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The Vascular Endothelial Growth Factor (VEGF) Isoforms: Differential Deposition into the Subepithelial Extracellular Matrix and Bioactivity of Extracellular Matrix-bound VEGF

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Vascular endothelial growth factor (VEGF)mRNA undergoes alternative splicing events that generate four different homodimeric isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, or $VEGF_{206}$. $VEGF_{121}$ is a nonheparin-binding acidic protein, which is freely diffusible. The longer forms, $VEGF_{189}$ or $VEGF_{206}$, are highly basic proteins tightly bound to extracellular heparin-containing proteoglycans. $VEGF_{165}$ has intermediate properties. To determine the localization of VEGF isoforms, transfected human embryonic kidney CEN4 cells expressing $VEGF_{165}$, $VEGF_{189}$, or $VEGF_{206}$ were stained by immunofluorescence with a specific monoclonal antibody. The staining was found in patches and streaks suggestive of extracellular matrix (ECM). $VEGF_{165}$ was observed largely in Golgi apparatus-like structures. Immunogold labeling of cells expressing $VEGF_{189}$ or $VEGF_{206}$ revealed that the staining was localized to the subepithelial ECM. VEGF associated with the ECM was bioactive, because endothelial cells cultured on ECM derived from cells expressing $VEGF_{189}$ or $VEGF_{206}$ were markedly stimulated to proliferate. In addition, ECM-bound VEGF can be released into a soluble and bioactive form by heparin or plasmin. ECM-bound $VEGF_{189}$ and $VEGF_{206}$ have molecular masses consistent with the intact polypeptides. The ECM may represent an important source of VEGF and angiogenic potential.

INTRODUCTION

Remodeling of the extracellular matrix (ECM) plays a key role in a variety of developmental processes (Matrisian and Hogan, 1990). During morphogenesis, cells derived from the various germinal layers interact with the ECM resulting in complex patterns of induction and suppression of differentiation or proliferation yielding the final mature organ. Extracellular proteolysis and degradation of the ECM are thought to be integral aspects of such processes. Cells are freed from the mechanical barrier imposed by the ECM and may proliferate and migrate in response to a variety of positive and negative signals. Such degradative mechanisms are thought to play a major role also in the local invasiveness and metastasis of tumor cells; the correlation between metastatic potential and expression of proteases (Garbisa *et al.*, 1992; Leto *et al.*, 1992; Sreenath *et al.*, 1992) and heparinases (Peretz *et al.*, 1990) is well established.

A growing body of evidence supports the concept that growth factors stored in the ECM and released in the course of its degradation are major mediators of such inductive processes. Numerous growth factors including fibroblast growth factor (FGF) (Vlodavsky *et al.*, 1987), platelet-derived growth factor (Raines and Ross, 1992), granulocyte-macrophage colony-stimulating factors (CSF) (Gordon *et al.*, 1987), tumor growth factor (TGF-β) (Andres *et al.*, 1989), and leukemia inhibitory factor (Rathjen *et al.*, 1990) have been shown to be associated with the ECM. Other growth factors, such as tumor necrosis factor-α (Perez *et al.*, 1990), TGF-α (Massagué, 1990) and CSF-1 (Stein *et al.*, 1990), or c-kit ligand (Flanagan *et al.*, 1991) are synthesized as membrane-bound precursors. In some of these instances, the growth factors may be released or activated via enzymatic cleavage, yielding an active product (Folkman *et al.*, 1988; Lopez *et al.*, 1991). Expression of

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enzymes that can act to synthesize or degrade the ECM may be regulated developmentally (Adler *et al.*, 1990; Flenniken and Williams, 1990) and by other growth factors (Bassols and Massagué, 1988; Matrisian and Hogan, 1990; Rifkin *et al.*, 1990). The most intensively studied of the matrix-stored growth factors is bFGF (Basilico and Moscatelli, 1992). Basic FGF is a cytosolic protein (Abraham *et al.*, 1986) that, if released because of injury or by other as yet unknown means (Kandel *et al.*, 1991), becomes localized to the basement membrane in a variety of tissues. Extracellular bFGF can be released from the basement membrane by heparin (Folkman *et al.*, 1988), heparinase (Vlodavsky *et al.*, 1992), or after proteolysis (Saksela and Rifkin, 1990). Furthermore, heparin-like molecules found in vivo either as cell surface phosphotidylinositol-linked or ECM-bound proteoglycans are required for the activity of bFGF in vitro (Yayon *et al.*, 1991).

Vascular endothelial growth factor (VEGF), known also as vascular permeability factor (Connolly *et al.*, 1989), is an endothelial-cell specific mitogen and angiogenesis inducer (Ferrara and Henzel, 1989; Plouet *et al.*, 1989) and may play an important role in normal and pathological processes (Ferrara *et al.*, 1992). VEGF-binding sites are localized exclusively to endothelial cells in adult rat tissues (Jakeman *et al.*, 1992). VEGF mRNA expression is temporally and spatially related to proliferation of blood vessels in the ovarian corpus luteum and in the developing embryo (Phillips *et al.*, 1990; Breier *et al.*, 1992). Furthermore, recent studies have shown that monoclonal antibodies (mAbs) specific for VEGF are able to suppress the growth of human tumor cell lines in nude mice, suggesting that VEGF is an important mediator of tumor angiogenesis (Kim *et al.*, 1993). VEGF mRNA undergoes alternative splicing events that lead to the production of four mature homodimeric proteins, each monomer having respectively 121, 165, 189, or 206 amino acids ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (Leung *et al.*, 1989; Houck *et al.*, 1991; Tischer *et al.*, 1991). Cells transfected with cDNAs encoding $VEGF_{121}$ or $VEGF_{165}$ secrete bioactive VEGF into the medium. In contrast, when $VEGF_{206}$ and $VEGF_{189}$ are expressed in mammalian cells, little or no VEGF can be found in the medium (Houck *et al.*, 1991). In a previous study we demonstrated that $VEGF_{189}$ or $VEGF_{206}$ are secreted but are almost completely bound to extracellular heparin-containing proteoglycans (Houck *et al.*, 1992). To better understand the biological significance of the VEGF isoforms, we decided to further investigate their localization in transfected human embryonic kidney CEN4 cells by a variety of techniques, including immunoelectron microscopy. Here we demonstrate that the longer forms of VEGF are bound to the ECM and are stably incorporated in this structure but can be released in a diffusible form by several agents. ECM derived from cells expressing $VEGF_{189}$ or $VEGF_{206}$ and, to a lesser extent, $VEGF_{165}$ promotes the growth of vascular endothelial cells, demonstrating that ECM-bound VEGF is bioactive.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless noted. Plasmin (20 casein units/mg) was purchased from KabiVitrum (Stockholm, Sweden). ^{35}S-methionine and Enlightning were from DuPont New England Nuclear (Boston, MA). Recombinant human $VEGF_{165}$ was purified from the conditioned medium of transfected Chinese hamster ovary cells as described (Ferrara *et al.*, 1991).

Culture of Transfected Cells

Human embryonic kidney CEN4 cell lines stably transfected with pHEBO23 expression vectors containing cDNA inserts encoding the four isoforms of VEGF were established as previously described (Houck *et al.*, 1992). Figure 1 illustrates the amino acid sequence of the VEGF isoforms. Cell lines were maintained in Ham's F12Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 200 μg/ml G418 (to maintain Epstein Barr virus antigen-I expression), and 200 μg/ml hygromycin B (complete medium). Stock cultures were passaged weekly at a split ratio of 1:10.

Preparation of ECM

To prepare isolated ECM, we exploited the fact that CEN4 cells adhere tenuously to the plastic substratum and can be removed easily, leaving behind a thin ECM. VEGF-expressing cells or cells transfected with vector alone were grown to confluency. Cultures were then washed four times with phosphate-buffered saline (PBS) to remove the cells. This process did not result in significant cell death as evidenced by >98% viability on the basis of trypan blue exclusion. Alternatively, cells were removed from the dishes by incubation in the presence of 125 mM NaCl, 5 mM KCl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 5 mM glucose, and 1 mM EDTA pH 7.4 for 10 min at 37°C, followed by washes with PBS. In both cases, a thin fibrillary network coating the surface of the dishes, consistent with ECM, was visible by phase contrast microscopy. The protein content of the isolated ECMs was ~2% of that of the intact monolayers, as determined by trichloracetic acid-precipitable counts in metabolically labeled cultures.

Immunofluorescence Microscopy

For immunofluorescence, cells were cultured on coverslips (VWR Scientific, Bridgeport, NJ). When indicated, cells were pretreated with

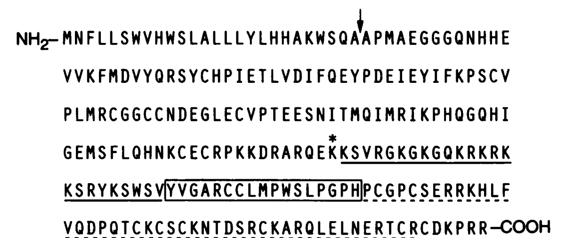

Figure 1. Amino acid sequences of the four VEGF isoforms. Boxed amino acids indicate the 17 amino acid insertion unique to $VEGF_{206}$. —, amino acids contained in both $VEGF_{206}$ and $VEGF_{189}$; ---, amino acids shared by $VEGF_{206}$, $VEGF_{189}$, and $VEGF_{165}$. In $VEGF_{165}$, the starred lysine residue is replaced by an asparagine. All remaining amino acids are shared by all four isoforms. The arrow marks the signal peptide cleavage site.

cycloheximide (100 μg/ml for 2 h) before staining. Confluent cultures were fixed for 30 min in 3.7% formalin, washed thrice with PBS, permeabilized with 0.1% Triton X-100, then incubated with anti-VEGF mAb A.4.6.1 at 10 μg/ml. The characteristics of this antibody, including its ability to recognize all isoforms of VEGF, have been described (Kim *et al.*, 1992). To assess the relative amount of extracellular staining, cultures were also stained in the absence of Triton X-100. They were washed thrice and stained with 1:200 dilution of rhodamine-conjugated goat anti-mouse IgG (Organon-Technika, Durham, NC) and washed and mounted in 90% glycerol, 10% PBS plus 1% (wt/vol) p-phenylenediamine to reduce bleaching. For immunofluorescence microscopy of ECM, confluent cultures were exposed to EDTA solution as described above, and the denuded ECM was then fixed exactly as the intact monolayers. All solutions used contained PBS pH 7.4 unless noted.

Immunoelectron Microscopy

Transfected cells expressing $VEGF_{189}$, $VEGF_{206}$, or vector-transfected cells were cultured in 35-mm dishes. At confluency, they were fixed for 2 h with periodate-lysine-paraformaldehyde (McLean and Nakane, 1974), washed thrice in PBS, incubated 1 h with mAb A.4.6.1 at 10 μg/ml, and washed three times again. The dishes were incubated for 1 h with a 1:10 dilution of 10 nm Au-conjugated goat anti-mouse IgG (Sigma) and then washed 10 times with PBS. The monolayers were fixed again with 1.5% glutaraldehyde, stained with 1% reduced osmium tetroxide, and counterstained with aqueous 1% uranyl acetate. They were dehydrated with a graded ethanol series, and the cells were removed from the dish with propylene oxide. The cells were pelleted, washed with propylene oxide, embedded in Epon, and sectioned. The grids were stained with uranyl acetate followed by lead citrate. The grids then were viewed at 80 kV on a Phillips 300 electron microscope (Mahwah, NJ).

Endothelial Cell Growth Assays

Bovine adrenal cortex-derived capillary endothelial cells were maintained in low glucose DMEM supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium) as previously described (Ferrara and Henzel, 1989). ECM-coated dishes were prepared from cells expressing each VEGF isoform or from vector-transfected cells grown to confluency in 6-well plates (Costar, Cambridge, MA) by EDTA wash as described above. Dishes were washed with PBS and stored at 4°C with 2 ml PBS. After removal of PBS, endothelial cells were seeded on ECM-coated dishes at 7000 cells/well in the presence of growth medium. The assay volume was 3 ml. Cell counts were determined 5 d later with a Coulter counter after dissociation with trypsin.

Metabolic Labeling and Immunoprecipitation of VEGF Isoforms

Cells expressing VEGF isoforms or vector-transfected cells were grown to confluency in 35-mm dishes in the presence of complete medium. They were washed once with serum-free DMEM lacking methionine (Sigma) and incubated in this medium for 30 min at 37°C. Cells were then incubated for 1 h in methionine-free DMEM containing 100 μCi/ml ^{35}S-methionine (labeling medium). Labeling media were removed, and cells were incubated for an additional 4 h at 37°C in the presence of complete medium containing serum. ECM was then prepared from half of the dishes as described. Dishes containing intact monolayers or ECM were extracted with 1 ml RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecylsulfate [SDS], 0.9% NaCl, 25 mM tris(hydroxymethyl) aminomethane [Trish], 1 mM EDTA pH 7.4). Extracts were centrifuged to remove debris. To each sample, 20 μl of rabbit anti-human VEGF antiserum were added and mixed for 1 h. Fifty microliters of Protein A agarose (Calbiochem, La Jolla, CA) was added to each tube and mixed an additional hour. Beads were pelleted in a microfuge and washed six times with RIPA buffer and once with 20 mM Tris pH 8.0. Thirty microliters of 2× Laemmli sample buffer was added to each sample. Samples were heated to 95°C for 1 min before electrophoresis on 17–27% gradient gels. Gels were fixed with 10% methanol, 5% acetic acid fixative for 30 minutes before they were impregnated with fluor (Enlightning, DuPont New England Nuclear) according to manufacturer's instructions. Gels were dried and subjected to fluorography at −80°C.

VEGF Enzyme-linked Immunoadsorbent Assay (ELISA)

VEGF ELISA was performed as previously described (Houck *et al.*, 1992). Briefly, microtiter plates were coated overnight with anti-VEGF mAb A.4.6.1. Plates were then blocked in the presence of bovine serum albumin. After washes with Tween-20 in PBS pH 7.4, 100 μl aliquots of each sample or standard ($rhVEGF_{165}$) were added. The plates were incubated for 2 h at room temperature with agitation and washed. They were then incubated with 100 μl of horseradish peroxidase-conjugated rabbit anti-VEGF polyclonal antiserum per well. The plates were washed, and color was developed for 15 min in the dark with 100 μl of 0.04% o-phenylenediamine dihydrochloride, 3% H_2O_2 in PBS. The reaction was stopped by addition of 100 μl of 2.25 M sulfuric acid. A_{490} was determined on a Vmax plate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated using rhVEGF as a standard, using a four parameter nonlinear regression curve fitting program.

RESULTS

$VEGF_{189}$ and $VEGF_{206}$ Are Localized to the ECM

Stable CEN4 cell lines expressing $VEGF_{206}$ (Figure 2, A and B), $VEGF_{189}$ (Figure 2, C and D), $VEGF_{165}$ (Figure 2, E and F), or control cultures transfected with vector alone (Figure 2G) were stained with an anti-VEGF mAb. Cells expressing $VEGF_{165}$ exhibited a discrete juxtanuclear staining pattern indicative of the Golgi complex (Figure 2E), which was largely abolished by cycloheximide (Figure 2F), suggesting that such staining probably merely represents biosynthetic intermediates. In contrast, cells expressing $VEGF_{189}$ or $VEGF_{206}$ demonstrated a staining pattern that extended beyond the cell borders and was largely unaffected by absence of detergent (Figure 2, B and D), indicating an extracellular localization consistent with the ECM or the cell surface. Vector-transfected cells showed no specific staining.

To determine whether VEGF immunostaining was actually associated with the ECM, isolated ECMs were prepared from cultures expressing the four VEGF isoforms or from cells transfected with vector alone and were fixed and processed for immunofluorescence. As shown in Figure 3, A and B, ECM derived from cell lines expressing $VEGF_{206}$ or $VEGF_{189}$ displayed an intense staining with anti-VEGF mAb. In contrast, ECM prepared from monolayers expressing $VEGF_{165}$, $VEGF_{121}$, or from control monolayers did not display any appreciable staining (Figure 3 C–E).

To conclusively identify the subcellular localization of $VEGF_{206}$ or $VEGF_{189}$, monolayers expressing such VEGF isoforms were processed for immunoelectron microscopy (Figure 4) and viewed on the electron microscope. Gold particles were clearly localized to the

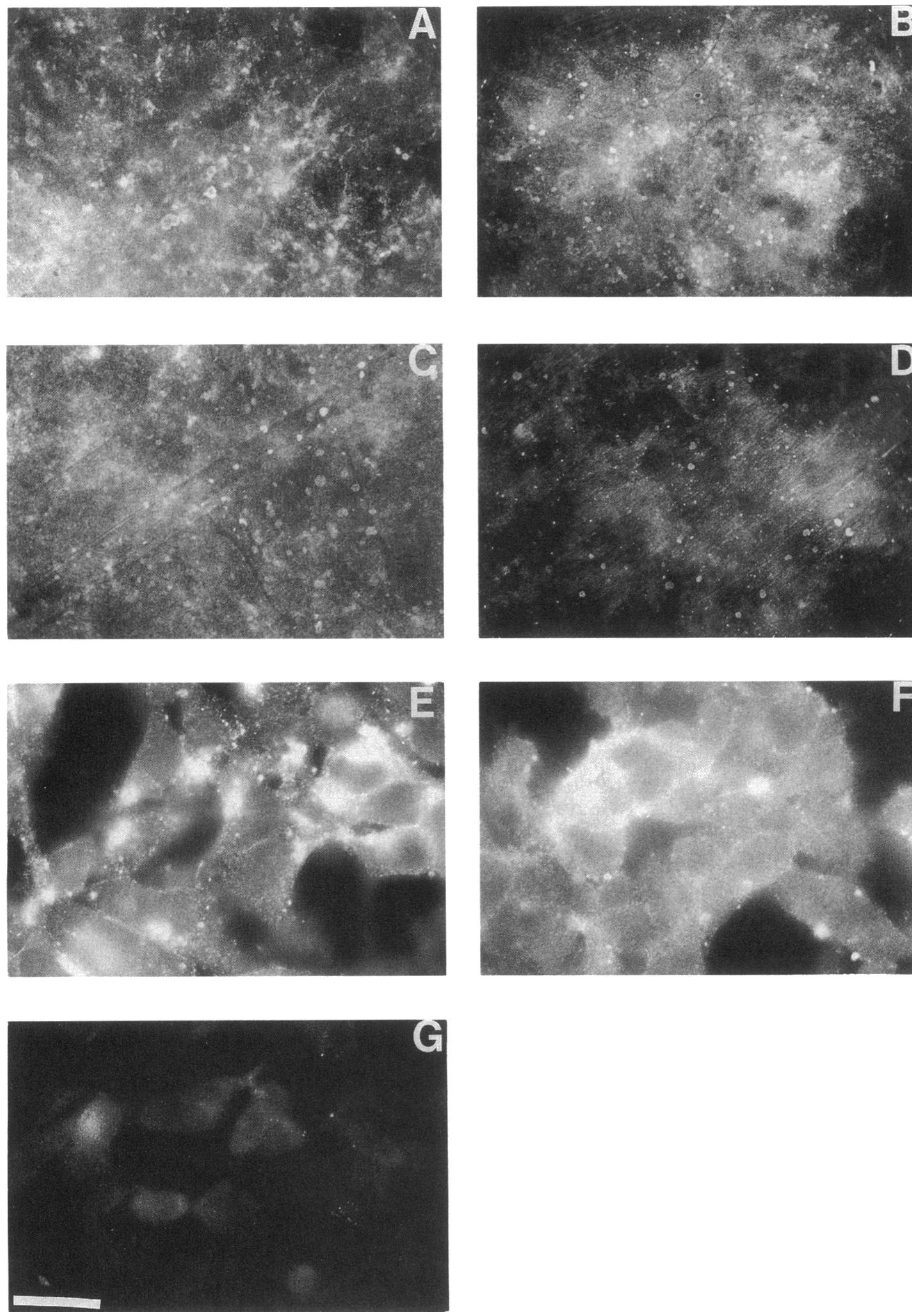

Figure 2. Immunofluorescent staining of monolayers expressing VEGF isoforms. Cells expressing $VEGF_{206}$ (A and B), $VEGF_{189}$ (C and D), $VEGF_{165}$ (E and F), or vector alone (G) were stained with an anti-VEGF mAb. In B and D cells were stained in the absence of detergent. Cells in F were treated with cycloheximide for 2 h before staining. Cells expressing $VEGF_{206}$ or $VEGF_{189}$ show a staining pattern that extends beyond the cell borders and is largely unaffected by the absence of detergent, indicating extracellular antigen localization. Cells expressing $VEGF_{165}$ show a discrete juxtanuclear staining pattern consistent with Golgi complex, which is largely abolished by cycloheximide. Vector-transfected cells showed no specific staining. Bar, 100 μm.

subepithelial ECM of the cells expressing both molecular species (Figure 4, A and B). However, no significant staining was evident on the cell surface. The lack of intracellular staining reflects the fact that the immunogold technique employed here is not suited to label intracellular antigens. No appreciable staining was observed on the ECM or the cell surface of cells transfected with vector alone (Figure 4C).

To elucidate the molecular nature of the ECM-bound VEGF, intact monolayers expressing the VEGF isoforms or isolated ECMs were immunoprecipitated with an anti-VEGF antibody and then subjected to electrophoresis and fluorography. As shown in Figure 5, monolayers contain strong immunoreactive bands having the predicted molecular size for the four mature VEGF isoforms: 29 kDa, 26 kDa, 23 kDa, or 17 kDa, respectively, in reducing conditions. ECM derived from cells expressing $VEGF_{189}$ or $VEGF_{206}$ contains significant amounts of immunoreactive material having the same molecular mass as the monolayer-associated proteins.

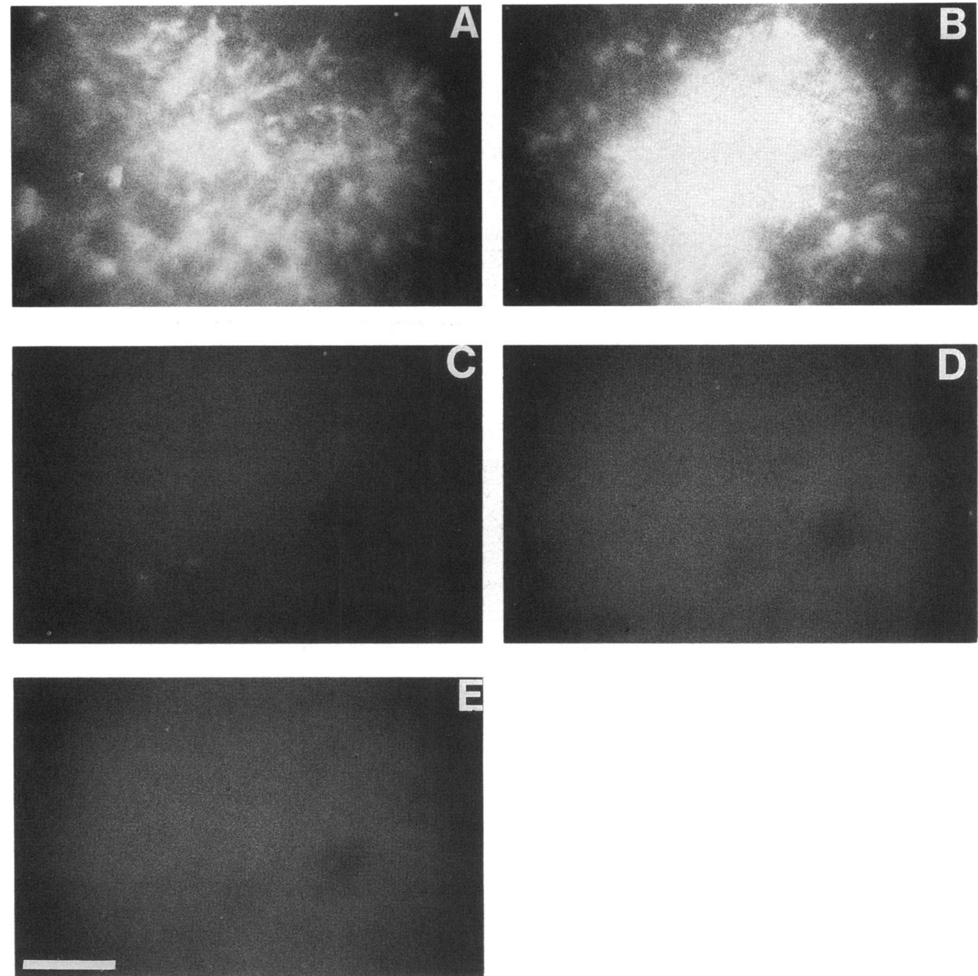

Figure 3. Immunofluorescent staining of isolated ECMs with an anti-VEGF mAb. Cells were grown to confluence on coverslips, and ECMs were prepared by EDTA incubation (see MATERIALS AND METHODS). Staining of ECM derived from cells expressing $VEGF_{206}$ (A), $VEGF_{189}$ (B), $VEGF_{165}$ (C), $VEGF_{121}$ (D), or vector alone (E) is shown. Bar, 100 μm.

In contrast, VEGF proteins were not detectable in the ECM derived from cells expressing $VEGF_{165}$, $VEGF_{121}$, or from vector-transfected cells.

ECMs Derived from Cells Expressing the VEGF Isoforms Differentially Promote Endothelial Cell Growth

To assess whether ECM-bound VEGF is bioactive, ECMs were isolated from cells expressing the four isoforms of VEGF or from vector-transfected cells. Capillary endothelial cells were then cultured on such ECM-coated dishes. As shown in Figure 6, the proliferation rate of endothelial cells cultured on ECM derived from CEN4 cells expressing $VEGF_{121}$ was not different from that of endothelial cells cultured on control ECM. In contrast, ECM derived from cells expressing $VEGF_{189}$ or $VEGF_{206}$ markedly stimulated endothelial cell growth. The proliferation rate achieved in the presence of such ECMs was similar to that obtained in control ECM in the presence of 2 ng/ml bFGF (not shown). A lower, albeit statistically significant, growth stimulation was measured when endothelial cells were cultured on ECM derived from $VEGF_{165}$-expressing cells.

Heparin and Plasmin Release VEGF from ECM

To directly test whether ECM-associated VEGF may be released in a diffusible form, intact monolayers or ECM-coated dishes derived from cells expressing $VEGF_{165}$ or $VEGF_{189}$ were incubated overnight in the presence or in the absence of 100 μg/ml heparin in serum-free medium (Figure 7). VEGF levels in the conditioned media were assayed by ELISA. Heparin induced a twofold increase in $VEGF_{165}$ release from intact monolayers in agreement with previous reports (Houck *et al.*, 1992). However, no measurable $VEGF_{165}$ was released from ECM either in the presence or in the absence of heparin. In contrast, ECM derived from cells expressing $VEGF_{189}$ released appreciable amounts of immunoreactive VEGF when challenged with heparin. However, little or no VEGF was released in the absence of heparin. The amount of $VEGF_{189}$ released by isolated ECM following heparin addition was ~25% of the amount released by

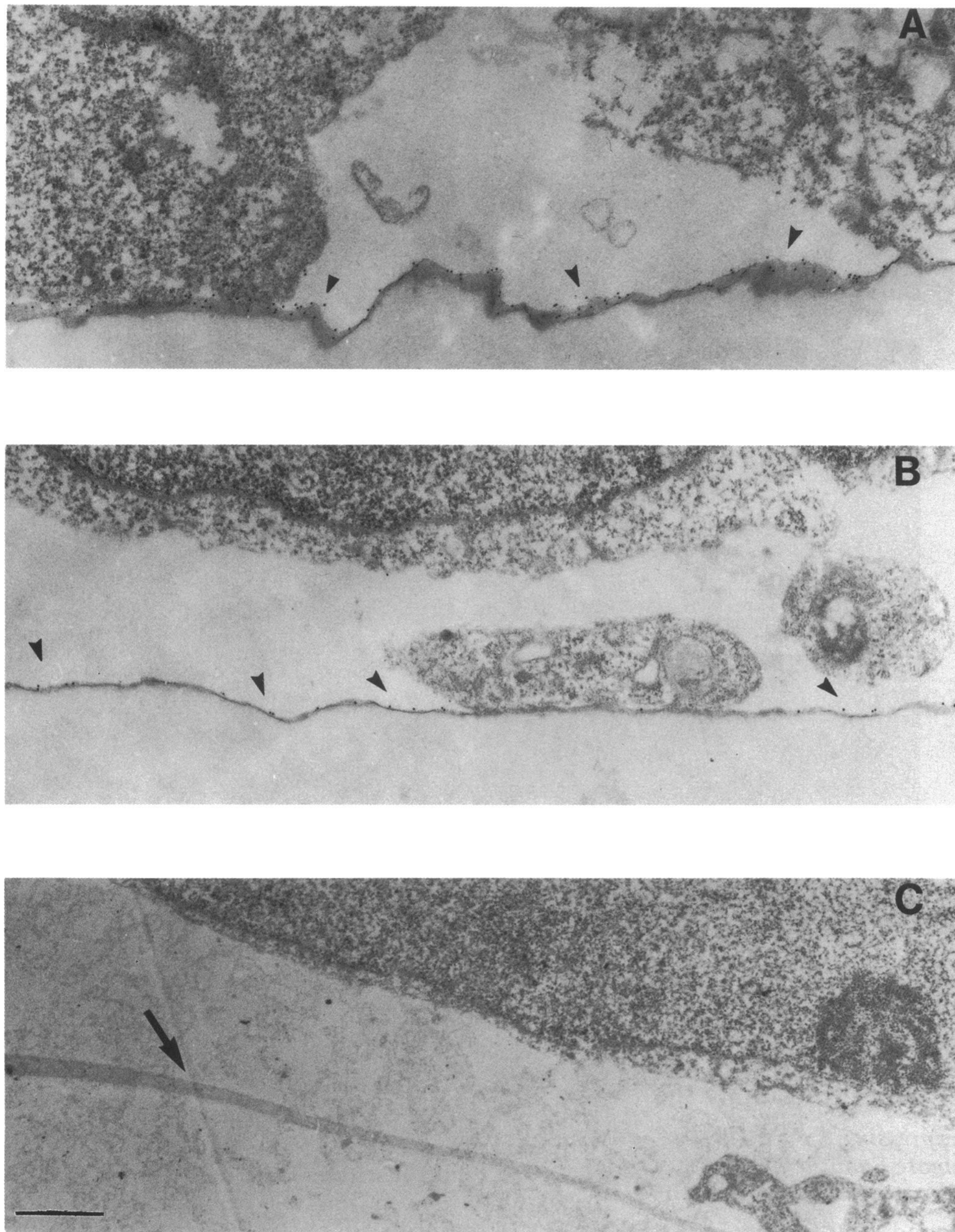

Figure 4. Electron microscopy of transfected cells expressing the longer VEGF isoforms. Cells expressing $VEGF_{206}$ (A), $VEGF_{189}$ (B), or vector alone (C) were fixed and stained with a specific anti-VEGF mAb followed by 10 nm colloidal Au-conjugated goat anti mouse IgG. They were embedded in Epon, sectioned, and viewed at 80 kV. Small arrowheads point to gold particles, indicating VEGF immunoreactivity. Large arrow indicates basement membrane of control cells. Bar, 1 μm.

the intact monolayers. That the release of $VEGF_{189}$ from ECM was not because of cells remaining in the dish is demonstrated by the fact that after a week in culture at most one colony per dish was present, as evidenced by crystal violet staining. Furthermore, the addition of cycloheximide did not prevent VEGF release. Heparin induced $VEGF_{189}$ release from ECM in a dose-dependent manner (Figure 8A). A maximal response was achieved with $\sim$10 μg/ml. We then sought to determine whether plasmin was also able to release ECM-bound VEGF. This physiologically relevant protease has been shown to cleave VEGF at the COOH terminus, releasing a 34 000 dalton bioactive fragment (Houck *et al.*, 1992). As illustrated in Figure 8B, plasmin was able to release VEGF from ECM derived from cells expressing $VEGF_{189}$ in a dose-dependent fashion.

DISCUSSION

Numerous studies have documented the interaction of growth factors with cell surface or ECM-bound proteoglycans (Vlodavsky *et al.*, 1987; Yayon *et al.*, 1991;

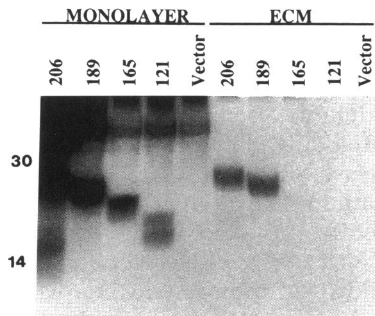

Figure 5. Characterization of ECM-bound VEGF. Cells expressing each isoform of VEGF or vector alone were metabolically labeled with ^{35}S-methionine as described. ECM was prepared from half of the dishes. Monolayers or ECM were extracted, immunoprecipitated with an anti-VEGF polyclonal antiserum, subjected to electrophoresis on 17–27% SDS-polyacrylamide gel electrophoresis, and fluorographed. Numbers indicate mass in kDa of standards.

Andres *et al.*, 1992). The binding may serve to target growth factors, store them in an inactive form, or aid in their binding to specific receptors. All of these possibilities appear to be true for one angiogenic factor, basic FGF. Similarly, it has been postulated that cell surface heparan sulfates are required for the binding of VEGF to its receptor(s) (Gitay-Goren *et al.*, 1992). In addition, VEGF is expected to interact with heparin-containing proteoglycans in the ECM. In fact, the VEGF isoforms, except the shortest form, $VEGF_{121}$, are known to bind to heparin with $VEGF_{189}$ and $VEGF_{206}$ having the highest affinity (Ferrara and Henzel, 1989; Houck *et al.*, 1992). Consistent with these properties, we demonstrate by biochemical and microscopic criteria that $VEGF_{189}$ and $VEGF_{206}$ are localized to the subepithelial

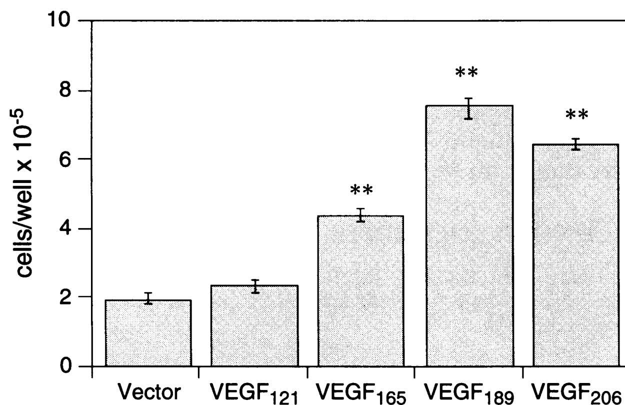

Figure 6. Proliferation of endothelial cells on ECM derived from cells expressing VEGF isoforms. ECM-coated plates were prepared from cells expressing $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$, $VEGF_{121}$, or from vector-transfected cells as described. Bovine adrenal cortex capillary endothelial were plated at 7000 cells/well, and cell numbers were determined 5 d later. Bars represent the average of quintuplicate determinations. *, values that are significantly different when compared to vector-derived ECM (Student's t-test, $p < 0.05$).

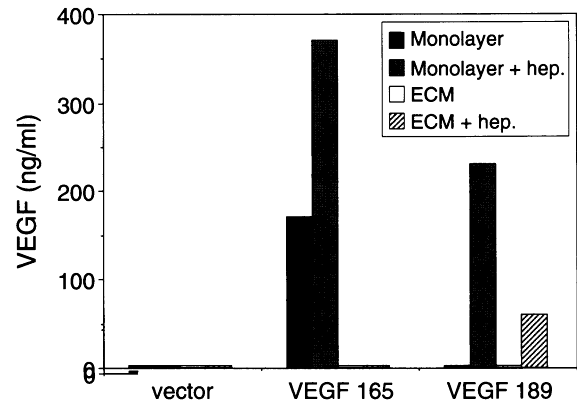

Figure 7. VEGF release from ECM vs. intact monolayers. ECM-coated dishes derived from vector-transfected cells or from cells expressing $VEGF_{165}$, $VEGF_{189}$, or intact monolayers were incubated overnight in the presence or in the absence of 100 μg/ml heparin in serum-free medium, as indicated. Media were collected, and VEGF levels were measured by ELISA.

ECM of transfected CEN4 cells. This deposition speaks for a protein-targeting system that is primarily dependent upon the 24-amino acid insertion unique to $VEGF_{189}$ or $VEGF_{206}$. Consistent with such localization, the ECM isolated from cells expressing $VEGF_{189}$ or $VEGF_{206}$ strongly supports endothelial cell growth. In

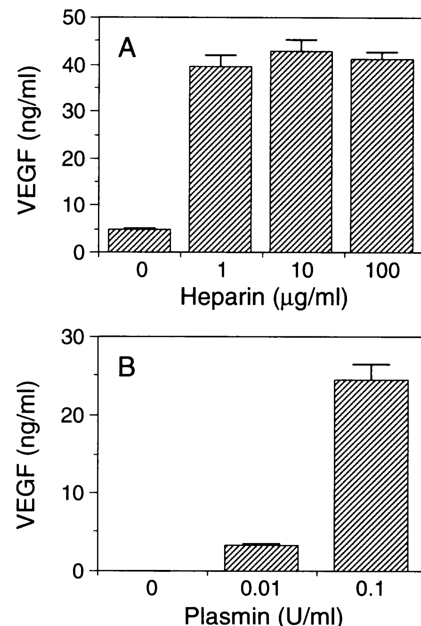

Figure 8. Release of $VEGF_{189}$ from ECM by heparin or plasmin. (A) ECM-coated dishes were incubated overnight with varying amounts of heparin in the presence of cycloheximide. (B) Dishes were treated with varying amounts of plasmin for 20 min at 37°C, and the reaction was then stopped with aprotinin. Media were assayed for VEGF concentrations by ELISA.

contrast, VEGF was biochemically undetectable in the ECM isolated from cells expressing $VEGF_{121}$ or even $VEGF_{165}$. Interestingly, however, ECM derived from cells expressing $VEGF_{165}$ was able to stimulate endothelial cell growth, although less effectively than that derived from the two longer isoforms, in spite of the fact that the VEGF protein was undetectable by the methods we employed. A possibility is that ECM-bound $VEGF_{165}$ may be more potent than $VEGF_{189}$ or $VEGF_{206}$. However, the inability to quantitate the amount of $VEGF_{165}$ remaining in the ECM and the fact that endothelial cells cultured on ECMs derived from cells expressing the longer isoforms were proliferating at a near maximal rate makes such a comparison difficult. Also, it is possible that $VEGF_{189}$ or $VEGF_{206}$, to exert mitogenic activity, must be released locally from their bound state via the action of proteases or other means whereas $VEGF_{165}$, being less strongly bound to the ECM, may exist in equilibrium with a soluble form. Therefore, even very low amounts of $VEGF_{165}$ bound to the ECM may be able to support endothelial cell growth.

Heparin or plasmin are very effective in releasing VEGF in a diffusible form from the ECM. This may be relevant to the in vivo situation, because the basement membrane of many epithelial cells is oriented toward blood vessels and therefore would be directly accessible to blood-borne factors. Plasminogen activation and generation of plasmin are critical steps of the angiogenesis cascade (Mignatti *et al.*, 1989), and VEGF has been shown to induce expression and synthesis of urokinase-type and tissue-type plasminogen activators from endothelial cells (Pepper *et al.*, 1991). Therefore, plasmin may play a major role in cleaving ECM-bound VEGF and releasing soluble and bioactive proteolytic fragments. Another potential proteolytic mechanism for release of the longer forms of VEGF is represented by degradation of the basement membrane after polarized secretion of proteases or heparinase by transformed epithelial cells. In this context, it has been shown that one of the events marking the acquisition of a transformed phenotype in prostatic cells is the inversion of polarity of protease secretion from the apical surface toward the basement membrane (Djakiew *et al.*, 1992). This mechanism might be significant in tumor angiogenesis, an event where VEGF may play a major role.

Interestingly, this study did not provide evidence for a plasma membrane binding of the longer isoforms of VEGF. Virtually all of the immunoreactive material observed microscopically was localized to the ECM. Cell-free ECMs released $\sim$25% as much $VEGF_{189}$ as the intact monolayers. If one takes into account that the conditions needed to insure that all cells have been removed inevitably result in loss of part of the ECM, such relatively high recovery strongly suggests that a very significant fraction of $VEGF_{189}$ is indeed bound to the ECM. Clearly, we cannot rule out the possibility that a percentage of VEGF is found in other cellular compartments that the microscopic methods employed here failed to detect.

Therefore, the longer forms of VEGF are likely to represent primarily a storage form of the growth factor, releasable after degradation of the ECM. Rapid changes in VEGF release, sensitive to the dynamic requirements of the surrounding microenvironment, may be instead more efficiently provided by the shorter, diffusible, forms.

It is unknown at the present time whether the targeting of the VEGF proteins described in this study occur also in nontransfected cells or in vivo. However, heparin-binding sites are integral components of the ECM produced by a variety of cells types. Therefore, the behavior of the long forms of VEGF produced by other cell types is expected to be similar to that described here. The VEGF mRNA is expressed in numerous normal and transformed cell types, including epithelial cells, and in numerous tissues and organs (Berse *et al.*, 1992; Ferrara *et al.*, 1992). Polymerase chain reaction analysis demonstrates expression of a transcript corresponding to $VEGF_{189}$ in the majority of cells and tissues expressing the VEGF gene, although substantial variations exist in the expression pattern of this VEGF isoform (Houck *et al.*, 1991; Ferrara *et al.*, 1992). Although a minor product in some cDNA libraries, in others, the transcript encoding $VEGF_{189}$ was almost as abundant as that encoding $VEGF_{165}$, the predominant isoform of VEGF. In contrast, $VEGF_{206}$ appears to be a rare form, so far identified only in a human fetal liver cDNA library (Houck *et al.*, 1991). Recent studies have shown that $VEGF_{188}$ is highly expressed in a variety of tissues in the rat embryo (Jakeman *et al.*, 1993). Intriguingly, proteolysis and breakdown of the ECM are especially prominent during morphogenesis and organ remodeling (Matrisian and Hogan, 1990). Therefore, the mechanisms that we describe here have the potential to operate in several physiological or pathological circumstances. The availability of more sensitive detection techniques and antibodies specific for the 24-amino acid insertion unique to the long forms of VEGF should allow us to confirm whether our observations can be extended to the in vivo situation.

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REFERENCES

Abraham, J., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D., and Fiddes, J. (1986). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science *233*, 545–548.

Adler, R.R., Brenner, C.A., and Werb, Z. (1990). Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells. Development. *110*, 211–220.

Andres, J.L., Stanley, K., Cheifetz, S., and Massagué, J. (1989). Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. J. Cell Biol. *109*, 3137–3145.

Andres, J.L., DeFalcis, D., Noda, M., and Massagué, J. (1992). Binding of two growth factor families to separate domains of the proteoglycan betaglycan. J. Biol. Chem. *267*, 5927–5930.

Basilico, C., and Moscatelli, D. (1992). The FGF family of growth factors and oncogenes. Adv. Cancer Res. *59*, 115–165.

Bassols, A., and Massagué, J. (1988). Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J. Biol. Chem. *263*, 3039–3045.

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

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

Connolly, D.T., Heuvelmann, D.M., Nelson, R., Olander, J.V., Delfino, J.J., Siegel, N.R., Leimgruber, R.M., and Feder, J. (1989). Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J. Clin. Invest. *84*, 1470–1478.

Djakiew, D., Pflug, B., Delsite, R., Lynch, J.H., and Onoda, M. (1992). Density dependent polarized secretion of a prostatic epithelial cell line. Prostate *20*, 15–27.

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

Ferrara, N., Leung, D.W., Cachianes, G., Winer, J., and Henzel, W.J. (1991). Purification and cloning of vascular endothelial growth factor secreted by pituitary folliculostellate cells. Methods Enzymol. *198*, 391–405.

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

Flanagan, J.G., Chan, D.C., and Leder, P. (1991). Transmembrane form of the kit ligand growth factor determined by alternative splicing and is missing in the Sl^d mutant. Cell *64*, 1025–1035.

Flenniken, A.M., and Williams, B.R. (1990). Developmental expression of the endogenous TIMP gene and a TIMP-lacZ fusion gene in transgenic mice. Genes & Dev. *4*, 1094–1106.

Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. (1988). A heparin-binding angiogenic protein-basic fibroblast growth factor is stored within basement membrane. Am. J. Pathol. *130*, 393–400.

Garbisa, S., Scagliotti, G., Masiero, L., Di-Francesco, C., Caenazzo, C., Onisto, M., Micela, M., Stetler-Stevenson, W.G., and Liotta, L.A. (1992). Correlation of serum metalloproteinase levels with lung cancer metastasis and response to therapy. Cancer Res. *52*(16), 4548–4549.

Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. (1992). The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J. Biol. Chem. *267*, 6093–6098.

Gordon, M.Y., Riley, G.P., Watt, S.M., and Greaves, M.F. (1987). Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature *326*, 403–405.

Houck, K.A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D.W. (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.

Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J. Biol. Chem. *267*, 26031–26037.

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

Jakeman, L.B., Armanini, M., Phillips, H.S., and Ferrara, N. (1993). Developmental expression of binding sites and mRNA for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology *133*, 848–859.

Kandel, J., Bossy-Wetzel, E., Radvanyi, F., Klagsbrun, M., Folkman, J., and Hanahan, D. (1991). Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell *66*, 1095–1104.

Kim, K.J., Li, B., Houck, K., Winer, J., and Ferrara, N. (1992). The vascular endothelial growth factor proteins, identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors *7*, 53–64.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature *262*, 841–844.

Leto, G., Gebbia, N., Rausa, L., and Tuminello, F.M. (1992). Cathepsin D in the malignant progression of neoplastic diseases (review). Anticancer Res. *12*, 235–240.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*, 1306–1309.

Lopez Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S., and Massagué, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell *67*, 785–795.

Massagué, J. (1990). Transforming growth factor-α. J. Biol. Chem. *265*, 21393–21396.

Matrisian, L.M., and Hogan, B.L. (1990). Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development. Curr. Top. Dev. Biol. *24*, 219–259.

McLean, L.W., and Nakane, P.K. (1974). Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J. Histochem. Cytochem. *22*, 1077–1083.

Mignatti, P., Tsuboi, R., Robbins, E., and Rifkin, D.B. (1989). In vitro angiogenesis in the human amniotic membrane, requirement for basic fibroblast growth factor-induced proteinase. J. Cell Biol. *108*, 671–682.

Pepper, M.S., Ferrara, N., Orci, L., and Montesano, R. (1991). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem. Biophys. Res. Commun. *181*, 902–906.

Peretz, T., Antebi, S.U., Beller, U., Horowitz, A.T., Fuks, Z., and Vlodavsky, I. (1990). Maintenance on extracellular matrix and expression of heparanase activity by human ovarian carcinoma cells from biopsy specimens. Int. J. Cancer. *45*, 1054–1060.

Perez, C., Albert, I., Defay, K., Zachariades, N., Gooding, L., and Kriegler, M. (1990). A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell to cell contact. Cell *63*, 251–258.

Phillips, H.S., Hains, J., Leung, D.W., and Ferrara, N. (1990). Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology *127*, 965–967.

Plouet, J., Schilling, J., and Gospodarowicz, D. (1989). Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. EMBO J. *8*, 3801–3806.

Raines, E.W., and Ross, R. (1992). Compartmentalization of PDGF on extracellular binding sites dependent on exon-6-encoded sequences. J. Cell Biol. *116*(2), 533–543.

Rathjen, P.D., Toth, S., Willis, A., Heath, J.K., and Smith, A.G. (1990). Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. Cell *62*, 1105–1114.

Rifkin, D.B., Moscatelli, D., Bizik, J., Quarto, N., Blei, F., Dennis, P., Flaumenhaft, R., and Mignatti, P. (1990). Growth factor control of extracellular proteolysis. Cell Differ. Dev. *32*, 313–318.

Saksela, O., and Rifkin, D.B. (1990). Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. J. Cell Biol. *110*, 767–775.

Sreenath, T., Matrisian, L.M., Stetler-Stevenson, W., Gattoni-Celli, S., and Pozzatti, R.O. (1992). Expression of matrix metalloproteinase genes in transformed rat cell lines of high and low metastatic potential. Cancer Res. *52*(18), 4942–4947.

Stein, J., Borzillo, G.V., and Rettenmier, C.W. (1990). Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood *76*, 1308–1314.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., and Abraham, J.A. (1991). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J. Biol. Chem. *266*, 11947–11954.

Vlodavsky, I., Eldor, A., Haimovitz-Friedman, A., Matzner, Y., Ishai-Michaeli, R., Lider, O., Naparstek, Y., Cohen, I.R., and Fuks, Z. (1992). Expression of heparanase by platelets and circulating cells of the immune system, possible involvement in diapedesis and extravasation. Invasion & Metastasis, *12*(2), 112–127.

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M.N. (1987). Endothelial cell-derived basic fibroblast growth factor, synthesis and deposition into subendothelial extracellular matrix. Proc. Natl. Acad. Sci. USA *84*, 2292–2296.

Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., and Ornitz, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell *64*, 841–848.