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Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites*

(angiogenesis/tumor cells)

YOSHINARI MYOKEN, YOSHIAKI KAYADA†, TETSUJI OKAMOTO†, MIKIO KAN, GORDON H. SATO, AND J. DENRY SATO‡

W. Alton Jones Cell Science Center, Inc., 10 Old Barn Road, Lake Placid, NY 12946

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ABSTRACT **A distinct family of endothelial cell mitogens that are homologous to platelet-derived growth factor has recently been identified. Unlike other known endothelial cell mitogens, these vascular endothelial cell growth factors (VEGFs) are secreted and appear to act specifically on endothelial cells. We have purified VEGF 2083-fold to apparent homogeneity from protein-free culture medium conditioned by A-431 human epidermoid carcinoma cells. This A-431-derived VEGF was characterized as a homodimer composed of 22-kDa subunits with an N-terminal sequence that was similar to VEGFs produced by human HL-60 leukemic and U-937 histiocytic lymphoma cells. A-431 VEGF was used to identify specific and saturable binding sites for VEGF on human umbilical vein endothelial cells (HUVEC). By affinity cross-linking, VEGF-binding site complexes of 230, 170, and 125 kDa were detected on HUVEC. VEGF specifically induced the tyrosine phosphorylation of a 190-kDa polypeptide, which was similar in mass to the largest binding site detected by affinity cross-linking.**

There is increasing evidence to support the hypothesis that the growth of solid tumors is dependent on angiogenesis (1–3). Tumor cells themselves induce the outgrowth of new capillary vessels by producing diffusible angiogenic factors that directly or indirectly stimulate the growth of normally quiescent endothelial cells (4–11). For example, basic fibroblast growth factor (bFGF) (10, 12), platelet-derived endothelial cell growth factor (PD-ECGF) (8, 13), and transforming growth factor α (TGF-α) (11, 14) have been isolated from tumor cells and are both angiogenic *in vivo* and mitogenic for endothelial cells *in vitro*.

We have purified a 44-kDa polypeptide growth factor from culture medium conditioned by A-431 human epidermoid carcinoma cells based on its mitogenic activity for human umbilical vein endothelial cells (HUVEC). N-terminal sequence analysis of the A-431-derived growth factor indicated that it was a member of a new family of platelet-derived growth factor (PDGF)-related mitogens with apparent specificity for vascular endothelial cells (15–18). These 40-kDa to 46-kDa dimeric vascular endothelial growth factors (VEGFs) have been isolated recently from medium conditioned by bovine pituitary folliculo-stellate cells (19, 20), rat glioma cells (21), and U-937 human histiocytic lymphoma cells (vascular permeability factor, VPF) (22). Unlike the endothelial cell mitogens bFGF (23) and platelet-derived endothelial cell growth factor (8), VEGFs are secreted (15–18) and therefore may play a role in normal angiogenic processes as well as in tumor angiogenesis. Vaisman *et al.* (24) have characterized VEGF receptors on bovine endothelial cells. Here we use A-431-derived VEGF to identify specific saturable VEGF membrane binding sites on HUVEC, and we provide evidence that VEGF induces the tyrosine phosphorylation of a 190-kDa polypeptide in intact HUVEC.

MATERIALS AND METHODS

Growth Factors. Heparin-binding growth factor 1 (HBGF-1, acidic FGF) was purified as described (25). Porcine transforming growth factor β_1 (TGF-β_1) was purchased from R & D Systems (Minneapolis). All other growth factors were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell Culture Conditions and Proliferation Assay. HUVEC were maintained in RD medium [1:1 (vol/vol) RPMI 1640 medium/Dulbecco's modified Eagle's medium (DMEM)] supplemented with five factors (bovine insulin at 10 μg/ml, human transferrin at 5 μg/ml, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, and 10 nM sodium selenite; Sigma) 10% (vol/vol) fetal bovine serum (FBS; HyClone), HBGF-1 at 10 ng/ml, and heparin at 10 μg/ml (Sigma) in collagen-coated dishes. In proliferation assays, HUVEC were plated at 1×10^4 per well into 24-well tissue culture plates (Falcon) coated with collagen and cultured in RD medium containing five factors and 10% FBS; 12 hr later samples to be assayed were added. On day 5, cell numbers were determined with a Coulter Counter. Cultures of human aortic smooth muscle cells (HASMC) (26), human foreskin fibroblasts (HFFB) (27), and human foreskin keratinocytes (HFK) (28) were isolated and maintained as previously described. Fetal bovine heart endothelial cells (FBHEC) were purchased from the American Type Culture Collection and were maintained in RD medium containing 10% FBS. The mitogenic activity of purified A-431 VEGF was tested on HASMC, HFFB, HFK, and FBHEC plated at an initial density of 5×10^3 cells per well into collagen-coated 24-well plates.

Preparation of Conditioned Medium. The A-431-AJC cell line was subcloned from A-431 human epidermal carcinoma cells (29) obtained from J. E. DeLarco (Monsanto). The

Abbreviations: PDGF, platelet-derived growth factor; VEGF, vascular endothelial cell growth factor; VPF, vascular permeability factor; HUVEC, human umbilical vein endothelial cells; DSS, disuccinimidyl suberate; FGF, fibroblast growth factor; bFGF, basic FGF; TGF, transforming growth factor; HBGF-1, heparin-binding growth factor (acidic FGF); HASMC, human aortic smooth muscle cells; HFFB, human foreskin fibroblasts; HFK, human foreskin keratinocytes; FBHEC, fetal bovine heart endothelial cells; EGF, epidermal growth factor; Tyr(*P*), phosphotyrosine.

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†Present address: Department of Maxillofacial Surgery I, Hiroshima University Dental School, Hiroshima City, Japan.
‡To whom reprint requests should be addressed.

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A-431-AJC cells were maintained in roller bottles (Costar) in DMEM/F-12 medium, 1:1 (vol/vol). After the cells reached confluence, conditioned medium was collected daily.

Purification Procedure. Twenty liters of conditioned medium were applied to a heparin-Sepharose CL-6B column (Pharmacia) that had been equilibrated in phosphate-buffered saline (PBS). The column was washed with PBS, and bound proteins were eluted with NaCl concentrations of 0.25, 0.3, 0.9, 1.5 and 2.0 M. Aliquots (20 μl) of 5-ml fractions were removed for cell proliferation assays, and bioactive fractions eluted in 0.3 M NaCl were pooled and dialyzed in distilled water and then lyophilized. The lyophilized sample was reconstituted with 0.1% CF_3COOH/10% CH_3CN and applied to a Vydac C_{18} HPLC column (0.46 × 25 cm; The Separations Group) equilibrated with 0.1% CF_3COOH/10% CH_3CN. The column was eluted with a linear gradient of CH_3CN (30–40% in 50 min) at a flow rate of 0.5 ml/min. Bioactive fractions were pooled, concentrated under vacuum, and applied to a Vydac C_{18} HPLC column equilibrated with 0.1% CF_3COOH in water. The column was eluted with a linear gradient of *n*-propanol (15–25% in 100 min) at a flow rate of 0.5 ml/min. Bioactive fractions were injected onto a TSK-G3000 SW (0.75 × 30 cm; Phenomenex, Torrance, CA) column preequilibrated with 0.1% CF_3COOH/35% CH_3CN. Separation was performed at a flow rate of 0.25 ml/min.

Protein Microsequencing. Approximately 30 pmol of protein from the most bioactive fractions obtained from the TSK-G3000 SW step were analyzed by using a model 420A amino acid analyzer and a model 470A gas-phase sequencer (Applied Biosystems) as described by Crabb *et al.* (30). All sequencer reagents and solvents were from Applied Biosystems.

Binding Assay with ^{125}I-Labeled VEGF (^{125}I-VEGF). Purified VEGF was iodinated to a specific activity of 6.1×10^5 cpm/ng by a modified chloramine-T method (31). HUVEC were seeded at 5×10^4 cells per well in collagen-coated 24-well tissue culture plates and allowed to grow for 48 hr. The monolayers then were washed with 0.5 ml of RD medium containing 0.1% bovine serum albumin, 1% FBS, and 25 mM Hepes (pH 7.4) (binding buffer) and were preincubated in this medium for 2 hr at 37°C. The cells were incubated in 0.3 ml of binding buffer containing ^{125}I-VEGF, unlabeled VEGF, or other growth factors for 3 hr at 22°C. Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled VEGF. After four washes in binding buffer, the cells were solubilized in 1% Triton X-100, and bound radioactivity was determined with a model 1274 LKB γ counter. Cell numbers were determined from duplicate wells. Binding data were analyzed by the method of Scatchard (32). In experiments testing internalization of bound ^{125}I-VEGF, surface-bound ^{125}I-VEGF was determined by extracting cells with 0.3 ml of 0.5 M sodium acetate (pH 3.5) for 30 min (33), and total ^{125}I-VEGF binding was determined by solubilizing the cells.

Affinity Labeling with ^{125}I-VEGF. Cross-linking of ^{125}I-VEGF to HUVEC was done as described by Massague (34) except that the cells were incubated with ligand and disuccinimidyl suberate (DSS) at 22°C. Cellular proteins were solubilized in 20 mM Hepes (pH 7.4)/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA and analyzed by SDS/PAGE (35).

***In Vivo* Labeling and Immunoprecipitation of Tyrosine-Phosphorylated Proteins.** Nearly confluent HUVEC were incubated at 37°C in phosphate-free RITC-807 medium (Kyokuto Pharmaceutical, Tokyo) with 0.25 mCi (1 Ci = 37 GBq) of [^{32}P]orthophosphoric acid (Amersham) per ml and 5% dialyzed PBS. After 4 hr, 200 pM VEGF was added for 10 min at 37°C. The cultures were washed twice with ice-cold TBS (Tris-buffered saline) containing 100 μM sodium orthovanadate and solubilized in 1 ml of lysis buffer (10 mM Tris/50 mM NaCl/5 mM EDTA/100 μM sodium orthovanadate/1% Triton X-100/30 mM sodium pyrophosphate/1 mM phenylmethylsulfonyl fluoride, pH 7.4). The lysates were centrifuged for 10 min at 13,000 × *g* at 4°C, and the supernatants were mixed with 5 μg of anti-phosphotyrosine [anti-Tyr(*P*)] monoclonal antibody (Upstate Biotechnology) for 2 hr at 4°C. Tyrosine-phosphorylated proteins were collected with protein A-Sepharose beads (Pharmacia) and were analyzed by SDS/PAGE and autoradiography.

RESULTS

Purification of A-431 VEGF. Serum-free medium conditioned by A-431-AJC cells stimulated the proliferation of HUVEC *in vitro* by 4- to 8-fold, and the mitogenic activity was found to bind to immobilized heparin. The majority of the mitogenic activity was eluted from heparin in 0.3 M NaCl, whereas a minor activity peak corresponding to bFGF (36) was eluted in 1.5 M NaCl (data not shown). Heparin affinity chromatography resulted in a 7-fold purification with a 22%

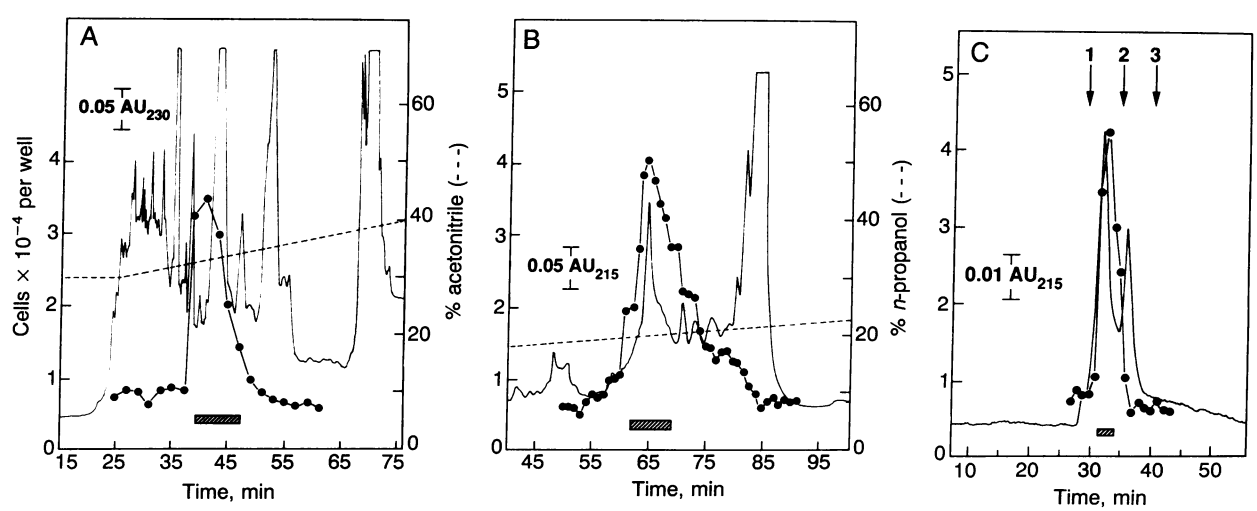

FIG. 1. Purification of VEGF. A-431 VEGF was purified as described. Mitogenic activity for HUVEC was monitored throughout the purification (●). Active eluate fractions pooled either for subsequent purification or for characterization are noted by the horizontal bar. (*A*) C_{18} reverse-phase HPLC (CH_3CN) of active material from heparin affinity chromatography. (*B*) C_{18} reverse-phase HPLC (*n*-propanol). (*C*) Size-exclusion HPLC. Mass markers were: 1, ovalbumin (43 kDa); 2, chymotrypsinogen A (25 kDa); 3, ribonuclease A (13 kDa). AU, absorption unit.

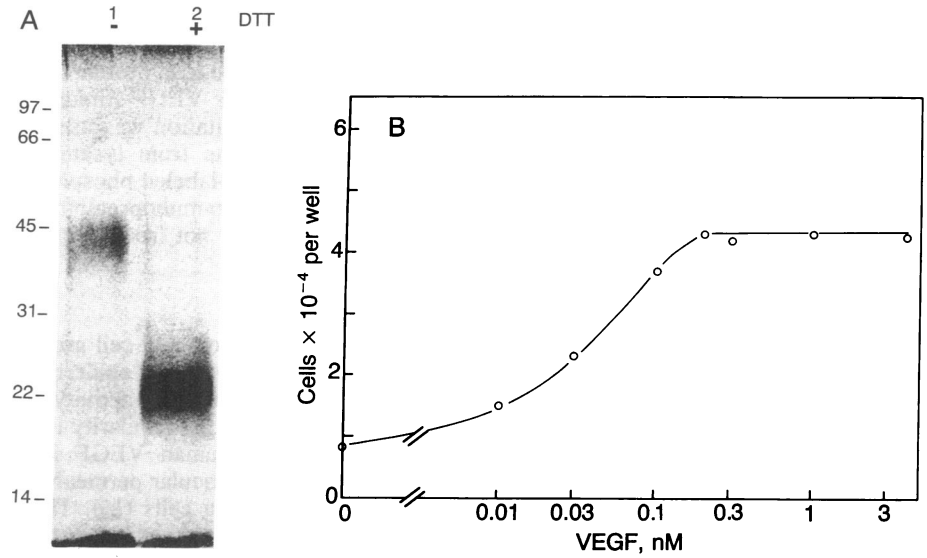

FIG. 2. Properties of A-431-derived VEGF. (*A*) SDS/PAGE of bioactive fractions purified by size-exclusion HPLC. Protein samples (20 μl) were treated in sample buffer under nonreducing (lane 1) or reducing (lane 2) conditions. The samples were electrophoresed in a 12% gel that was subsequently stained with silver nitrate. The positions of molecular mass markers are indicated. DDT, dithiothreitol. (*B*) Dose–response of HUVEC growth to purified VEGF. HUVEC were seeded at a density of 1×10^4 cells per well, and the indicated amounts of VEGF were added. Cells were counted after 5 days. Individual points are the mean of triplicate determinations.

recovery of mitogenic activity from 300 mg of protein. The heparin-binding mitogenic activity was eluted in 33% CH_3CN when fractionated by C_{18} reverse-phase HPLC (Fig. 1*A*); 6.2% of the total mitogenic activity was recovered with a further 15-fold increase in specific activity. In a second C_{18} reverse-phase HPLC step, the mitogenic activity was eluted in 20% 1-propanol (Fig. 1*B*); 4.3% of the total activity was recovered with a further 5-fold purification. Final purification of the HUVEC mitogenic activity was attained by size-exclusion HPLC: a bioactive peak corresponding to a single UV absorbance peak was obtained with a 4-fold increase in specific activity (Fig. 1*C*). Overall 5.5 μg of A-431 VEGF was purified 2083-fold from conditioned medium with a 3.8% recovery of activity.

Physical and Biological Characterization of A-431 VEGF. Microsequencing of 30 pmol of purified VEGF revealed an N-terminal amino acid sequence that was at least 83% similar to that deduced for human VEGF from HL-60 leukemia cells (15) and human VPF from U-937 histiocytic lymphoma cells (16). Residues 1–23 of A-431 VEGF were identified as Ala-Pro-Met-Ala-Glu-Gly-Gly-Gly-Gln-Asp-Trp-Xaa-Glu-Val-Val-Lys-Phe-Met-Xaa-Val-Tyr-Gln-Arg, while the corresponding sequences in HL-60 VEGF and U-937 VPF contained the amino acids Asn-His-His at positions 10–12 and aspartic acid at position 19. Purified A-431 VEGF had an estimated molecular mass of 44 kDa when electrophoresed under nonreducing conditions and an apparent mass of 22 kDa under reducing conditions (Fig. 2*A*). From these data, it appeared that the purified mitogen consisted of a dimeric molecule composed of identical subunits. Purified VEGF half-maximally stimulated HUVEC proliferation at 41 pM (1.8 ng/ml) and maximally stimulated HUVEC growth at 200 pM (8.8 ng/ml) (Fig. 2*B*). The activity of A-431 VEGF was not affected by heat (100°C for 5 min) or acidification (pH 2.5 for 5 hr), but it was sensitive to treatment with reducing agents. In addition heparin did not significantly enhance the activity of VEGF (data not shown). The bioactivity of the growth factor was also tested on different cell types; appreciable activity was observed only with HUVEC, while HFFB, HASMC, HFK, and FBHEC failed to show any significant proliferative response (results not shown).

Characterization of VEGF Binding Sites on HUVEC. VEGF and other growth factors were tested for the ability to compete with ^{125}I-VEGF in binding to HUVEC. VEGF but not epidermal growth factor, TGF-β, bFGF, or PDGF inhibited the binding of ^{125}I-VEGF in a concentration-dependent manner (Fig. 3*A*). These results indicated that ^{125}I-VEGF bound specifically to target cells and that binding occurred to binding sites that did not recognize other growth factors.

Binding isotherms obtained with ^{125}I-VEGF indicated that saturation of VEGF binding sites on HUVEC at 22°C was achieved at 600–800 pM VEGF (Fig. 3*B Inset*). A Scatchard plot of the ^{125}I-VEGF binding data was curvilinear (Fig. 3*B*), which was consistent with a model involving two affinity classes of binding sites. The high-affinity sites had a dissociation constant of 5–13 pM with ≈3000 binding sites per cell while the low-affinity sites had a dissociation constant of 72–120 pM with ≈12,000 binding sites per cell.

Time and Temperature Dependence of ^{125}I-VEGF Binding to HUVEC. Fig. 4 illustrates the kinetics of ^{125}I-VEGF association with cellular binding sites on HUVEC at 22°C and 37°C. At 22°C (Fig. 4*A*), maximal cell-associated radioactivity was

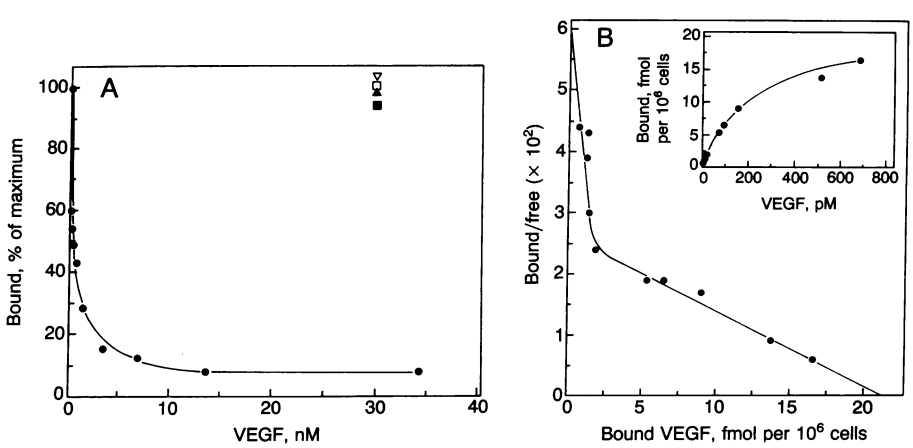

FIG. 3. Specificity of ^{125}I-VEGF binding to HUVEC. (*A*) HUVEC (9.2×10^4 cells per well) were incubated for 3 hr at 22°C in the presence of ^{125}I-VEGF and the indicated concentrations of VEGF (●), epidermal growth factor (▽), TGF-β (□), bFGF (▲), and PDGF (■). Results are the average of duplicate determinations and are expressed as the percentage of ^{125}I-VEGF bound to untreated cells. (*B*) Scatchard plot of ^{125}I-VEGF binding to HUVEC. Increasing concentrations of ^{125}I-VEGF were incubated with HUVEC (9.1×10^4 cells per well) at 22°C for 3 hr in the presence or absence of 100-fold molar excess of unlabeled VEGF (*Inset*).

obtained after 2 hr of incubation, and the binding appeared to have reached a plateau. There was no significant difference between the surface binding (acid-extractable) and the total binding (detergent-extractable). At 37°C (Fig. 4*B*), total binding was maximal after 2 hr and gradually decreased on prolonged incubation, while surface binding was maximal by 3 hr, followed by a gradual decrease in binding. Maximal levels of internalized ^{125}I-VEGF were obtained after 1 hr and then decreased.

Affinity-Labeling of HUVEC and Different Cell Types with ^{125}I-VEGF. To examine whether the binding observed in the Scatchard analyses correlated with specific classes of cell-surface binding sites, affinity-labeling of VEGF binding sites on HUVEC was carried out. By autoradiography, three molecular mass species of radiolabeled complexes of 230 kDa, 170 kDa, and 125 kDa were detected (Fig. 5*A*, lane 2). These labeled complexes were not observed in cells that were incubated with ^{125}I-VEGF but not exposed to DSS (Fig. 5*A*, lane 1) or in experiments in which incubations with ^{125}I-VEGF were performed with excess unlabeled VEGF (Fig. 5*A*, lane 3). The mobilities of these labeled species were identical in the absence of reducing agents. Using cross-linking techniques, we similarly examined HFFB, HASMC, HFK, and FHBEC for their ability to specifically bind to ^{125}I-VEGF. None of them exhibited significant binding to [^{125}I]VEGF (data not shown).

Stimulation by VEGF of Protein Tyrosine Phosphorylation in HUVEC. Anti-Tyr(*P*) antibodies have been shown to be sensitive reagents for the identification of proteins phosphorylated on tyrosine residues (37). HUVEC starved of HBGF-1 were stimulated with VEGF, and then cell lysates were prepared and subjected to SDS/PAGE and immunoblotting with anti-Tyr(*P*) monoclonal antibodies: VEGF induced the specific tyrosine phosphorylation of a 190-kDa protein (data not shown). In an alternative assay for VEGF-stimulated tyrosine phosphorylation immunoprecipitation was used to isolate phosphotyrosine-bearing proteins from lysates of [^{32}P]orthophosphate-labeled cells. A ^{32}P-labeled phosphotyrosine-bearing 190-kDa protein was immunoprecipitated from HUVEC stimulated by VEGF but not from unstimulated cultures (Fig. 5*B*).

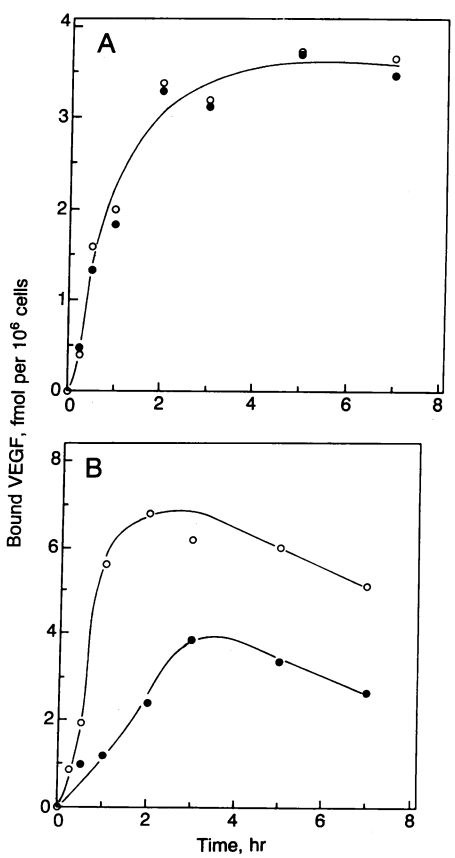

FIG. 4. Time-course and temperature-dependent binding of ^{125}I-VEGF to specific binding sites. HUVEC (9.0×10^4 cells per well) were incubated with 100 pM ^{125}I-VEGF at 22°C (*A*) or 37°C (*B*) for the indicated times. Surface-bound ^{125}I-VEGF (●) and total cell-associated ^{125}I-VEGF (○) were determined.

DISCUSSION

We have identified and purified an endothelial cell growth factor from culture medium conditioned by A-431 epidermoid carcinoma cells on the basis of its mitogenic activity for HUVEC. This growth factor had significant similarity in its N-terminal amino acid sequence to human VEGF from HL-60 leukemia cells (15) and human vascular permeability factor from U-937 histiocytic lymphoma cells (16). These VEGFs and those isolated from bovine and rat sources have been found to exhibit homology to PDGF (15–18). In contrast to FGFs, which are mitogenic for cell types in addition to vascular endothelial cells (12), VEGFs stimulated the growth of vascular endothelial cells with apparent specificity (19–22). Similarly, A-431-derived VEGF stimulated the proliferation of HUVEC, but it did not bind to or act on HFFB, HASMC, HFK, or FBHEC. The mitogenic, angiogenic, and permeability-enhancing properties of VEGF (19–22) suggest that the secretion of VEGF by tumorigenic cells may promote tumor angiogenesis and metastasis.

Polypeptide growth factors and hormones elicit pleiotropic effects by binding to and activating cell-surface receptors (38). Our results demonstrate the presence of high-affinity specific binding sites for VEGF on HUVEC. The binding of ^{125}I-VEGF to these binding sites was time- and temperature-dependent, saturable and specifically inhibited by VEGF.

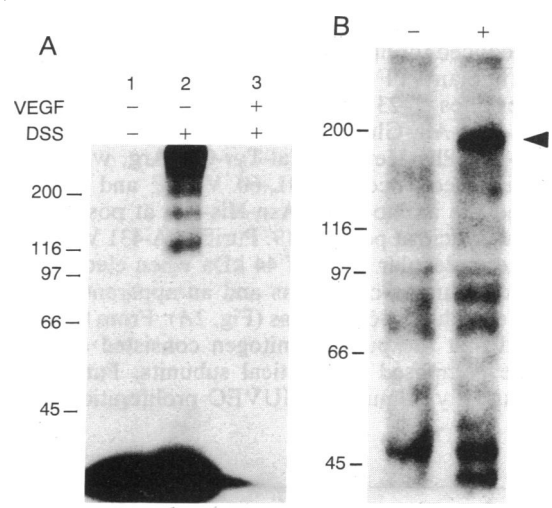

FIG. 5. Visualization of VEGF–receptor complexes and VEGF-induced tyrosine phosphorylation in HUVEC. (*A*) HUVEC (4×10^5 cells) were incubated with 150 pM ^{125}I-VEGF at 22°C for 3 hr in the absence (lane 2) or presence of 100-fold molar excess of unlabeled VEGF (lane 3) followed by DSS crosslinking. Lane 1 was not treated with DSS. Binding site–ligand complexes were resolved by SDS/PAGE and visualized by autoradiography. (*B*) Stimulation of tyrosine phosphorylation by VEGF in HUVEC labeled *in vivo* with [^{32}P]orthophosphate. HUVEC (4.6×10^5) were metabolically labeled with [^{32}P]orthophosphate and incubated at 37°C for 10 min in the absence (−) or presence (+) of 200 pM VEGF. Cell lysates were prepared and immunoprecipitated with anti-Tyr(*P*) monoclonal antibody. The arrowhead indicates a polypeptide phosphorylated on tyrosine in response to VEGF.

^{125}I-VEGF binding to HUVEC generated a curvilinear Scatchard plot (Fig. 3*B*), which was consistent with two classes of binding sites with apparent dissociation constants in the picomolar range. These properties are similar to those reported for bovine VEGF receptors (24). As a maximal growth response to VEGF occurred at concentrations of 200 pM or more (Fig. 2*B*), the occupancy of all VEGF binding sites may be necessary for maximum activity. This conclusion then raises the question of the functional significance of the two apparent classes of VEGF binding sites. Whether or not these binding sites have distinct physiological roles remains to be determined. ^{125}I-VEGF at a sufficiently high concentration to bind both affinity classes of binding sites was internalized at 37°C, and VEGF binding sites, like those of other growth factors (33, 39, 40), were down-regulated by ligand (Fig. 4*B*).

To identify the binding sites present on HUVEC, ^{125}I-VEGF was cross-linked covalently to its binding sites by using disuccinimidyl suberate. Three macromolecular complexes with molecular masses of 230 kDa, 170 kDa, and 125 kDa were specifically labeled by ^{125}I-VEGF (Fig. 5*A*). The molecular masses of these labeled complexes were the same in the presence or absence of reducing agents. The structural relationships between the three ^{125}I-VEGF cross-linked macromolecular species are unknown. Connolly *et al.* (41) have reported that ^{125}I-labeled VPF formed a >330-kDa complex with an HUVEC surface protein by using cross-linking. The basis for the discrepancy between our data is not clear, but it may be important that the cross-linking experiments were done at different temperatures. Affinity-labeled complexes of 225 kDa, 195 kDa, and 170 kDa were detected in bovine aortic arch endothelial cells (24).

It has been observed that when some growth factors bind to their receptors, a tyrosine-specific protein kinase activity is stimulated, and a mitogenic signal is transmitted (38). Immunoblot analysis using anti-Tyr(*P*) monoclonal antibodies suggested that VEGF specifically induced tyrosine phosphorylation of a 190-kDa protein in HUVEC. Immunoprecipitation of ^{32}P-labeled proteins with monoclonal anti-Tyr(*P*) antibodies confirmed that this protein was phosphorylated on tyrosine residues in HUVEC (Fig. 5*B*). This VEGF-induced phosphotyrosine-bearing protein was similar in mass to the 230-kDa ^{125}I-VEGF-binding site complex minus the mass of VEGF. VEGF-induced tyrosine phosphorylation of the remaining two ligand binding complexes was not detected. Tyrosine-phosphorylation of certain cellular components other than receptors, such as phospholipase C-γ, may occur after growth factor stimulation (42). However, these results raise the possibilities that VEGF induces the phosphorylation of its receptor and that VEGF receptors possess intrinsic tyrosine kinase activity.

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