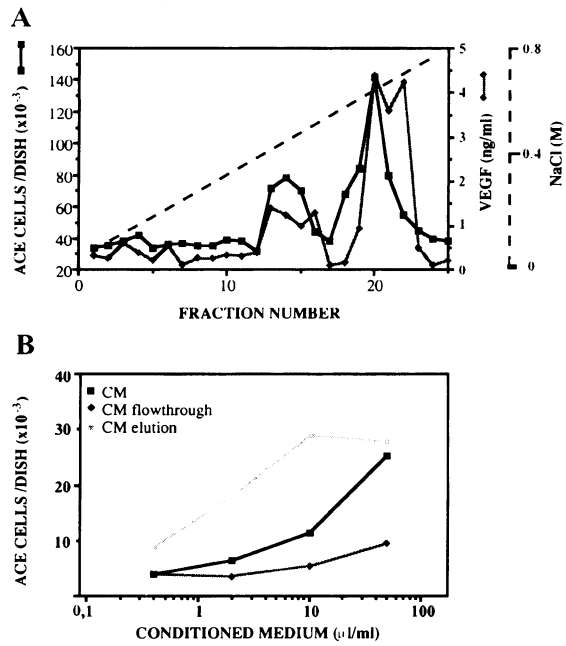

Figure 3. *Purification of the conditioned medium by affinity chromatography for heparin* (A) *and anti-VEGF IgG* (B). A: *Concentrated conditioned medium was loaded on a heparin affinity column (0.2 × 0.4 cm) and eluted by a NaCl stepwise gradient (·····). The 0.25-ml fractions were collected and assayed for their VEGF content by radioreceptor assay (◆) and mitogenic activity for ACE cells (■) as described in the text.* B: *Conditioned medium was loaded on an anti-VEGF IgG affinity column, and the flow-through and eluted fractions were assayed for their mitogenic activity for ACE cells.*

were immunolabeled with anti-VEGF antibody on granular cytoplasmic areas disseminated uniformly within the cytoplasm (Figure 1). Staining with the same batch of IgG depleted of specific anti-VEGF IgG did not show any labeling (data not shown). NHA cells were also labeled with an antibody directed against α-actin, whereas HUVECs remained negative for VEGF and α-actin. In contrast to NHA cells, HUVECs appeared positive for the endothelial markers vWF and S-endo-1 and for UEA1 binding.

Expression of VEGF by NHA Cells

The immunodetection of VEGF in cultured NHA cells prompted us to document the expression of VEGF transcripts in these cells. Northern blot analysis of HUVEC and NHA cell mRNA showed that only NHA cells expressed a 4-kb transcript (Figure 2A). We then determined which splice variants were expressed. Reverse transcribed total mRNA was probed with primers overlapping the sequences of exons 1 and 8 of VEGF. Two bands of 474 and 606 bp, corresponding to transcripts of the mature 121- and 165-amino-acid forms of VEGF, generated after cleavage of the signal peptide, were visualized in the three cases examined (Figure 2B). The specificity of the amplification reaction was ascertained by the cleavage by *Nco*I leading to a shift of 86 bp, generating fragments at the expected sizes of 356 and 488 bp, respectively.

The presence of VEGF 121 and 165 mRNAs prompted us to see whether they were transcribed in diffusible growth factors. NHA-conditioned medium (12 mg) was chromatographed on a heparin-Sepharose affinity column and eluted in a stepwise NaCl gradient. Each fraction was tested for its ability to compete for the binding of iodinated VEGF to its receptors on ACE cells and to promote endothelial cell growth. As shown in Figure 3A, the major bulk of VEGF-like bioactivity was recovered at 0.8 mol/L NaCl. The mitogenic activity was recovered in the same 20 to 22 fractions. An affinity chromatography for anti-VEGF IgG showed that most (>90%) of the mitogenic activity for ACE cells was removed by this chromatography step (Figure 3B). An acidic treatment of the column eluted 60% of the bioactivity, confirming that it was immunologically related to VEGF.

Expression of VEGF Receptors by NHA Cells

We determined the expression of the VEGF receptors KDR and flt-1 by PCR. Reverse transcribed total mRNA was probed with an upper primer located in the immunoglobulin-like domain and a lower primer located in the kinase insert domain. The amplification generated the predicted fragment of 1221 bp (Figure

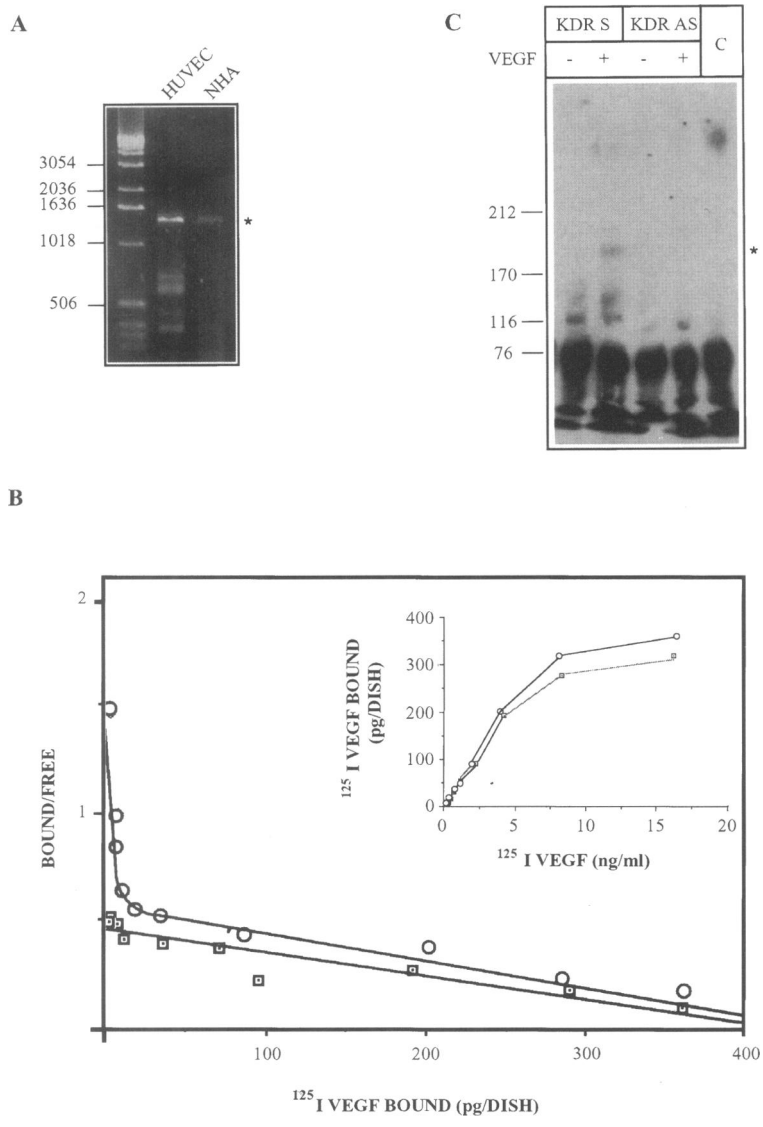

Figure 4. *Functional expression of KDR in NHA cells.* A: *A 0.2-μg amount of NHA and HUVEC cell cDNA was prepared as described in the legend of Figure 2 and subjected to PCR amplification with KDR primers. Products from each of the reactions were resolved on a 1.5% agarose gel. Their size (*) was monitored by comparison with the 1-kb ladder.* B: *Subconfluent NHA cells seeded in 24-well cluster plates were transfected with 2 μmol/L antisense (□) or sense (○) KDR oligonucleotides, and 24 hours later, the cells were incubated 2 hours at 4°C in the presence of increasing concentrations of ^{125}I-labeled VEGF in a final volume of 500 μl. The nonspecific binding was determined in parallel dishes containing 500 ng of unlabeled VEGF and subtracted from the values. Data are presented according to the Scatchard's procedure.* Inset: *Binding isotherms.* C: *NHA cells were stimulated for 5 minutes at 37°C with 1 nmol/L VEGF, lysed in RIPA buffer, and immunoprecipitated with anti-phosphotyrosine monoclonal IgG. The immune complexes were collected on protein-A-Sepharose beads, electrophoresed, transferred to nitrocellulose, and further probed with anti-flk-1 IgG.*

4A). By contrast to HUVECs, no *flt*-1 mRNA could be detected in NHA cells (results not shown). To determine whether *KDR* mRNA was translated in a functional product, VEGF binding studies were performed on NHA cells. The cells were transfected with oligonucleotides targeted on the translation initiation codon of *KDR* to see whether the *KDR* gene was expressed and translated in a functional product. Transient transfection with a 15-mer sense oligomer (0.2 to 10 μmol/L) did not affect the binding of iodinated VEGF. The binding was saturable, and half-maximal and maximal binding occurred at 22 and 150 pmol/L, respectively (Figure 4B, inset). The dissociation constants calculated according to the LIGAND fitting program from the slopes obtained on a Scatchard's representation were 1 and 96 pmol/L, respectively, for the two binding sites (Figure 4B), assuming an apparent molecular mass of 46 kd for VEGF. The two-site *versus* the one-site computation was highly significant. The abscissa intercepts in this representation indicated the presence of 350 and 10,500 binding sites per NHA cell. In contrast, transfection with the corresponding antisense oligomer led to a reduction of less than 10% of the VEGF maximal binding capacity. Scatchard's representation of the data demonstrated that only the higher-affinity binding site was abolished; the affinity and number of the second binding site remained unaffected. NHA cells were exposed to 2 nmol/L VEGF for 5 minutes at 37°C and probed for tyrosine phosphorylation to see whether the reduction of VEGF binding was due to the down-regulation of *KDR*. Cell

Table 2. *Effect of KDR Down-Regulation on NHA Cell Proliferation*

	NHA		ACE	
	KDR S	KDR AS	KDR S	KDR AS
Control	3,850 ± 220	2,220 ± 140	14,230 ± 1,120	13,240 ± 1,090
VEGF	10,380 ± 690	2,600 ± 350	45,720 ± 2,310	15,780 ± 1,000
PIGF	3,970 ± 540	2,260 ± 280	16,220 ± 980	15,380 ± 1,490
Anti-IdJ	12,780 ± 1,110	4,230 ± 540	68,000 ± 4,550	12,680 ± 990
FGF2	71,780 ± 5,680	82,670 ± 7,810	74,350 ± 4,560	65,890 ± 3,400
FGF2 Ab	1,450 ± 180	1,100 ± 220	11,450 ± 1,100	12,100 ± 660

Subconfluent NHA or ACE cells seeded in 24-well cluster plates were transfected 8 hours with 2 μmol/L antisense (AS) or sense (S) KDR oligonucleotides, and 24 hours later, the cells were incubated with 2 ng/ml VEGF, FGF2 or PIGF or 40 ng/ml Anti-IdJ. In the last experiment 30 μ/ml of anti-FGF2 Ab were added. At 18 hours later, the cells were thymidine labeled for 6 hours and processed as described in the text. Values are the means of cpm counts and standard deviation.

lysates were immunoprecipitated by anti-phosphotyrosine IgG, reduced, electrophoresed, and transferred on nitrocellulose, which was further probed with anti-flk-1 IgG. As shown in Figure 4C, equal loading of each well, represented by anti-phosphotyrosine revelation, was ascertained by the intensity of a 120-kd band and of a smear migrating at 80 to 60 kd seen in each lane, including that performed in the absence of cell lysate. Sense transfected NHA cell lysates, but not their antisense transfected counterparts, revealed the presence of a 140-kd band that might represent a truncated form of *KDR* presumably phosphorylated by endogenous VEGF. A 190-kd band was observed only upon VEGF stimulation, which migrates at the expected size for tyrosine-phosphorylated *KDR*.[24,25] This band was not observed in VEGF-stimulated antisense transfected cells.

KDR *Activation Mediates the Mitogenic Signal of VEGF for NHA Cells*

To elucidate whether VEGF triggered a mitogenic signal, we exposed NHA cells to VEGF or to specific VEGF receptor agonists, namely PlGF for *flt*-1 and Anti-IdJ for *KDR*. VEGF and Anti-IdJ induced a mitogenic response but PlGF was ineffective (Figure 5A). Anti-VEGF neutralizing IgG reduced the basal proliferation of NHA cells by 20% and abolished almost totally the effects of exogenously added VEGF or Anti-IdJ. Accordingly VEGF and Anti-IdJ induced a strong mitogenic signal in ACE cells which was abolished by anti-VEGF neutralizing IgG (Figure 5B). As expected, PlGF was ineffective.[38] In contrast, the basal proliferation of ACE cells, which do not secrete bioactive VEGF,[19] was not affected by anti-VEGF IgG. To further confirm that VEGF and its receptor KDR contributed to an autocrine loop of proliferation, NHA and ACE cells were transfected with sense or antisense oligonucleotides targeted on the *KDR* translation initiation codon. Sense transfected cells responded to exogenously added VEGF or Anti-IdJ, but their antisense counterparts no longer responded (Table 2). Accordingly, the basal growth of NHA cells was reduced by 45%, but that of ACE cells

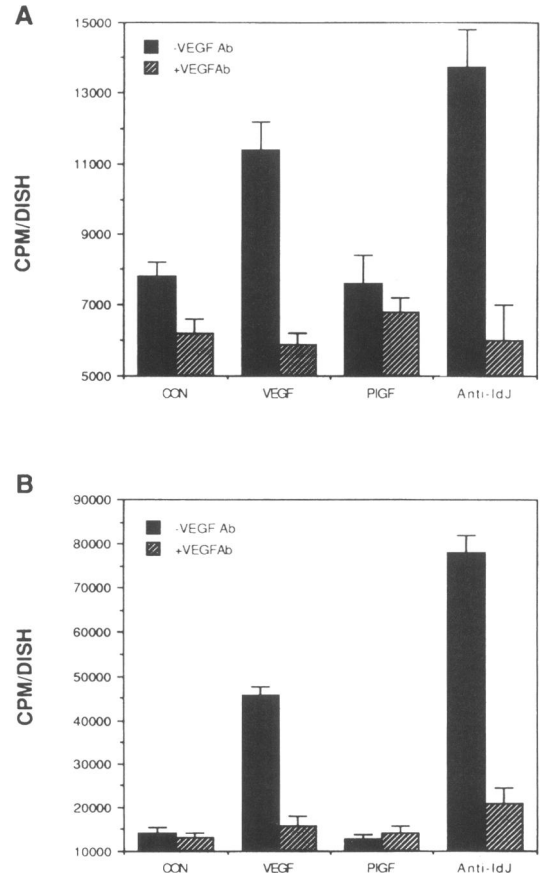

Figure 5. *Effect of anti-VEGF IgG on NHA* (**A**) *and ACE* (**B**) *cell proliferation. Subconfluent NHA or ACE cells seeded in 24-well cluster plates were growth arrested by 24 hours of exposure to serum-free conditions. Triplicate dishes were then incubated with 2 ng/ml VEGF or PlGF or 40 ng/ml Anti-IdJ in the absence or presence of 30 μg/ml anti-VEGF IgG, and 18 hours later, the cells were thymidine labeled for 6 hours and processed as described in the text. Values are the means of cpm counts and standard deviation.*

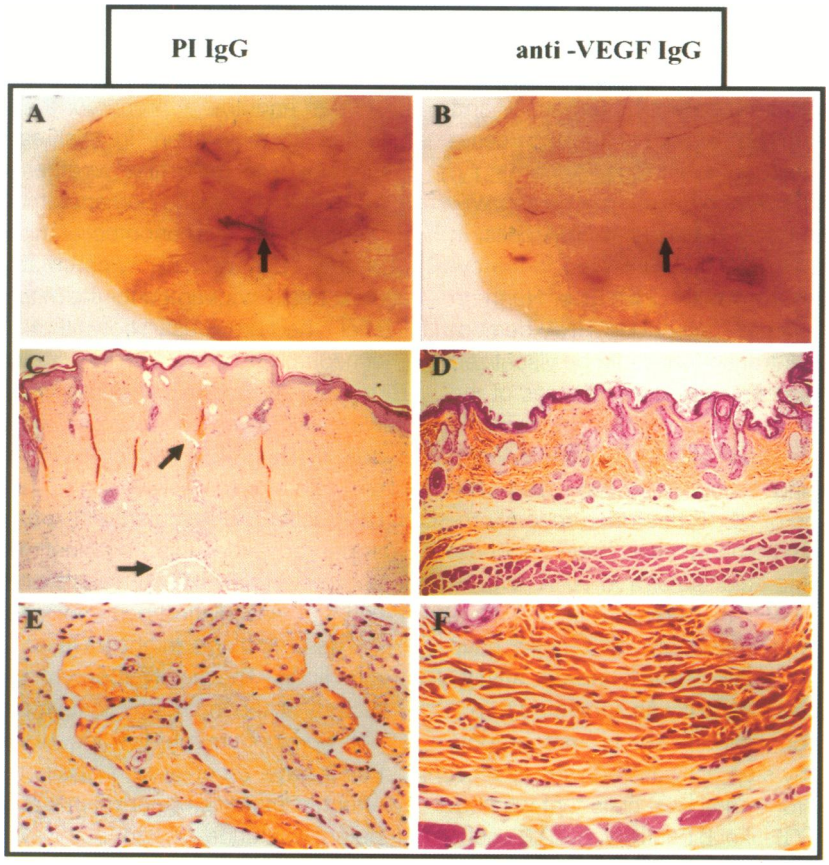

Figure 6. *Immunoneutralization of VEGF in NHA cells grafted in nude mice. A total of 2 × 10⁶ cells were injected subcutaneously in the dorsal areas of 8- to 10-week-old female athymic mice* (arrow). *Each group of five received 200 μg of either anti-VEGF or preimmune IgG twice weekly by intraperitoneal injection. After 2 weeks, the mice were killed and the skin at the injection sites was photographed. Thin sections were stained with H&E eosin and saffron and examined with a light microscope. Magnification, ×44* (C *and* D) *and ×200* (E *and* F).

was unaffected. Exogenously added FGF2 exerted a strong mitogenic response on NHA and ACE cells. Addition of anti-FGF2 IgG strongly reduced the basal growth of NHA cells (70%) and that of ACE cells only slightly (20%). *KDR* down-regulation did not modify the responses to exogenously added FGF2.

Immunoneutralization of VEGF Reduces Angiogenesis in NHA Grafts

To confirm that the NHA-derived VEGF-like bioactivity would act as an inducer of angiogenesis, NHA cells were grafted in nude mice that received, twice a week, an intraperitoneal injection of preimmune IgG or anti-VEGF IgG. Macroscopic examination performed after 15 days showed the presence of microvessels at the injection site, which were absent in the skin of animals treated with anti-VEGF IgG (Figure 6, A and B). Microscopic examination showed a stromal reaction containing numerous vascular spaces organized or not in capillary structures in preimmune-IgG-treated mice (Figure 6, C and E). In contrast, anti-VEGF IgG treatment led to a reduction of these capillary-like structures (Figure 6, D and F).

Discussion

The present study indicates that human neonatal stromal hemangioma cells express VEGF, which acts as an autocrine growth factor through the activation of its KDR receptor and as a paracrine factor for endothelial cells.

Hemangiomas are formed or capillary-like vascular spaces delimited by thin-walled channels of endothelial cells surrounded by stromal cells. Although their pathogenesis is not well understood, these benign primary tumors of the vasculature offer an attractive model to study angiogenesis. Immunohistochemistry and *in situ* studies have recently focused the attention on the expression of VEGF and its receptors in hemangiomas[32,46] as well as in the malignant related disorders hemangioblastomas and

hemangiopericytomas.[47–49] To understand the functional relationships existing between endothelial and stromal cells, we cultured the stromal NHA cells *in vitro*. They exhibited an apparent homogeneity and were strongly labeled with α-actin antibody, a cellular marker of the fibroblastic lineage. Accordingly, none of the three endothelial markers vWF, S-endo-1, and UEA1 was detected in NHA cells, in contrast to HUVECs. VEGF was detected in the cytoplasm of NHA cells but not in HUVECs. Northern blot and PCR of reverse transcribed RNA demonstrated that two VEGF transcripts could be visualized at 473 and 605 bp, consistent with the sizes of the corresponding 121- and 165-amino-acid products. Although the expression of alternatively spliced mRNA corresponding to the 121-, 165-, and 189-amino-acid isoforms of VEGF has been recently reported in cerebellum hemangiomas,[47] we could not detect the 189-amino-acid isoform in NHA. We investigated whether VEGF mRNAs were actively transcribed. Several angiogenic growth factors have been purified on the basis of their heparin affinity. The NHA-conditioned medium was subjected to purification using heparin-Sepharose chromatography. This chromatography step allowed recovery of most of the mitogenicity for endothelial cells that was coeluted with the VEGF-like bioactivity by 0.8 mol/L NaCl. The observation that most of the mitogenic activity bound to anti-VEGF IgG confirmed that it was immunologically related to VEGF.

Surprisingly, we found that NHA cells expressed a functional *KDR* product and proliferated upon VEGF addition. The use of selective agonists for each VEGF receptor suggested that this mitogenic effect was mediated by *KDR*, thus confirming recent reports.[40,50,51] *KDR* antisense oligonucleotide transfection reduced the overall binding of iodinated VEGF to NHA cells by only 10%, but this reduction appeared to lie on the higher-affinity binding sites. This transfection also induced the abolition of *KDR* tyrosine phosphorylation upon VEGF addition and prevented the mitogenic effect of VEGF. As the basal growth was also significantly inhibited by *KDR* down-regulation, it is likely that the VEGF-dependent activation of *KDR* acts as an autocrine loop of proliferation in NHA cells. In fact, the addition of neutralizing anti-VEGF IgG only weakly reduced the basal growth (20%) as compared with *KDR* down-regulation (45%), suggesting that the interaction of VEGF and *KDR* might occur at least in part inside the cell and create an intracrine loop inaccessible to antibodies.

The expression of *KDR/flk*-1 has been reported in hemangioblasts during the early stages of vasculogenesis and in vascular endothelial cells in the late stages of embryonic development[24] and in hemangiomas.[46] Its presence in neonatal hemangioma stromal cells suggests that these cells might represent an endothelial precursor that has not yet completed its maturation. In this respect, it would be interesting to determine whether the expression of *KDR* fades when the hemangioma regresses.

As the secreted endothelial mitogen was retained on anti-VEGF IgG affinity chromatography, VEGF almost totally accounted for the mitogenic activity. Kim et al[52] had previously shown that systemic injections of neutralizing anti-VEGF IgG could inhibit tumor progression in nude mice grafted with transformed cells. We investigated whether systemic injections of neutralizing anti-VEGF antibodies inhibited the angiogenic response observed in nude mice grafted with NHA cells. The vascular structures almost totally disappeared under this treatment. This confirmed the paracrine role of VEGF in this endothelial proliferation that was elicited by tumor cells.

These findings might have an important clinical relevance. The involvement of angiogenic growth factors in the pathophysiology of hemangiomas is not well documented. Although large amounts of FGF2 have recently been detected in the urine of affected patients,[53] our results show that FGF2 is a potent autocrine growth factor synthesized by NHA cells, although no release could be detected *in vitro*, even by activating putative latent forms present in the conditioned medium after ammonium sulfate precipitation.[54] However, an as yet unknown secretory pathway might occur *in vivo*. The recent observation that transduction of a plasmid coding for VEGF in human ischemic arteries induced not only a beneficial effect on vasodilatation but also the formation of cutaneous hemangioma that regressed when VEGF bioavailability decreased[55] confirmed the pathophysiological role of VEGF in this disease. Designing drugs interfering with VEGF or its receptors might open up a new field in hemangioma treatment. We are currently evaluating the usefulness of vascular immunotoxins targeted to angiogenic endothelial cells designed by conjugation of toxins with the KDR-specific VEGF anti-idiotypic antibody used in this study.

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