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Research Article

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Expression of Vascular Endothelial Growth Factor Does Not Promote Transformation but Confers a Growth Advantage In Vivo to Chinese Hamster Ovary Cells

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Abstract

Vascular endothelial growth factor (VEGF) is a mitogen with a specificity for endothelial cells in vitro and an angiogenic inducer in vivo. We tested the hypothesis that VEGF may confer on expressing cells a growth advantage in vivo. Dihydrofolatereductase$^-$ Chinese hamster ovary cells were transfected with expression vectors which direct the constitutive synthesis of VEGF. Neither the expression nor the exogenous administration of VEGF stimulated anchorage-dependent or anchorage-independent growth of Chinese hamster ovary cells in vitro. However, VEGF-expressing clones, unlike control cells, demonstrated an ability to proliferate in nude mice. Histologic examination revealed that the proliferative lesions were compact, well vascularized, and nonedematous. Ultrastructural analysis revealed that capillaries within the lesions were of the continuous type. These findings indicate that the expression of VEGF may confer on cells the ability to grow in vivo in the absence of transformation by purely paracrine mechanisms. Since VEGF is a widely distributed protein, this property may have relevance for a variety of physiological and pathological proliferative processes. (*J. Clin. Invest.* 1992. 91:160–170.) Key words: vascular endothelial growth factor • vascular permeability factor • angiogenesis • paracrine growth regulation • tumor growth

Introduction

Angiogenesis, the development of a microvascular bed, is required for a variety of important physiological processes, such as normal growth and differentiation, wound healing, compensatory hyperplasias, corpus luteum formation, etc. (1–3). The new blood vessels provide an enhanced supply of oxygen and nutrients, which allows the tissues to meet increased metabolic demands, and also carry critical regulatory molecules (4). However, aberrant angiogenesis can be a significant pathogenic component of a variety of disorders such as cancer, diabetes mellitus, atherosclerosis, or rheumatoid arthritis (1–3). In view of the clinical relevance of cancer, an especially large amount of work has been dedicated to tumor angiogenesis (5–7). The new capillaries provide nourishment to the growing tumor and also allow the tumor cells to establish continuity with the vasculature of the host. In support of the hypothesis that angiogenesis plays an important role in the development of a malignancy, it has been recently shown that the acquisition of an angiogenic phenotype marks the transition from hyperplasia to neoplasia (7).

Several factors of both peptide and nonpeptide nature have been shown to induce angiogenesis in vivo: epidermal growth factor, transforming growth factor-alpha and beta, tumor necrosis factor-alpha, angiogenin, acidic fibroblast growth factor, basic FGF (bFGF), platelet-derived endothelial cell growth factor, PGE_2, and monobutyrin (1–3). Recently, a family of endothelial cell mitogens and angiogenic factors known as vascular endothelial growth factor (VEGF)[1] (8–12) or vascular permeability factor (13–16) has been identified and the respective cDNAs have been cloned. By alternative splicing of mRNA, four different molecular species of VEGF are generated. These have 121, 165, 189, and 206 amino acids, respectively ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (9, 11, 12, 17). $VEGF_{165}$ is the most abundant molecular species in the majority of cells and tissues (11, 17). The two longer forms are characterized by the presence of a 24 amino acid insertion highly enriched in basic residues ("nuclear targeting site"). The two shorter forms are soluble after secretion, while the longer ones are mostly insoluble (17). Recent studies have provided evidence that the *flt* protein is a receptor for VEGF (18). These factors display several properties which suggest that they may be important and specific mediators of endothelial cell differentiation and angiogenesis. First, $VEGF_{121}$ and $VEGF_{165}$, unlike FGFs or platelet-derived endothelial cell growth factor, which are mostly intracellular (19–21), are secreted proteins. Second, VEGFs are endothelial cell-specific mitogens in vitro (8, 10, 21). Third, VEGF promotes angiogenesis in several in vivo models (9, 14, 22). Fourth, the VEGF mRNA is expressed in a variety of abundantly vascularized tissues such as pituitary, brain, lung, heart, or kidney (12, 23, 24). In the rat and primate ovary, the expression of the VEGF mRNA is temporally and spatially related to the proliferation of capillaries into the corpus luteum, providing circumstantial evidence that VEGF is a physiological mediator of angiogenesis (25, 26). This hypothesis is supported by recent findings that demonstrate expression of VEGF mRNA in the ventricular neuroectoderm in the mouse embryo, temporally and spatially related to the development of capillaries from the perineural plexus (27).

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CHO, Chinese hamster ovary; DHFR, dihydrofolatereductase; G-H-T, glycine-hypoxanthine-thymidine; rh, recombinant human; VEGF, vascular endothelial growth factor.

An additional property of this family of factors is the ability to induce vascular leakage (13–16) as evidenced by blue Evans extravasation in the guinea pig skin-based Miles assay (28). Vascular permeability factor was identified from the conditioned medium of tumor cell lines on the basis of this property. It was proposed that such a factor is a tumor-specific protein, involved in the increased permeability to plasma proteins displayed by tumor vessels (13). It has been suggested that high vascular permeability to proteins such as fibrinogen plays a significant role in the process of tumor angiogenesis and metastasis; an extravascular fibrin gel would provide an optimal substratum for both tumor and endothelial cell growth (29).

Transfection of mammalian cells with expression vectors carrying specific cDNAs and introduction of such cells into a living host represents an important tool to investigate the in vivo effects of gene products in general and to test the hypothesis that such gene products may confer on a given cell the ability to grow in vivo in particular (30–33). In the present study, we describe the effects of the introduction of expression vectors which direct the constitutive synthesis of VEGF in a dihydrofolatereductase $(DHFR)^-$ clone of Chinese hamster ovary (CHO) cells. The transfected cells were characterized for their ability to grow in anchorage-dependent or anchorage-independent fashion. We also injected transfected or parental cells into nude mice. The proliferative lesions, which developed from CHO cells expressing VEGF, were characterized for their morphological features and for VEGF expression. The ultrastructure of capillaries within the tumors was also elucidated.

Methods

Reagents. Tissue culture reagents, media, and sera were purchased from Gibco Laboratories (Grand Island, NY) through the Genentech media facility. Tissue culture plates were from Costar Corp. (Cambridge, MA) or Nunc (Kamstrup, Denmark). Recombinant human (rh) $VEGF_{165}$ was purified from conditioned medium of transfected CHO cells as described (34). The purity of the material was confirmed by the presence of a single NH_2-terminal amino acid sequence and by a silver-stained SDS/PAGE gel. The avidin-biotin complex kit for immunoperoxidase staining was from Vector Laboratories (Burlingame, CA). Eponete 12 was from Ted Pella Inc. (Redding, CA). [^{35}S]methionine was purchased from Amersham Corp. (Arlington Heights, IL). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Establishment of constructs and transfection of CHO cells. An expression vector carrying the full length $VEGF_{165}$ cDNA insert (9) was constructed. This vector is a variant of PCISh (35) which carries a DHFR gene. The $VEGF_{165}$ cDNA (~800 bp) was subcloned into the EcoRI site in the polylinker region of the vector in such a way that the transcription of the VEGF cDNA is driven by an SV40 promoter. The transcription of DHFR is also driven by an SV40 promoter. The $VEGF_{121}$ cDNA (9) was ~650 bp. A $DHFR^-$ clone of CHO cells (DP_{12}) was used for transfection. Cells were cultured in 10-cm tissue culture dishes in the presence of low glucose DMEM/F12 (1:1) plus 2% dialyzed fetal bovine serum, 2 mM glutamine, glycine (10 mg/ml), hypoxanthine (15 mg/ml), thymidine (5 mg/ml) (G-H-T) and antibiotics. Subconfluent cultures were transfected with expression vectors carrying VEGF cDNA or vector alone by the calcium phosphate coprecipitation method (36). 48 h after transfection, cells were placed in selection medium. This was identical to the growth medium except for the absence of G-H-T. After approximately a week, colonies were picked by a pipette tip and transferred into individual wells of 24 multiwell plates in the presence of selection medium. Subconfluent clones were screened for their ability to release in the medium mitogenic activity for capillary endothelial cells. Clones which displayed the highest bioactivity were expanded and amplified (37) in the presence of increasing concentrations of methotrexate. Clones proliferating in the presence of 1 μm methotrexate were used in the present study.

Proliferation assays. Mitogenic assays on bovine adrenal cortex-derived capillary endothelial cells were performed as previously described (8). Various aliquots of conditioned media or $rhVEGF_{165}$ were added to cells 4–6 h after plating. Cells were counted in a counter after 4–5 d (Coulter Corp., Hialeah, FL).

To assess the effects of VEGF on the growth of CHO cells, parental or transfected cells were seeded at the density of 20,000/well in 12 multiwell plates in the presence of DMEM/F12 supplemented with 2% dialyzed fetal bovine serum, G-H-T, 2 mM glutamine, and antibiotics. Various concentrations of $rhVEGF_{165}$ were added in 10-μl aliquots. Cells were dissociated by exposure to trypsin and counted in a counter after 5 d (Coulter Corp.). To determine proliferation as a function of time, CHO cells were plated in 12 multiwell plates as described above and counted every other day.

VEGF ELISA. 96-well microtiter plates were coated with a monoclonal antibody directed against $rhVEGF_{165}$ (mAb 4.6.1.) (38) by incubation at 4°C with 100 μl/well of antibody at 2.5 μg/ml in 50 mmol/liter of sodium carbonate, pH 9.6, and then blocked with 0.5% BSA in PBS for 1 h at room temperature. $rhVEGF_{165}$ (between 0.78 and 50 ng/ml) or test samples were added to the coated wells and then incubated for 2 h at room temperature. Plates were then incubated with horseradish peroxidase-labeled monoclonal antibody 3.13.1. (38). Substrate solution (0.04% *o*-phenyenediamine dihydrochloride in PBS plus 0.4 ml/liter of 30% hydrogen peroxide) was added to plates. After 15 min in the dark, the reaction was stopped by the addition of 2.25 mol/liter sulfuric acid and absorbance at 490 nm was determined on a plate reader. Between each step, plates were washed six times with PBS containing 0.1% Tween 20. A standard curve was generated by plotting absorbance vs log of $rhVEGF_{165}$ concentration, using a four-parameter nonlinear regression curve fitting program. The limit of sensitivity of the assay was 0.4 ng/tube.

Metabolic labeling of cells. For metabolic labeling, cells were passaged into 24 multiwell tissue culture plates at the density of 10,000 cells/well. As soon as cells were confluent, they were washed three times with PBS and then incubated for 1 h in the presence of serum-free methionine-cysteine free DMEM/F12 containing 100 μCi [^{35}S]-methionine. The medium was removed and replaced with fresh serum-free medium containing methionine and cysteine. After 5 h, media were removed and aliquots were subjected to SDS/PAGE gel (12.5%), in both reducing and nonreducing conditions. The gel was then dried and processed for fluorography.

Soft agar colony formation assay. The soft agar assay was performed essentially as previously described (39). 4 ml of high glucose DMEM supplemented with 10% FBS, 2 mM glutamine, G-H-T, and antibiotics (complete medium) plus 0.5% (wt/vol) agar (Difco Laboratories, Inc., Detroit, MI) were added to 60-mm tissue culture dishes and allowed to solidify. 30,000 cells in a 0.1-ml vol were then added slowly, followed by 3 ml of complete medium containing 0.25% agar. Half of the dishes seeded with parental or vector-transfected cells also received $rhVEGF_{165}$ at the final concentration of 20 ng/ml. Cultures were incubated in a humidified atmosphere at 37°C in the presence of 5% CO_2. After 8 d, 1 ml of complete medium containing 0.23 mg of dimethylthiazol diphenyl tetrazolium bromide was added to each dish to identify viable colonies (40). After overnight incubation, stained colonies (> 100 μm) were counted in triplicate in a grid and photographed in a Nikon dissection microscope.

Injection into nude mice. Cells were dissociated from stock plates by exposure to trypsin. Serum-containing medium was added to neutralize the trypsin and cells were pelleted by centrifugation at 100 *g* for 5 min. Cells were then washed twice with PBS and resuspended at the appropriate density in serum-free medium. Cells were injected in groups of five into 8–10-wk old female nude athymic mice (nu/nu[CD-1]BR) obtained from Charles River Breeding Laboratories (Wilmington, MA). Injections were performed subcutaneously in the

dorsal areas in a volume of 0.2 ml at various final densities (Table III). Length and width of tumors were measured twice weekly. After 4–6 wk, animals were killed by CO_2 inhalation and tissues were dissected and immersed in the appropriate fixative.

Light level histological examination. The lung, liver, spleen, and injection sites were removed from each animal immediately after euthanasia and immersion-fixed in 10% neutral buffered phosphate formalin. Tissues were processed for paraffin embedding, sectioned at 6 μm, and stained with hematoxylin-eosin.

In situ hybridization. Tumors (clones 9 or 24) were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate, pH 7.2, for 1 h at 4°C. After fixation, specimens were infiltrated overnight with 20% sucrose, frozen, and sectioned on a cryostat at 12 μm. Sections were thaw mounted onto Vectabond-coated slides (Vector Laboratories, Burlingame, CA), dried, and stored at −70°C. In situ hybridization was performed as described (26) using a 1-kb probe representing residues 924–1920 of the coding region of the human VEGF sequence (9). For specificity control, adjacent sections were incubated with sense probe.

Immunocytochemistry. Sections of tumors derived from clone 9 were stained for VEGF by the avidin-biotin-peroxidase method. Deparaffinized sections were incubated overnight with a murine monoclonal anti-VEGF (mAb 4.6.1) (38) at the concentration of 10 μg/ml at 4°C. Slides were then reacted with biotinylated equine anti-mouse IgG diluted 1:100 for 30 min, according to the instructions of the vendor. Negative control consisted of slides incubated with a murine monoclonal antibody of the same class (IgG_1) with inappropriate specificity.

Electron microscopy. Tumors (clones 9 or 24) were fixed with 1% paraformaldehyde 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at 4°C. After washing, the samples were postfixed in 2% osmium in the same buffer, washed in distilled water, en bloc stained in ethanolic uranyl acetate overnight at 4°C, dehydrated through graded ethanols and propylene oxide, and embedded in Eponete 12 (Ted Pella Inc.). Ultrathin sections were cut on a microtome (Ultracut E; Reichert Scientific Instruments, Buffalo, NY) and counterstained with ethanolic uranyl acetate and lead citrate, examined at 80 kV, and photographed on a transmission electron microscope (CM 12; Phillips Scientific, Mahwah, NJ).

Results

Expression of VEGF cDNAs in CHO cells. Transfected cells were selected for their ability to proliferate in the absence of exogenous G-H-T and amplified in the presence of methotrexate. Medium conditioned by parental or vector-transfected CHO cells (tested up to 200 μl/ml) had essentially no mitogenic activity on endothelial cells. In contrast, media conditioned by VEGF-expressing cells was strongly mitogenic. The amount of conditioned medium required to induce a maximal growth effect ranged from 0.5–2 μl/ml (clones 9 and 21) to 5–10 μl/ml (clones 1, 24) (Fig. 1). Using a sensitive ELISA, the VEGF levels after a 24-h incubation ranged between 101 and 520 ng/ml in various $VEGF_{165}$-expressing clones (Table I). However, VEGF was undetectable in the conditioned medium of parental or vector transfected cells. That the bioactivity was actually caused by VEGF was demonstrated by the neutralization of such activity by a rabbit polyclonal antiserum raised against purified $rhVEGF_{165}$ (Fig. 1). This antiserum did not affect endothelial cell growth in response to bFGF. Fig. 2 demonstrates the presence of a protein band of the appropriate molecular size (46 kD in nonreducing and 23 kD in reducing conditions) (8, 10) in the conditioned medium of clone 9, our most extensively characterized VEGF-expressing cell line. This band was not detectable in the conditioned medium of parental or vector-transfected cells. A similar band was identified in the medium of clones 1 or 21. In the conditioned medium of clone 24, expressing $VEGF_{121}$, a band of ~ 34 kD in nonreducing and ~ 17 kD in reducing conditions was observed, in agreement with previous studies (17) (data not shown).

VEGF is not an autocrine growth factor nor does it promote anchorage-independent growth of CHO cells. The addition of purified $rhVEGF_{165}$ in a concentration range between 1 and 20 ng/ml had no effect on the final density of parental, vector-transfected, or VEGF-expressing cells (Fig. 3 *A*). Growth curves revealed that the proliferation rate of clone 9 was about twofold lower than that of parental cells. Clone 24 proliferated at the same rate as the parental cells. Cells transfected with vector alone exhibited the highest growth rate (Fig. 3 *B*).

Cell lines were also tested for their ability to grow in an anchorage-independent manner in soft agar. Table II summarizes the results of such assay. Parental cells did not form colonies. Also, the addition of 20 ng/ml of $rhVEGF_{165}$ did not promote colony formation. Vector-transfected cells demonstrated instead an ability to form colonies. This was not affected by the addition of $rhVEGF_{165}$. Among cell lines expressing $VEGF_{165}$, clones 9 and 21 failed to form colonies. Clone 1 demonstrated a moderate ability to grow in soft agar, which was still lower than that exhibited by the vector-transfected

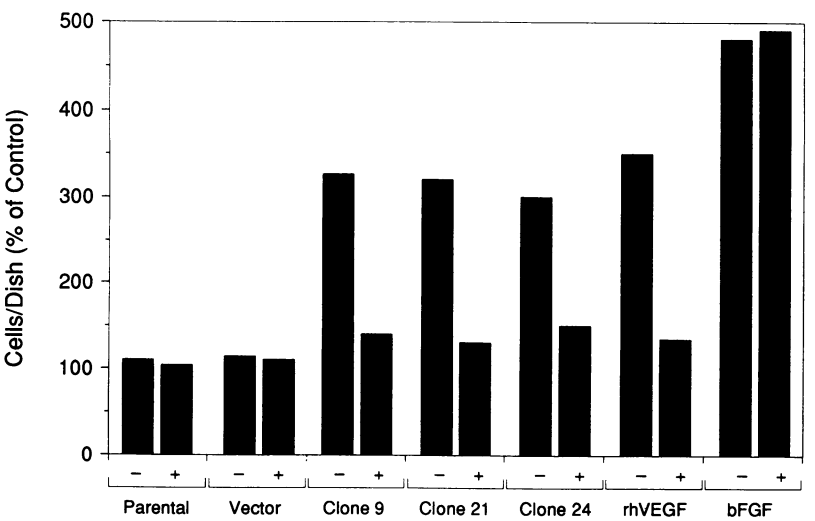

Figure 1. Mitogenic activity of media conditioned for 48 h by parental or VEGF-expressing CHO cells and immunoneutralization of the activity by a VEGF-specific antiserum. Conditioned medium of parental or vector-transfected cells was added to capillary endothelial cells at 200 μl/ml. Data shown for the VEGF-expressing cells reflect the response to aliquots of medium which induce a maximal mitogenic effect: 1 μl/ml clone 9; 2 μl/ml clone 21; 10 μl/ml clone 24. Half of the wells were incubated with a polyclonal VEGF antiserum at the final dilution of 1:500. As a positive control, $rhVEGF_{165}$ was added at the concentration of 5 ng/ml. bFGF was tested at the concentration of 2 ng/ml. Plus and minus signs denote the presence or absence of antiserum.

Table I. Concentration of $VEGF_{165}$ by ELISA in the Conditioned Medium of Parental or Transfected CHO Cells

Clone	VEGF
	ng/ml
Parental	ND
Vector	ND
1	101
9	520
21	305

Cells were cultured in 10-cm tissue culture dishes. As soon as cultures were subconfluent, media were replaced with fresh media. After 24 h, media were collected and aliquots were subjected to ELISA. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%. The limit of sensitivity of the assay was 0.4 ng/tube. (ND, not detectable).

cells. The $VEGF_{121}$-expressing clone examined (clone 24) formed few colonies. These findings indicate that the neither VEGF expression nor the exogenous administration of VEGF promoted anchorage-dependent or -independent growth.

VEGF expression confers a growth advantage to CHO cells in nude mice. In two independent experiments, none of the mice injected with parental or vector-transfected cells developed tumors for the entire duration of the experiment. In contrast, all VEGF-expressing cell lines tested in nude mice demonstrated an ability to proliferate, although differences existed in the incidence of tumor formation among various clones. Clones 9 and 21 gave the highest incidence of tumors. The results of the two experiments are summarized in Table III. Tumor size reached a plateau at 4–6 wk. None of the animals appeared cachectic. No evidence of metastasis was found. Tissues and organs examined microscopically appeared normal.

Morphology of tumors at the light microscopy level. Tumors were observed in the subcutaneous tissue at the injection site. The tumors were well circumscribed lesions with thin fibrous encapsulation (Fig. 4, *A* and *B*). They were comprised of interlacing sheets and cords of spindle-shaped cells which had large oval-shaped vesiculated nuclei, prominent nucleoli, and a

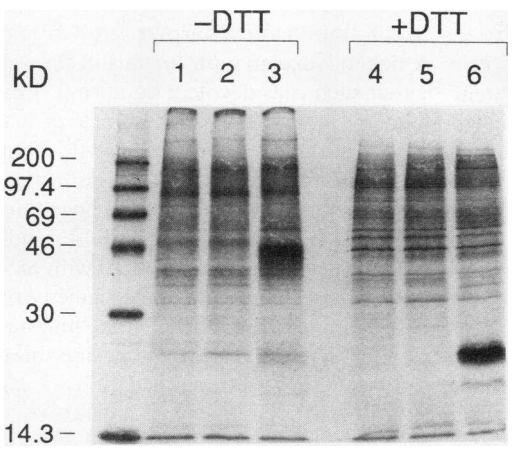

Figure 2. Analysis of conditioned media of parental (1, 4), vector-transfected (2, 5), or clone 9 ($VEGF_{165}$) cells (3, 6). Cells were metabolically labeled as described in Methods. Aliquots of conditioned medium were then subjected to SDS/PAGE gel in both nonreducing (−DTT) and reducing (+DTT) conditions. The gel was then dried and processed for fluorography.

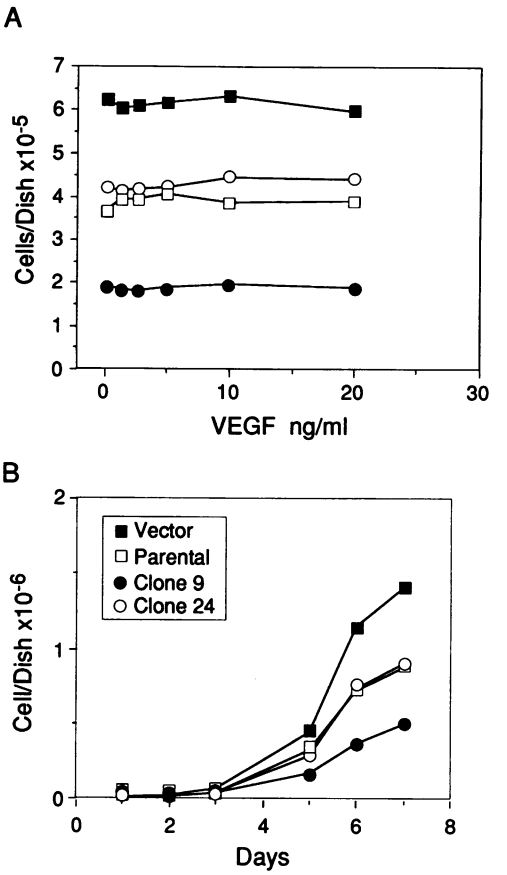

Figure 3. Proliferation of parental or transfected CHO cells as a function of VEGF concentration (*A*) or time (*B*). (*A*) Cells were plated as described at the density of 20,000/well. $rhVEGF_{165}$ (between 1 and 20 ng/ml) was added. Cells were counted after 5 d. (*B*) Cells were seeded as above and counted every day in a counter (Coulter Corp.). The variation from the mean did not exceed 10%.

Table II. Formation of Colonies in Soft Agar by CHO Cell Clones

Clone	Colonies/cm²
Parental	0
Parental + VEGF	0
Vector	10±2
Vector + VEGF	10±1
1	8±2
9	0
21	0
24	5±1

Cells were plated in soft agar as described in Methods. Half of dishes seeded with parental or vector-transfected cells received $rhVEGF_{165}$ (20 ng/ml). After 8 d, dimethylthiazol diphenyl tetrazolium bromide was added to dishes. Stained colonies (> 100 μm) were counted after overnight incubation. Values shown are means±S.D.

Table III. Tumor Formation in Nude Mice by Parental or Transfected CHO Cells

Clone	Cells injected/mouse	3 wk	4 wk	5 wk	6 wk
Experiment 1					
Par	1×10^5	0/5	0/5	0/5	0/5
	5×10^5	0/5	0/5	0/5	0/5
VC	1×10^5	0/5	0/5	0/5	0/5
	5×10^5	0/5	0/5	0/5	0/5
1	1×10^5	0/5	1/5 0.5 × 0.5	2/5 0.9 × 0.7	2/5 1.1 × 1.0
	5×10^5	1/5 0.4 × 0.4	2/5 0.7 × 0.6	3/5 1.0 × 0.9	3/5 1.2 × 1.1
21	1×10^5	0/5	2/5 0.5 × 0.6	3/5 1.1 × 0.9	4/5 1.3 × 1.1
	5×10^5	2/5 0.5 × 0.5	3/5 0.8 × 0.7	4/5 1.2 × 1.0	4/5 1.4 × 1.3
Experiment 2		1 wk	2 wk	3 wk	4 wk
Par	1×10^6	0/5	0/5	0/5	0/5
	3×10^6	0/5	0/5	0/5	0/5
VC	1×10^6	0/5	0/5	0/5	0/5
	3×10^6	0/5	0/5	0/5	0/5
9	1×10^6	2/5 0.4 × 0.4	3/5 0.6 × 0.5	3/5 0.7 × 0.6	3/5 0.8 × 0.6
	3×10^6	4/5 0.5 × 0.4	5/5 0.7 × 0.5	5/5 0.75 × 0.7	5/5 0.9 × 0.7
24	1×10^6	1/5 0.3 × 0.3	2/5 0.6 × 0.6	2/5 0.7 × 0.7	2/5 0.9 × 0.75
	3×10^6	2/5 0.7 × 0.5	3/5 0.8 × 0.5	3/5 0.8 × 0.6	3/5 0.9 × 0.9

The table reports the incidence of tumors in mice injected with parental (Par), vector-transfected (VC), and various VEGF-expressing clones in two separate experiments. Clones 1, 9, and 21 express $VEGF_{165}$; clone 24 expresses $VEGF_{121}$. Tumor sizes at the end of each week are indicated as averages of width and length in centimeters. In experiment 1, no tumors were detectable during the first 2 wk.

moderate amount of cytoplasm. Cell membranes were indistinct. The mitotic index was high. There was evidence of capillary ingrowth into the tumor masses. Capillaries were well represented within the sections. A mild to moderate infiltration of leukocytes, predominantly lymphocytes and macrophages, was associated with some tumors. There was mild edema in the subcutaneous connective tissue surrounding the tumor in some animals; there was however no evidence of vascular leakage within the tumors. No significant histologic differences could be appreciated among tumors derived from different clones.

In situ hybridization. In situ hybridization revealed that the VEGF mRNA was strongly expressed by the majority of tumor cells. Essentially similar results were obtained with tumors originating from either clone 9 or clone 24. Fig. 5 *A* illustrates the VEGF mRNA expression in a section from a clone 9–derived tumor and adjacent host tissue. VEGF mRNA expression appeared uniform throughout the extent of the lesion. Host tissues and the fibrous capsule surrounding the tumor were invariably negative for VEGF hybridization signal. Higher magnification examination revealed that the hybridization signal was not associated with endothelial cells within the lesion. No appreciable hybridization was observed in sections incubated with sense probe (Fig. 5 *B*).

Immunocytochemistry. Approximately 80% of tumor cells in lesions induced by clone 9 demonstrated cytoplasmic and membranous staining for VEGF when probed with a specific monoclonal antibody (Fig. 6 *A*). The specificity of the reaction was demonstrated by the lack of immunostaining when a control monoclonal antibody was used (Fig. 6 *B*).

Ultrastructural analysis. Transmission electron microscopy examination of tumor tissues derived from either clone 9 or clone 24 revealed than most of the capillaries were of the continuous type. Endothelial cells contained plasmalemmal vesicles opening at both apical and basal side. Close examination failed to show any apparent alterations in the ultrastructure in both endothelial cells and pericytes. Fig. 7 illustrates the morphology of a typical capillary from a tumor derived from clone 9.

Discussion

The present results indicate that the expression of VEGF does not confer a growth advantage in vitro on CHO cells. In fact, the highest expressing cell lines had even slower growth rate than parental cells. A possible explanation for this decreased proliferative ability is that such cells devote a significant fraction of their metabolic energy to high level synthesis of a recombinant protein which does not affect their own growth. That VEGF is not an autocrine growth factor for such cells is further supported by their lack of proliferative response to exogenous $rhVEGF_{165}$ as well as by their lack of specific ^{125}I-VEGF binding (data not shown). These results are in agreement with previous studies, which provided evidence for the specificity of VEGF for endothelial cells in culture (8, 10). These findings have been recently extended to the in vivo situation, since high affinity binding sites for VEGF were identified in vascular endothelial cells but not in other cell types in adult rat tissue sections (41).

However, expression of either secreted molecular species of VEGF confers on transfected CHO cells the property to form vascularized proliferative lesions in nude mice. The finding that no correlation existed between the ability to grow in soft agar and VEGF expression and also that the clones associated with the highest incidence of tumors failed to form colonies in

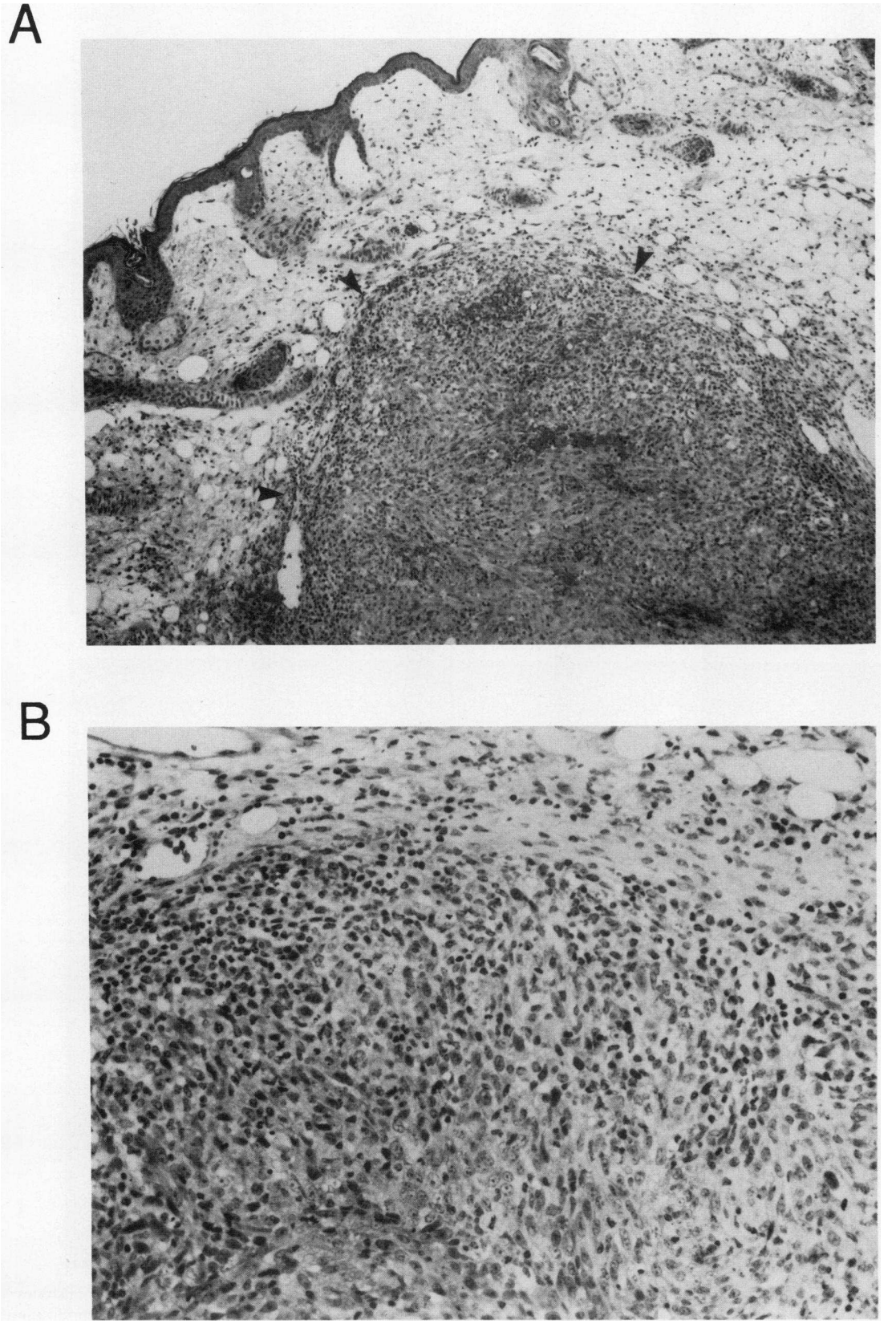

Figure 4. (*A*) Photomicrograph of a tumor derived from clone 9. Arrowheads point to the edge of the lesion. The mass is well circumscribed and nonedematous (×40). (*B*) Higher magnification (×250) of the periphery of the tumor. The tumor is composed of spindle cells with large vesiculated nuclei and prominent nucleoli. There is a moderate infiltration of lymphocytes into the tissues at the margin of the tumor.

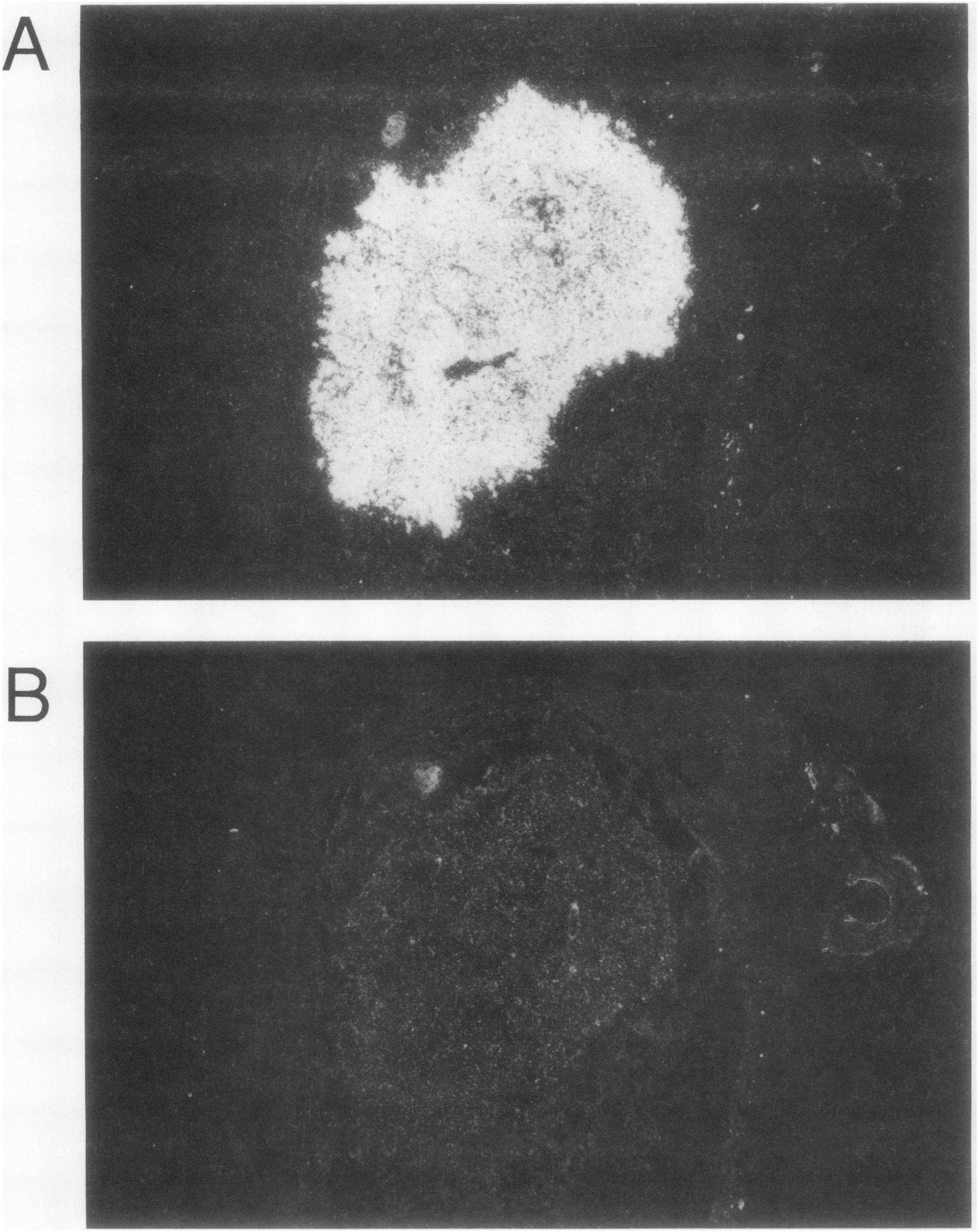

Figure 5. In situ hybridization of sections from a clone 9–derived tumor with a VEGF-specific probe. (*A*) Dark-field examination demonstrates the high and uniform expression of VEGF mRNA in a section incubated with antisense probe. Note the absence of appreciable hybridization in adjacent host tissues. (*B*) An adjacent section incubated with control sense probe.

soft agar provides evidence that the ability to grow in vivo was not related to transformation, at least as assessed by conventional in vitro criteria (39).

Therefore, it appears that such an advantage in vivo is accounted for by paracrine mechanisms. This situation is unlike that observed with other growth factors such as transforming

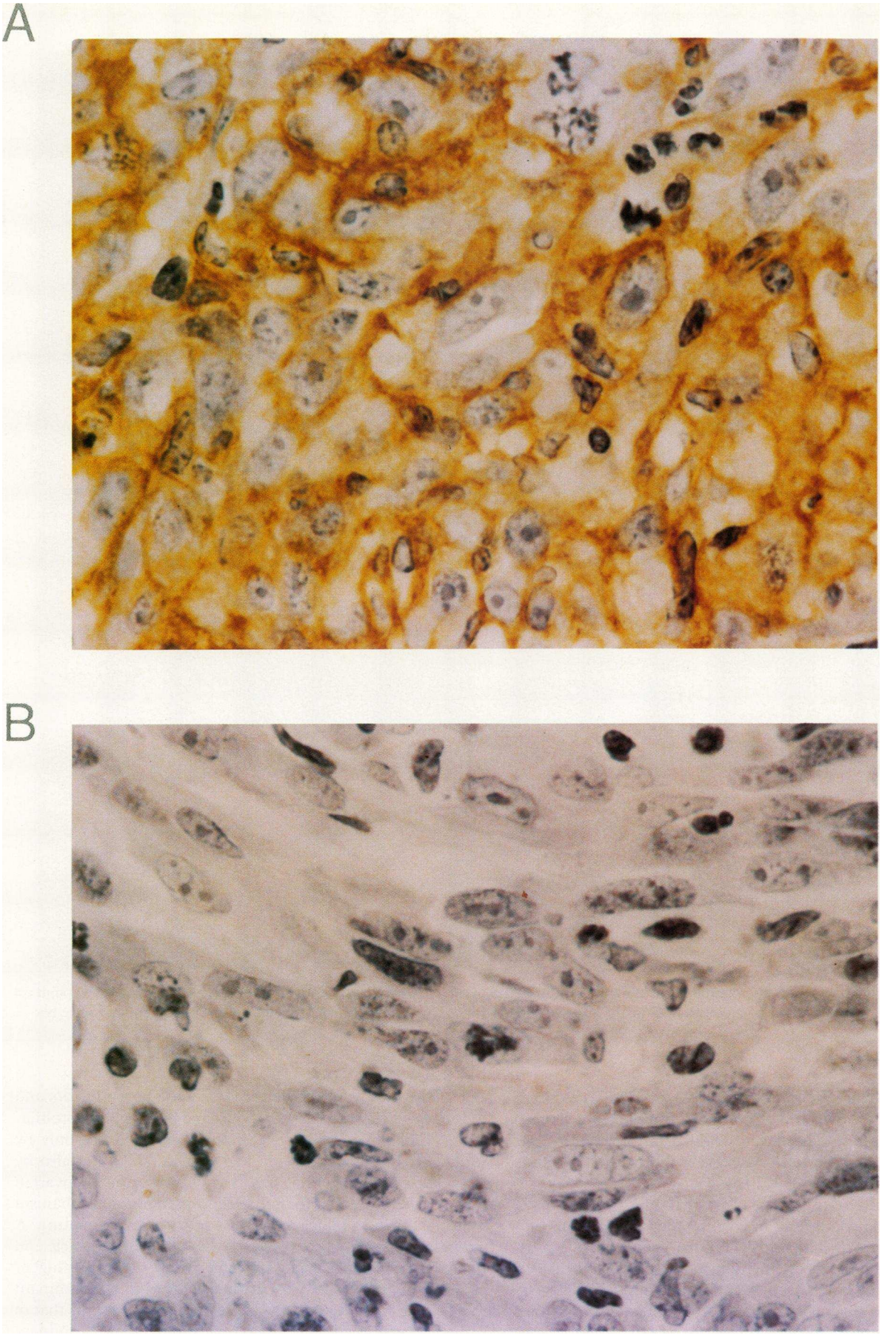

Figure 6. Immunocytochemical stain of tumor cells (clone 9) with a VEGF-specific monoclonal antibody (*A*) or with a control antibody (*B*). Staining for VEGF is present in the tumor cells cytoplasm and at the plasma membrane (×1,800).

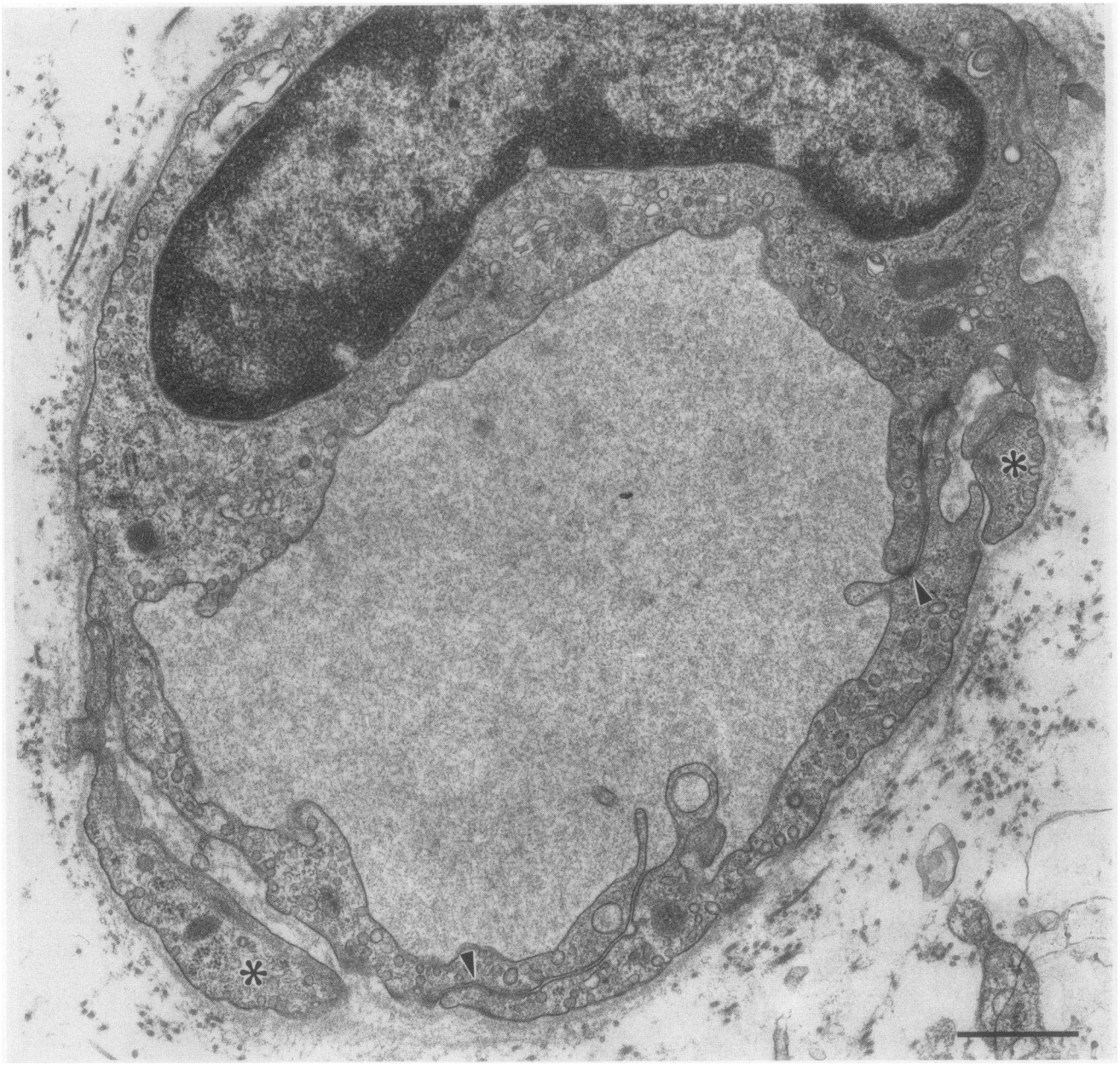

Figure 7. Transmission electron micrograph illustrating the ultrastructure of a typical capillary within a tumor (clone 9). Arrowheads point to junctional complexes. Asterisks are on pericytes. Note the absence of fenestrations and the regular morphology of the endothelium. (Scale bar = 1 μm).

growth factor-alpha (30), acidic FGF (32), or bFGF fused to a signal peptide (33) which also confer to transfected cells the ability to grow in vivo. These agents induce transformation and are thought to promote tumorigenesis by both autocrine and paracrine mechanisms (30, 32, 42–44).

We hypothesize that the in vivo growth of VEGF-expressing cells is mediated, at least in part, by the direct angiogenic properties of the growth factor. VEGF has been shown to promote angiogenesis in several in vitro and in vivo systems (8–10, 14, 22). That VEGF was truly available to elicit such effects is demonstrated by the high expression of the mRNA and by the immunocytochemical localization of the VEGF protein in the majority of cells in the lesion. The invasion of capillaries is expected to result in the delivery of blood-borne factors and nutrients which would in turn trigger and maintain cell proliferation. That this model is plausible is suggested not only by studies on tumor progression and growth (6, 7) but also by physiological angiogenesis like that occurring in the ovarian follicle. The process of follicle selection requires a mechanism by which a single follicle continues to survive and mature. A strong correlation exists between density of microvessels and follicular dominance in the primate ovary (45). The higher capillary density results in a greater delivery to the dominant follicle of gonadotropins, plasma factors, and nutrients that in turn support growth and differentiation, while nondominant follicles undergo atresia. In this context, it is very interesting to

observe that recent studies have provided evidence that only the selected follicle contains cells that intensely express the VEGF mRNA in the primate ovary (26).

A further mechanism that might potentially contribute to the proliferative process is angiogenesis and/or mitogenesis mediated by macrophage-derived factors. Recent studies provided evidence that VEGF has the ability to promote monocyte chemotaxis (46). Infiltration of macrophages and other inflammatory cells was observed in VEGF-expressing tumors, although it was not prominent in most of the lesions.

It is important to emphasize that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis. The lesions formed by VEGF-expressing CHO cells were essentially benign and did not grow beyond a relatively small size. This is in agreement with the notion that stimulation of angiogenesis is necessary but not sufficient for malignant growth (3).

Since VEGF/vascular permeability factor has been also characterized as a protein associated with tumor vascular permeability (13–16), and, more recently, it has been implicated in the development and maintenance of fenestrations in capillaries (27), it would be of significant interest to elucidate the ultrastructure of microvessels after continual exposure to the growth factor. Proliferative lesions derived from transfected cells provide a unique opportunity. However, capillaries in tumors expressing either secreted molecular species of VEGF did not exhibit fenestrations or other ultrastructural features consistent with unusually high permeability, such as intercellular gaps, high number of pinocytotic vesicles, or abnormalities in the junctional complex (47–50). The endothelium was of the continuous type and its ultrastructural features were consistent with a normal capillary (49). Even though tracer studies would be required to provide a direct measurement of vascular permeability (51, 52), very little or nothing in the morphology of the lesions was consistent with leakage. These findings clearly do not rule out the possibility that VEGF may promote extravasation in different experimental circumstances or that leakage may have occurred in the tumors at an earlier time point. The hypothesis that this factor is a mediator of vascular permeability was based largely on its potent activity in the Miles assay (28). Further studies are required to fully assess the significance and relevance of this activity.

In conclusion, our findings provide evidence that expression of VEGF confers on cells a growth advantage in vivo and suggest that a purely paracrine mediator may be sufficient to trigger the complex chain of events leading to a proliferative process. Since VEGF is a widely distributed secreted protein, such an inductive mechanism has the potential to operate in a variety of physiological circumstances such as normal growth, ovarian function, or wound healing. Furthermore, a variety of tumor cells express the VEGF mRNA at high level and secrete VEGF (12), suggesting that this soluble protein may facilitate tumor growth through its direct angiogenic effects. Our findings are consistent with this hypothesis.

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References

1. Folkman, J., and M. Klagsbrun. 1987. Angiogenic factors. *Science (Wash. DC)*. 235:442–445.

2. Klagsbrun, M., and P. A. D'Amore. 1991. Regulators of angiogenesis. *Annu. Rev. Physiol.* 53:217–239.

3. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931–10934.

4. Renkin, E. M. 1989. Microcirculation and exchange. *In* Textbook of Physiology. H. D. Patton, A. F. Fuchs, B. Hille, A. M. Scher, and R. Steiner, editors. Philadelphia: W. B. Saunders Co., Philadelphia, PA. 21:860–878.

5. Greenblatt, M., and P. Shubik. 1968. Tumor angiogenesis, transfilter diffusion studies in the hamster by the transparent chamber technique. *J. Natl. Cancer Inst.* 41:111–124.

6. Folkman, J. 1990. What is the evidence that tumors are angiogenesis-dependent? *J. Natl. Cancer Inst.* 82:4–6.

7. Folkman, J., K. Watson, D. Ingber, and D. Hanahan. 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature (Lond.)*. 339:58–60.

8. Ferrara, N., and W. J. Henzel. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851–859.

9. Leung, D. W., G. Cachianes, W.-J. Kuang, D. V. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Wash. DC)*. 246:306–309.

10. Conn, G., D. Soderman, M.-T. Schaeffer, M. Wile, V. B. Hatcher, and K. A. Thomas. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma cell line. *Proc. Natl. Acad. Sci. USA*. 87:1323–1327.

11. Tisher, E., R. Mitchell, T. Hartmann, M. Silva, D. Gospodarowicz, J. Fiddes, and J. Abraham. 1991. The human gene for vascular endothelial growth factor. *J. Biol. Chem.* 266:11947–11954.

12. Ferrara, N., K. Houck, L. Jakeman, and D. W. Leung. 1992. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr. Rev.* 13:18–32.

13. Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (Wash. DC)*. 219:983–985.

14. Connolly, D. T., D. M. Heuvelman, R. Nelson, J. V. Holander, B. L. Eppley, J. J. Delfino, R. N. Siegel, R. S. Leimgruber, and J. Feder. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84:1470–1478.

15. Keck, P. J., S. D. Kauser, G. Krivi, K. Sanzo, T. Warren, J. Feder, and D. T. Connolly. 1989. Vascular permeability factor, an endothelial cells mitogen related to PDGF. *Science (Wash. DC)*. 246:309–311.

16. Connolly, D. T. 1991. Vascular permeability factor, a unique regulator of blood vessel function. *J. Cell. Biochem.* 47:219–223.

17. Houck, K. A., N. Ferrara, J. Winer, G. Cachianes, B. Li, and D. W. Leung. 1991. The vascular endothelial growth factor family. Identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* 5:1806–1814.

18. deVries, C., J. A. Escobedo, H. Ueno, K. Houck, N. Ferrara, and L. T. Williams. 1992. The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science (Wash. DC)*. 255:989–991.

19. Abraham, J., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Jerrild, D. Gospodarowicz, and J. Fiddes. 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science (Wash. DC)*. 233:545–547.

20. Jaye, M., R. Howk, W. Burgess, G. A. Ricca, I. M. Chiu, M. W. Ravera, S. G. O'Brien, W. S. Modi, T. Maciag, and W. N. Drohan. 1986. Human endothelial cell growth factor, cloning, nucleotide sequence and chromosomal localization. *Science (Wash. DC)*. 233:541–544.

21. Ishikawa, F., K. Miyazono, U. Hellman, H. Drexler, C. Westernrdt, K. Hagiwara, K. Usuki, F. Takaku, W. Risau, and C.-H. Heldin. 1989. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature (Lond.)*. 338:557–561.

22. Ploüet, J., J. Shilling, and D. Gospodarowicz. 1989. Isolation and characterization of a newly identified endothelial cell mitogen produced by At-T20 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3801–3807.

23. Ferrara, N., D. W. Leung, and H. S. Phillips. 1991. Molecular characterization and distribution of vascular endothelial growth factor. *In* Neuroendocrine Perspectives. E. E. Müller and R. B. MacLeod, editors. Springer-Verlag, New York Inc. 9:127–161.

24. Berse, B., L. F. Brown, L. Van De Vater, H. F. Dvorak, and D. R. Senger. 1992. Vascular permeability factor (vascular endothelial growth factor) gene is differentially expressed in normal tissues, macrophages and tumors. *Mol. Biol. of the Cell.* 3:211–220.

25. Phillips, H. S., J. Hains, D. W. Leung, and N. Ferrara. 1990. Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology.* 127:965–968.

26. Ravindranath, N., L. Little-Ihrig, H. S. Phillips, N. Ferrara, and A. J. Zeleznick. 1992. Vascular endothelial growth factor mRNA expression in the primate ovary. *Endocrinology.* 131:254–260.

27. Breier, G., U. Albrecht, S. Sterrer, and W. Risau. 1992. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cells differentiation. *Development (Camb.).* 114:521–532.

28. Miles, A. A., and E. M. Miles. 1952. Vascular reactions to histamine, histamine liberators or leukotoxins in the skin of the guinea pigs. *J. Physiol. (Lond.).* 265:228–246.

29. Dvorak, H. 1986. Tumors, wounds that do not heal. *N. Engl. J. Med.* 315:1650–1659.

30. Rosenthal, A., P. B. Lindquist, T. S. Bringman, D. V. Goeddel, and R. Derynck. 1986. Expression in rat fibroblasts of a human transforming growth factor-alpha cDNA results in transformation. *Cell.* 46:301–309.

31. Selden, R. F., M. J. Skoskiewicz, K. B. Howie, P. S. Russell, and H. M. Goodman. 1987. Implantation of genetically engineered fibroblasts into mice. Implications for gene therapy. *Science (Wash. DC).* 236:714–718.

32. Jaye, M., R. M. Lyall, R. Mudd, J. Schlessinger, and N. Sarver. 1988. Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:963–969.

33. Rojely, S., R. A. Weinberg, P. Fanning, and M. Klagsbrun. 1988. Basic fibroblast growth factor fused to a signal peptide transform cells. *Nature (Lond.).* 331:173–175.

34. Ferrara, N., D. W. Leung, G. Cachianes, J. Winer, and W. J. Henzel. 1991. Purification and cloning of vascular endothelial growth factor secreted by pituitary folliculostellate cells. *Methods Enzymol.* 198:391–404.

35. Gorman, C., D. R. Gies, and G. D. McCray. 1990. Transient production of proteins using an adenovirus transformed cell line. *DNA and Protein Engineering Techniques.* 2:3–10.

36. Gorman, C. 1985. Introduction of DNA in mammalian cells. *In* DNA Cloning I. D. M. Glover, editor. IRL Press Ltd., Oxford. 143–190.

37. Kaufman, R. J. 1990. Selection and coamplification of heterologous genes in mammalian cells. *Methods Enzymol.* 185:537–566.

38. Kim, J., B. Li, J. Weiner, K. Houck, and N. Ferrara. 1992. The vascular endothelial growth factor family. Identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors.* 7:53–64.

39. Rizzino, A. 1987. Soft agar growth assays for transforming growth factors and mitogenic peptides. *Methods Enzymol.* 146:341–352.

40. Neufeld, G., R. Mitchell, P. Ponte, and D. Gospodarowicz. 1988. Expression of human basic fibroblast growth factor cDNA in baby hamster kidney-derived cells results in transformation. *J. Cell Biol.* 106:1385–1394.

41. Jakeman, L. B., J. Winer, G. L. Bennett, C. A. Altar, and N. Ferrara. 1992. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J. Clin. Invest.* 89:244–253.

42. Sporn, M. B., and G. Todaro. 1980. Autocrine stimulation and malignant transformation of cells. *N. Engl. J. Med.* 303:878–892.

43. Sporn, M. B., and A. Roberts. 1985. Autocrine growth factors and cancer. *Nature (Lond.).* 313:745–747.

44. Goustin, A. S., E. B. Leof, G. D. Shipley, and H. L. Moses. 1986. Growth factors and cancer. *Cancer Res.* 46:1015–1029.

45. Zeleznick, A. J., H. M. Schuler, and L. E. Reichert, Jr. 1981. Gonadotropin-binding sites in the monkey ovary: role of the vasculature in the selective distribution of human chorionic gonadotropin to the preovulatory follicle. *Endocrinology.* 109:356–362.

46. Clauss, M., M. Gerlach, H. Gerlach, J. Brett, F. Wang, P. C. Familletti, Y.-C. Pan, J. V. Olander, D. T. Connolly, and D. Stern. 1990. Vascular permeability factor, a tumor-derived polypeptide that induces endothelial cells and monocyte procoagulant activity and promotes monocyte migration. *J. Exp. Med.* 164:470–479.

47. Madri, J. A. 1990. Inflammation and healing. *In* Anderson's Pathology. 9th edition. J. M. Kissane, editor. Mosby Yearbook, Inc., St. Louis, MO. 67–110.

48. Majno, J. 1964. Mechanisms of abnormal vascular permeability by acute inflammation. *In* International Symposium on Injury, Inflammation and Immunity. L. Thomas, J. W. Uhr, and L. Grant, editors. Williams & Wilkins Co., Baltimore, MD.

49. Simionescu, N., and M. Simionescu. 1988. The Cardiovascular System. *In* A Textbook of Histology. Cell and Tissue Biology. 6th edition. L. Weiss, editor. Urban & Shwarzenberg, Inc., Baltimore, MD. 355–400.

50. Costello, P., and R. Del Maestro. 1990. Human cerebral endothelium: isolation and characterization of cells derived from microvessels of non-neoplastic and malignant glial tissue. *J. Neurooncol.* 8:231–243.

51. Karnovsky, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* 35:213–219.

52. Dvorak, H. F., J. A. Nagy, J. T. Dvorak, and A. M. Dvorak. 1988. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am. J. Pathol.* 133:95–109.