Regulation of Vascular Endothelial Growth Factor Expression by Insulin-Like Growth Factor I

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Insulin-like growth factor I (IGF-I) and vascular endothelial growth factor (VEGF) levels are correlated with retinal ischemia-associated intraocular neovascularization in humans. Since VEGF is required for iris and retinal neovascularization in animal models of retinal ischemia, we tested whether IGF-I could act as an indirect angiogenic factor by increasing VEGF gene expression. IGF-I increased retinal pigment epithelial (RPE) cell VEGF mRNA in a concentration-dependent manner with an EC_{50} of 7 nmol/l (53.6 ng/ml). RPE and bovine smooth muscle cells exposed to 50 nmol/l (383 ng/ml) IGF-I achieved peak VEGF mRNA expression within 2 h. IGF-I–treated RPE cells increased VEGF protein levels in conditioned media and stimulated capillary endothelial cell proliferation. Blockade of the IGF-I receptor with a neutralizing antibody abrogated the VEGF increases in RPE cells. Further, hypoxia-mediated and IGF-I–mediated increases in VEGF mRNA and protein levels were additive in RPE cells, and the hypoxia-induced VEGF increases were independent of endogenous IGF-I. VEGF promoter activity was enhanced by IGF-I in RPE cells, but VEGF transcript half-life was unaltered. In summary, the supplementation of RPE and smooth muscle cell cultures with IGF-I at 5–100 nmol/l increased VEGF mRNA and secreted protein levels. The VEGF increases in RPE cells occurred primarily through enhanced transcription of the VEGF gene and via the IGF-I receptor. Elevated IGF-I levels may promote neovascularization through increased retinal VEGF gene expression. *Diabetes* 46:1619–1626, 1997

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BSA, bovine serum albumin; DMEM, Dulbecco's modified essential medium; EC_{50}, median effective concentration; IGF-I, insulin-like growth factor I; nt, nucleotide; RPE, retinal pigment epithelial; SMC, smooth muscle cell; TPA, 12-*O*-tetradecanoylphorbol-13 acetate; VEGF, vascular endothelial growth factor.

Clinical observations and laboratory data support a pathogenic link between insulin-like growth factor I (IGF-I) and intraocular neovascularization. In 1953, it was first reported that ablation of the pituitary gland was associated with the regression of retinal neovascularization in a diabetic patient (1). Subsequent reports implicated pituitary growth hormone in the process. Growth hormone–deficient dwarfs (2) and prepubescent children (3) were demonstrated to have a lower incidence and severity of proliferative diabetic retinopathy than diabetic adults with normal growth hormone levels. More recently, attention has shifted to IGF-I, the primary mediator of growth hormone activity. Serum and vitreous IGF-I levels have been demonstrated to correlate in timing and degree with the neovascularization of proliferative diabetic retinopathy (4–6). In 1993, these findings were extended by Meyer-Schwickerath et al. (6) when they demonstrated that elevated IGF-I levels were correlated with a wide variety of ischemic retinal disorders linked to neovascularization of the retina and iris. Grant and colleagues have provided a potential mechanism by demonstrating that IGF-I, in concert with serum or progression factors, can act as a vascular endothelial cell mitogen (7) and migration factor (8) in vitro and an angiogenic factor in vivo (9,10). Multiple injections of IGF-I into normal nondiabetic eyes have stimulated retinal neovascularization in animals (10).

Vascular endothelial growth factor (VEGF) is an angiogenic and permeability-enhancing peptide that is causally linked to retinal ischemia-associated neovascularization (11–15). VEGF is expressed at high levels in response to hypoxia (16), and increased VEGF mRNA levels are present in ischemic retinas in vivo (17–19). VEGF is the primary endothelial cell mitogen produced and secreted by hypoxic retinal cells in vitro (20) and preferentially binds to high-affinity receptors on retinal endothelial cells (21). Up to four different VEGF isoforms are derived through the alternative splicing of mRNA (22–24). The isoforms differ in their affinity for heparin, with the smaller isoforms ($VEGF_{121}$ and $VEGF_{165}$) binding less avidly than the larger isoforms ($VEGF_{189}$ and $VEGF_{206}$) (24). $VEGF_{121}$ and $VEGF_{165}$ are the predominant forms made in ischemic retina (25). The critical role played by VEGF in developmental angiogenesis and vasculogenesis is demonstrated by the fact that deletion of the genes for VEGF, or its high-affinity receptors flk-1 and flt-1, results in abnormal blood vessel development and death in utero (26–29).

In the human eye, elevated vitreous and aqueous VEGF levels strongly correlate with retinal ischemia-associated neovascularization in diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity (18,30–32). Further, VEGF has been directly demonstrated to be causative for retinal ischemia-associated intraocular neovascularization in animal models. The injection VEGF into normal primate eyes is sufficient to produce retinal and iris vessel growth and neovascular glaucoma (33,34). Moreover, the inhibition of intraocular VEGF suppresses retinal ischemia-associated iris (14) and retinal neovascularization (15).

Although hypoxia is a potent stimulus for VEGF gene expression (16,35), additional factors may serve to modulate VEGF levels in vivo (36,37). Since the presence and severity of retinal ischemia-associated intraocular neovascularization varies as a function of intraocular IGF-I levels, we tested the hypothesis that IGF-I indirectly stimulates neovascularization through increases in VEGF gene expression. We now report that IGF-I potently increases VEGF gene expression in vitro and that the effect is additive with hypoxia. The IGF-I–mediated increases in VEGF gene expression in retinal pigment epithelial (RPE) cells are primarily mediated through increases in VEGF transcription.

RESEARCH DESIGN AND METHODS

Materials. Human recombinant IGF-I and mouse anti-human IGF-I neutralizing antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Human recombinant $VEGF_{165}$ and the neutralizing monoclonal anti-human IGF-I receptor antibody were purchased from R&D Systems (Minneapolis, MN). The neutralizing monoclonal anti-human gp120 antibody was a gift of Dr. Napoleone Ferrara, Genentech (South San Francisco, CA). Bovine serum albumin (BSA), actinomycin D, and 12-*O*-tetradecanoylphorbol-13 acetate (TPA) were purchased from Sigma (St. Louis, MO).

Cell culture. Human RPE cells were immortalized through the stable integration of a cytomegalovirus-driven simian-virus-40 large T-antigen expression cassette and cultured on noncoated plates as previously described (38,39). The cells contain pigment, grow in a monolayer and increase VEGF mRNA during hypoxia in a manner identical to the parent cell line (39). Bovine smooth muscle cells (SMCs) were obtained from Dr. Patricia D'Amore and have been previously characterized and described (40). The RPE cells and SMCs were maintained until experimentation in Dulbecco's modified essential medium (DMEM) (Sigma) containing 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mmol/l L-glutamine. Cells were plated into six-well plastic plates (Falcon-Becton Dickinson Labware, Franklin Lakes, NJ) and used for experiments when they reached 80–100% confluence. Fresh serum-free media were placed on the cells 24 h before the experiments. All reagents were added directly to the wells in a volume of 10–100 µl phosphate buffered saline (Sigma) or DMEM. For the protein quantitation and cell proliferation and transfection experiments, each condition was prepared in triplicate, and the experiments were carried out at least twice with reproducible results. No changes in cell morphology were noted on visualization of the cells before RNA extraction or collection of conditioned media.

RNA isolation and Northern blot analysis. Total RNA was isolated from cultured cells using RNAzol B (Biotec Laboratories, Houston, TX) by the method of Chomczynski and Sacchi (41). RNA (15 µg) was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nylon filters (Gene Screen Plus, New England Nuclear, Boston, MA). The filters were prehybridized in buffer containing 50% deionized formamide, 5× sodium chloride–sodium phosphate–EDTA buffer, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, 10% dextran sulfate (Pharmacia Biotech, Uppsala, Sweden), and denatured salmon sperm DNA (100 g/ml) and hybridized at 42°C in fresh buffer without salmon sperm DNA. The hybridization buffer contained either a 520-bp *Nco I/Bgl* II fragment of the human VEGF cDNA or a 575-bp fragment encompassing the entire coding region of the mouse VEGF cDNA. The blots were stripped and reprobed with a 400-bp fragment encompassing the 3′UTR of the human β-actin cDNA. The cDNA probes were labeled with a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) using [α-^{32}P]deoxy-CTP (New England Nuclear, Boston, MA) and had specific activities of 1–2 × 10^5 cpm/µg DNA. Filters were washed twice in 0.5–2.0× sodium chloride–sodium phosphate–EDTA buffer and 0.1% sodium dodecyl sulfate for varying times and at increasing temperatures. The washes were titrated for maximum signal to noise ratio. The hybridized and washed filters were exposed to Kodak X-Omat AR X-ray film with an intensifying screen at –70°C for 12–72 h. Each Northern blot experiment was repeated at least three times with similar results. A representative blot was chosen from each experiment for the figures. VEGF values were obtained by subtracting background signal in each lane from the corresponding band signal in that lane. Normalization was done using β-actin values derived in the same manner. Densitometry was performed on all blots with IS-1000 Digital Imaging System (version 1.97 software, Alpha Inotech, Torrence, CA) within the linear range of signal quantitation.

RNase protection assay. The VEGF riboprobe was produced by subcloning the coding sequence of the human $VEGF_{121}$ cDNA into the *Sma* I site of the Bluescript vector (Stratagene, La Jolla, CA). Transcription by T7 RNA polymerase after linearization by *Nco* I resulted in a probe of 496 nucleotides (nts). This probe would protect a 416-nt fragment of $VEGF_{121}$ and a 338-nt fragment of $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, as depicted in Fig. 3*A*. The human β-actin probe was produced by transcribing the human β-actin cDNA template pTRI-β-actin-h (Ambion, Austin, TX), using T3 RNA polymerase, and was labeled 1/20 as radioactive as the VEGF probe. Full-length protection of this probe results in a 245-nt fragment. The assay was performed as previously described (42). Ten micrograms of total cellular RNA was hybridized with ^{32}P-labeled antisense VEGF, β-actin, and 18S rRNA riboprobes with specific activities of 0.2–1.5 × 10^9 cpm/µg RNA and was incubated overnight at 42°C in 30 µl hybridization buffer. Hybridized RNA was digested with nuclease P1 (20 µg/ml) and RNase T1 (2 µg/ml) for 1 h at 25°C in 300 µl digestion buffer. Digestions were terminated by addition of 20 µl of 10% sodium dodecyl sulfate and 50 µg proteinase K for 15 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, the protected fragments were resolved on 6% polyacrylamide, 7 mol/l urea gels, and visualized with autoradiography. Quantitation was performed using a Molecular Dynamics PhosphorImager.

Conditioned media VEGF measurements. Conditioned media VEGF levels were determined using an a sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MA). RPE cells were counted at the end of the experiment to ensure there was no difference among the different treatment groups.

Endothelial cell proliferation assay. Assays were performed as previously described (43). Briefly, low passage bovine capillary endothelial cells, the kind gift of Dr. Judah Folkman and Ms. Catherine Butterfield, were isolated from bovine adrenal glands and grown on gelatin-coated dishes as described by Folkman and Haudenschild (44). Cells were plated on 24-well plates at a density of 10^4 cells/well in DMEM containing 10% calf serum, 2 mmol/l glutamine, and antibiotics. After 24 h at 37°C, the medium is replaced with the same medium with only 5% calf serum, together with conditioned media of treated RPE cells (serum-free), resulting in a 2.5% serum concentration. Treated RPE cells were counted to ensure no statistically significant difference in cell number between the treatment groups after media collection. At this time, a few wells are trypsinized to determine the zero-hour cell count. After 96 h, all remaining wells were trypsinized, and the cell number was determined with a Coulter counter.

Hypoxia/normoxia. Twelve hours after changing to serum-free media, RPE cells were placed in a sealed chamber (Billups-Rothenberg, Del Mar, CA) and exposed to continuous hypoxia (3% O_2, 5% CO_2, and 92% N_2) or normoxia (21% O_2, 5% CO_2, and 74% N_2) for 6 h as previously described (20). The 6-h time period was chosen to give a submaximal response of both the hypoxia effect and IGF-I response to be better able to study the simultaneous response to both IGF-I and hypoxia.

Transient transfection assay. The 1.7 kb of rat VEGF 5′-flanking sequence (*Eco* R *V-Pst* I) was subcloned 5′ to the luciferase gene in the luciferase containing eukaryotic expression vector pXP2, as previously described (45). The plasmid pT81LUC contains 81 bp of the thymidine kinase minimum promoter constructed 5′ to the luciferase gene of the pXP2 expression vector. Plasmid DNAs were transfected into RPE cells with LipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's protocol. The day before transfection, 1 × 10^6 RPE cells were plated into a 10-cm^2 tissue culture dish. Serum-free medium (400 µl) containing 12 µg plasmid DNA was mixed with another 400 µl serum-free medium containing 20 µl of LipofectAMINE reagent. After 15 min of incubation at 25°C, 3.2 ml of serum-free medium was added to the 0.8-ml DNA-liposome mixture. The diluted DNA-liposome complex was applied to the RPE cell monolayer for a 10-h incubation at 37°C. The transfected cells were pooled and subsequently evenly plated into 60-mm dishes and cultured in serum-free medium for 24 h before the treatment with or without IGF-I for an additional 24 h. Cell extracts were prepared and luciferase activity was then determined according to the manufacturer's protocol (Analytical Luminescence Laboratory, San Diego, CA) in a monolight 2010 luminometer.

Actinomycin D transcriptional inhibition. RPE cells were pretreated for 60 min with actinomycin D (5 µg/ml), followed by continued actinomycin D exposure with or without 50 nmol/l IGF-I for 2 h. For half-life measurements, RPE cells were treated with 50 nmol/l IGF-I for 2 h and then exposed to actinomycin D and 50 nmol/l IGF-I for 0, 1, 2, 3, and 4 h. Total RNA was collected at the indicated time points and processed as described above for the RNAase protection assay.

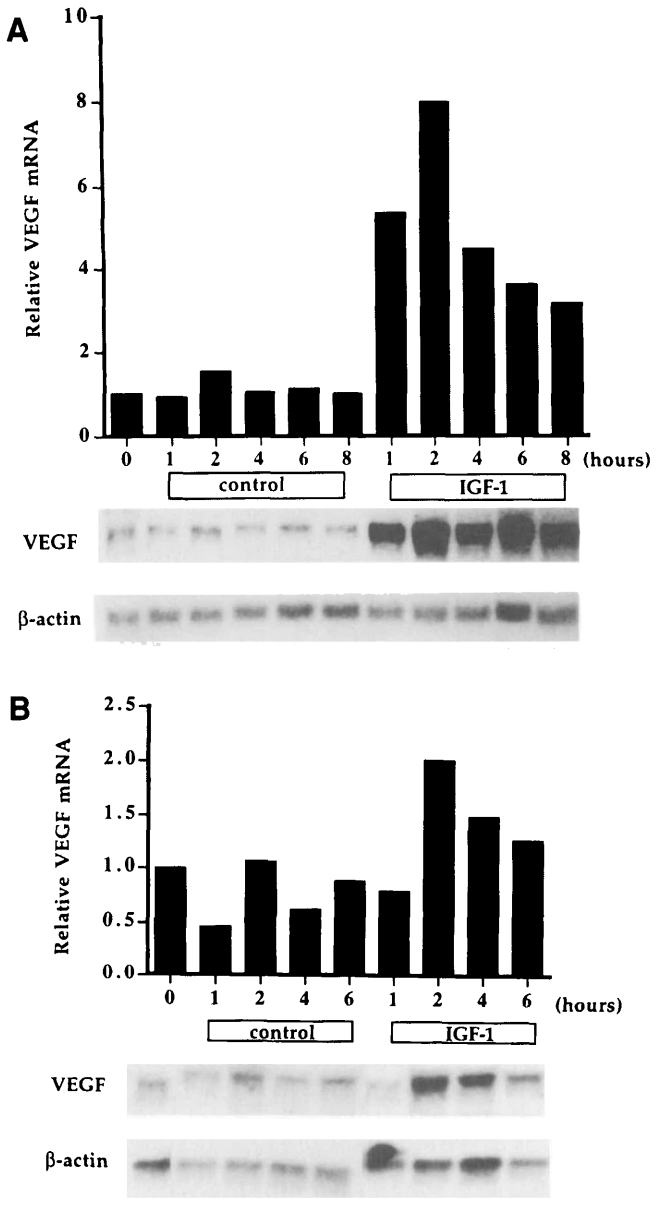

FIG. 1. VEGF mRNA temporal response to IGF-I. Northern blot analysis of VEGF mRNA from RPE cells (*A*) and SMCs (*B*) exposed to 50 nmol/l IGF-I for the indicated times. Control wells received a gram equivalent amount of BSA (383 ng/ml) in an equal volume of DMEM.

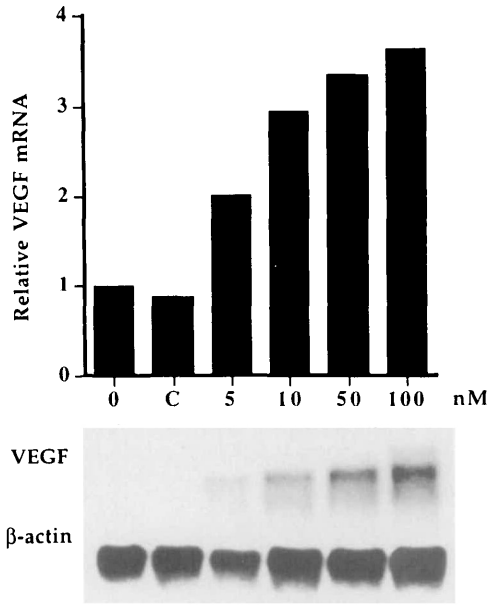

FIG. 2. VEGF mRNA dose-response to IGF-I. Northern blot analysis of VEGF mRNA from RPE cells exposed to increasing concentrations of IGF-I for 2 h. 0, vehicle (PBS); C, BSA alone in gram equivalent concentration to 100 nmol/l IGF-I (766 ng/ml).

RESULTS

VEGF mRNA temporal response to IGF-I. The effect of IGF-I on steady state VEGF mRNA levels was determined using Northern blotting for VEGF and β-actin. Human RPE cells (Fig. 1*A*) and bovine smooth muscle cells (Fig. 1*B*) exposed to 50 nmol/l IGF-I increased steady-state VEGF mRNA levels. Peak VEGF mRNA levels were observed in both cell types 2 h after exposure to IGF-I and remained elevated the duration of the experiment. Equivalent volumes of DMEM and bovine serum albumin (BSA) in gram equivalent amounts equal to 50 nmol/l IGF-I were added to parallel wells at the same time as IGF-I had no effect on RPE cell and SMC VEGF mRNA levels.

VEGF mRNA dose response to IGF-I. The concentration of IGF-I required to increase steady-state VEGF mRNA levels at 2 h was determined in RPE cells with Northern blotting. IGF-I at 5 nmol/l increased steady-state VEGF mRNA levels. Peak expression levels were observed at 50–100 nmol/l IGF-I (Fig. 2).

VEGF mRNA isoforms are coordinately increased by IGF-I. Several different VEGF isoforms can be derived from the alternative splicing of mRNA (Fig. 3*A*). To determine which isoforms of VEGF mRNA were increased by IGF-I, ribonuclease protection assays were performed using a riboprobe corresponding to $VEGF_{121}$ (416 nt protected fragment). This probe also detects three other isoforms of VEGF as a single protected fragment (338 nt; Fig. 3*A*). Normalization for probe length allowed for the quantification of the relative abundance of $VEGF_{121}$ versus other VEGF isoforms in the RPE cell VEGF mRNA pool. $VEGF_{121}$ contributed 54% of the VEGF mRNA pool, whereas $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ accounted for the remaining 46%. At 2 h, there was an increase of 4.7 ± 0.5 fold (n = 3) in the level of $VEGF_{121}$ mRNA in 50 nmol/l IGF-I–treated RPE cells (Fig. 3*B*). The band corresponding to $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ was increased to a slightly lesser degree (3.9 ± 0.3 fold, n = 3).

IGF-I increases VEGF protein in the conditioned media of RPE cells. The ability of IGF-I to stimulate the release of VEGF into the conditioned media of RPE cells was tested. Human RPE cells exposed to IGF-I for 24 h increased conditioned media VEGF protein in a concentration-dependent manner, with a median effective concentration (EC_{50}) of 7 nmol/l (Fig. 4*A*). The addition of increasing amounts of an anti-human IGF-I neutralizing antibody abolished the increase in conditioned media VEGF (Fig. 4*B*). The addition of equivalent amounts of a nonspecific protein (BSA) did not block the IGF-I–induced increases in VEGF protein (Fig. 4*B*). The addition of the IGF-I neutralizing antibody to cells treated with 10 nmol/l TPA did not block the increases in conditioned media VEGF levels (Fig. 4*B*).

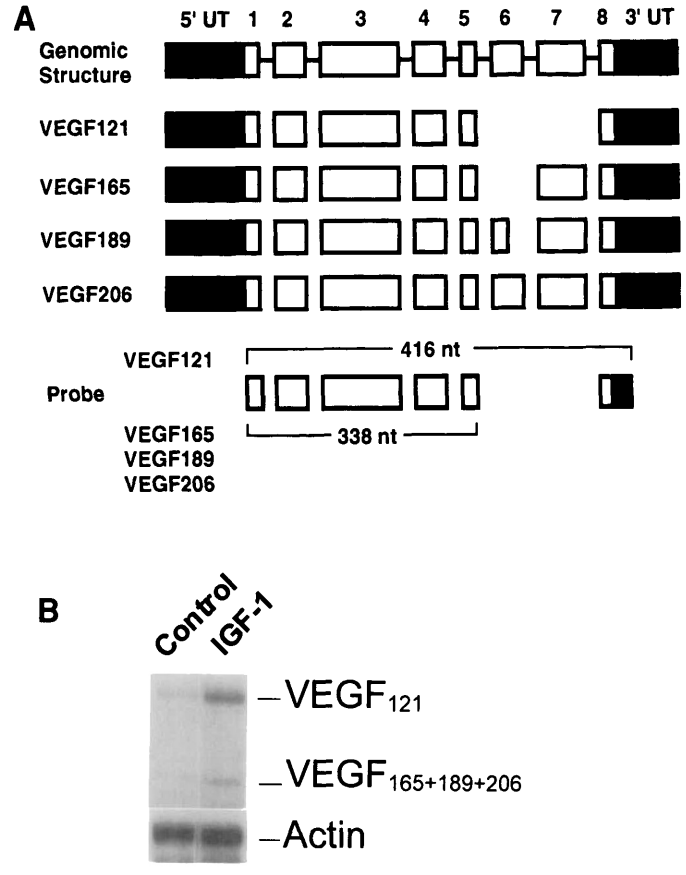

FIG. 3. VEGF mRNA isoforms are coordinately increased by IGF-I. *A*: diagram shows exon structure and differential splicing of VEGF mRNA to produce the 121, 165, 189, and 206 amino acid isoforms. The riboprobe template corresponding to $VEGF_{121}$ and the protected fragment length are shown. *B*: ribonuclease protection assay shows protected fragments of expected size. The human β-actin mRNA was used to normalize the samples for quantification of isoform expression. IGF-I = 50 nmol/l IGF-I.

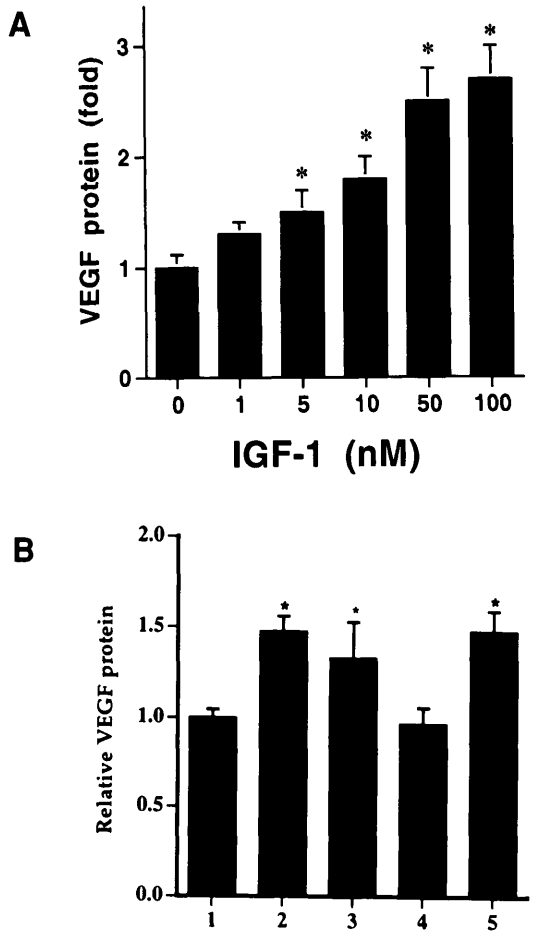

FIG. 4. IGF-I increases VEGF protein levels in the conditioned media of RPE cells. *A*: fold induction of immunoreactive VEGF in the conditioned media of IGF-I–treated human RPE cells, compared with parallel cultures of receiving 100 µl vehicle alone (PBS). Conditioned media were collected after 24 h. All IGF-I–treated wells were significantly elevated above control ($P < 0.05$). The concentration of VEGF in the conditioned media of PBS alone treated cells was 3.1 ± 0.2 ng/ml ($n = 3$). *B*: effect of anti-IGF-I antibody on VEGF increases. Conditioned media was collected after 24 h of exposure. Error bars represent SDs. A large asterisk indicates significant difference ($P < 0.01$) from BSA alone (*lane 1*) values, and a small asterisk indicates significant difference ($P < 0.05$) from BSA alone. Lane 1, 383 µg/ml BSA; lane 2, 383 ng/ml IGF (50 nmol/l) + 383 µg/ml BSA; lane 3, 383 ng/ml IGF-I + 96 µg/ml anti-IGF-I antibody + 287 µg/ml BSA; lane 4, 383 ng/ml IGF-I + 383 µg/ml anti-IGF-I antibody; lane 5, 65 ng/ml TPA (phorbol ester, positive control) + 383 µg/ml anti-IGF-I antibody. A total of 96 µg/ml antibody is 25% of complete neutralizing dose, and 383 µg/ml of antibody is required for complete neutralization of 383 ng/ml (50 nmol/l) of IGF-I.

Increased capillary endothelial cell proliferation with conditioned media of IGF-I–treated RPE cells. The ability of IGF-I–treated RPE cell conditioned media to stimulate capillary endothelial cell proliferation was tested. RPE cells were treated with 50 nmol/l IGF-I or BSA for 24 h and the conditioned media placed on BCE for 96 h. The conditioned media of the IGF-I–treated RPE cells increased the proliferation of bovine capillary endothelial cells 1.6-fold above control (BSA-treated) cells (Fig. 5*A*). To test if the added IGF-I was responsible for the increased BCE cell proliferation, IGF-I in the same concentration used on the RPE cells was added directly to BCE cells for 96 h. The addition of 50 nmol/l recombinant human IGF-I directly to endothelial cells did not increase endothelial cell proliferation (Fig. 5*B*).

Hypoxia-induced VEGF increases are independent of endogenous IGF-I. To determine if endogenous IGF-I is responsible for the increased VEGF in hypoxic RPE cells, a monoclonal anti-human IGF-I receptor antibody was used to block endogenous IGF-I bioactivity (Fig. 6). Ten micrograms per milliliter of either the anti-human IGF-I receptor antibody or an isotype control anti-human gp120 antibody were added 30 min before exposure to the stimuli (either 3% hypoxia or the addition of 50 nmol/l IGF-I). In a control experiment, exogenous IGF-I–stimulated VEGF increases in RPE cell conditioned media were partially blocked with the anti-IGF-I receptor antibody, demonstrating the efficacy of the antibody and mediation of the IGF-I–induced VEGF increases through the IGF-I receptor. The control anti-gp120 antibody did not inhibit the stimulatory effect of exogenous IGF-I on VEGF production (Fig. 6). Three percent O_2 increased RPE cell VEGF protein, but the effect was not inhibited by the IGF-I receptor antibody.

IGF-I–induced and hypoxia-induced VEGF mRNA and protein increases are additive. To determine if hypoxia and IGF-I can work in concert to increase VEGF gene expres-

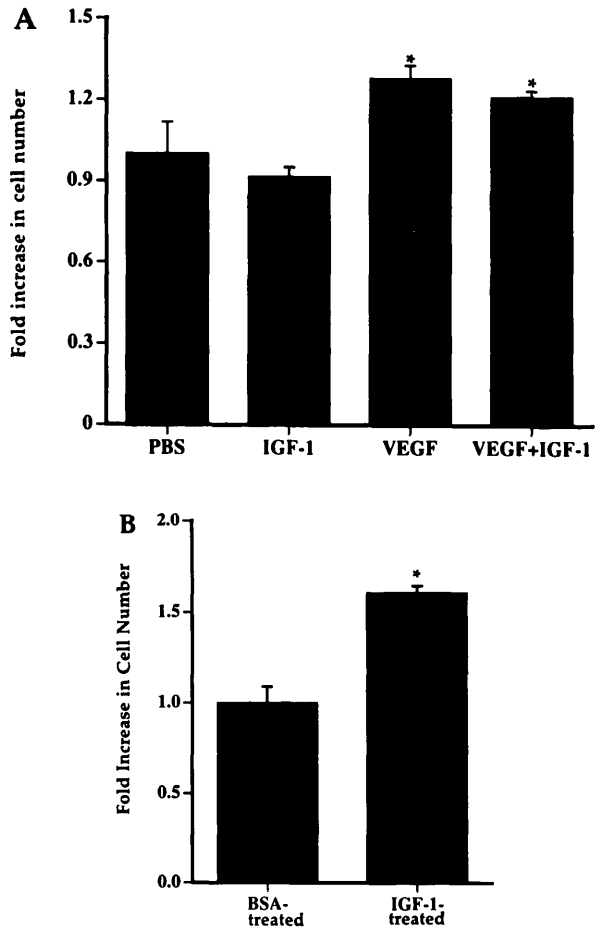

FIG. 5. Conditioned media of IGF-I–treated RPE cells increase capillary endothelial cell proliferation. *A*: conditioned media from RPE cells exposed to 383 ng/ml (50 nmol/l) IGF-I or 383 ng/ml BSA for 24 h was collected and added to low passage BCE cells. BCE cell values are shown as relative to BSA-treated values. Differences were significant ($P < 0.01$). Error bars represent SDs. *B*: IGF-I on endothelial cells does not stimulate endothelial cell proliferation. Lane 1, 100 µl PBS (vehicle alone); lane 2, 383 ng/ml (50 nmol/l) IGF-I in 100 µl PBS; lane 3, 20 ng/ml VEGF; lane 4, 383 ng/ml (50 nmol/l) IGF-I + 20 ng/ml VEGF. Proliferation increases are normalized to PBS alone. *$P < 0.05$ with respect to PBS alone.

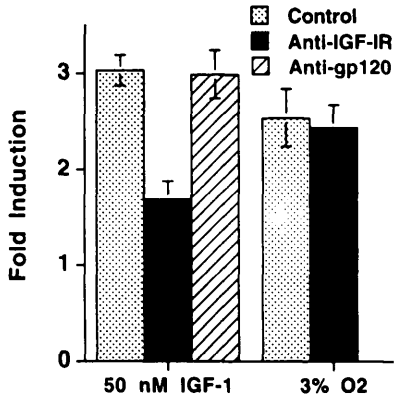

FIG. 6. IGF-I increases VEGF through the IGF-I receptor and hypoxia-induced VEGF mRNA increases are independent of endogenous IGF-I. Ten micrograms per milliliter of anti-human IGF-I receptor antibody or anti-human gp120 antibody was added to human RPE cells 30 min before stimulation with either 50 nmol/l IGF-I or 3% O_2. Exogenous IGF-I–induced VEGF synthesis in the RPE cell conditioned media was reduced from 3.03 ± 0.16– to 1.69 ± 0.19–fold. The isotype control anti-gp120 antibody did not inhibit the stimulatory effect of exogenous IGF-I on VEGF. Three percent O_2 stimulated a 2.54 ± 0.30–fold increase in VEGF, which was not inhibited by the IGF-I receptor antibody (2.44 ± 0.24–fold).

sion, they were tested alone and in combination. Human RPE cells were exposed to hypoxia or normoxia for 6 h, with or without 50 nmol/l IGF-I. Hypoxia alone and IGF-I alone increased steady-state VEGF mRNA 6.7- and 4.1-fold, respectively (Fig. 7*A*). The combination of hypoxia and IGF-I increased VEGF mRNA 13.5-fold (Fig. 7*A*) and conditioned media VEGF 5.7-fold (Fig. 7*B*).

IGF-I enhances VEGF promoter activity but not transcript stability. To examine whether the increases in VEGF mRNA levels by IGF-I are due to an enhancement of VEGF transcription, the effect of IGF-I on VEGF promoter activity was examined in transient transfection assays. IGF-I (50 nmol/l) stimulated VEGF promoter activity by an average of 2.6 ± 0.2-fold ($n = 3$ experiments). As a control, 50 nmol/l IGF-I did not affect the activity of the 81-bp thymidine kinase promoter (1.0 ± 0.1-fold, $n = 3$) (Fig. 8*A*). To determine if the increases in steady-state VEGF mRNA levels were due to an increase in transcript stability, actinomycin D was used to inhibit transcription and ribonuclease protection assays were used to quantitate the decay of transcript levels over time (Fig. 8*B*). IGF-I did not significantly affect the half-life of VEGF mRNA in RPE cells (1.06 ± 0.21 vs. 0.98 ± 0.09 h; $n = 3$; $P > 0.05$). This finding was confirmed by the observation that actinomycin D completely abolished the IGF-I–stimulated increases in VEGF expression (Fig. 8*C*).

DISCUSSION

VEGF is an important angiogenic molecule. Its systemic inactivation prevents both normal developmental (26,27) and adult pathological (46) angiogenesis. In ocular angiogenic diseases, VEGF is expressed at high levels in the nonendothelial cells of the retina and appears to act in a paracrine manner (14,18,19). The inhibition of VEGF potently reduces retinal ischemia-associated iris (14) and retinal (15) neovascularization in animal models. The injection of recombinant human VEGF into normal primate eyes is sufficient to stimulate the growth of iris (34) and retinal vessels (33). Although multiple growth factors are likely involved in the process of ocular neovascularization, these experimental studies indicate that the inhibition of VEGF prevents and/or lessens the degree of retinal ischemia-associated intraocular neovascularization (14,15).

Ischemic hypoxia is widely considered to be a functionally relevant stimulus for VEGF gene expression in vivo. Hypoxia potently increases VEGF gene expression in vitro (16) and oxygen microelectrode measurements have confirmed that ischemic retina is hypoxic in vivo (47). The reversal of hypoxia in ischemic retinas significantly lowers retinal VEGF mRNA levels (35). However, complete reversal of the oxygen deficit does not completely reverse ischemic retinal VEGF gene expression in vivo (35). Thus, hypoxia per se may not be the sole stimulus for VEGF expression in vivo. Numerous other potentially relevant stimuli can increase VEGF gene expression in vitro and in vivo, including hypoglycemia (36) and reactive oxygen intermediates (37). The functional relevance of these

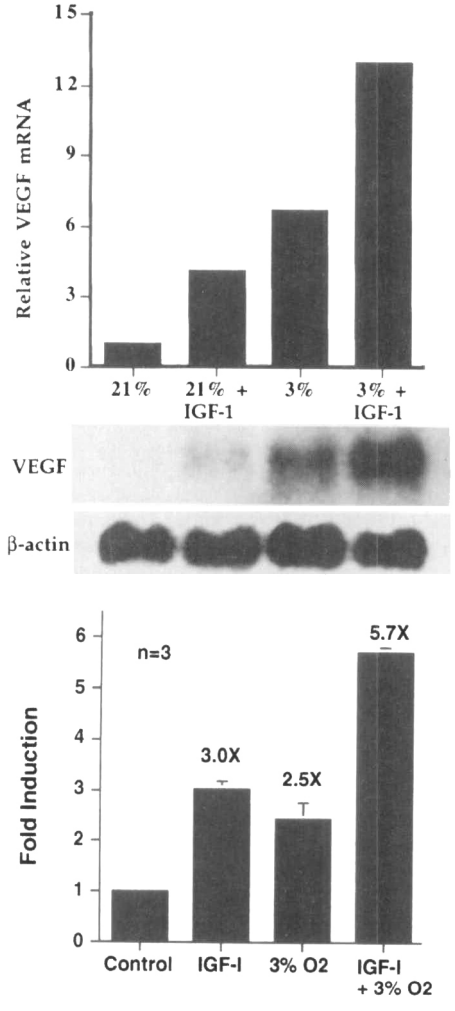

FIG. 7. Hypoxia-induced VEGF mRNA increases are additive with exogenous IGF-I. RPE cells were exposed to the following for 6 h: 21%, normaoxia; 3%, hypoxia; IGF-I, 50 nmol/l IGF-I. Non-IGF-I–treated conditions received an equivalent volume of IGF-I vehicle (PBS). VEGF steady-state mRNA (*A*) and conditioned media protein (*B*) levels were increased in an additive fashion by hypoxia and IGF-I.

stimuli in retinal disease remains to be determined.

IGF-I is a likely stimulus for increased retinal VEGF gene expression in vivo. Elevated IGF-I levels are strongly linked to proliferative diabetic retinopathy and other neovascular disorders secondary to retinal ischemia (5,6). Although Grant and colleagues have shown that IGF-I can act as a direct angiogenic factor (9,10), the possibility remains that IGF-I may mediate angiogenesis through increases in VEGF gene expression. The data presented herein support such a role. RPE cells were chosen for in vitro experimentation, since these cells provide a stable neuroectodermally derived cell population that has been a good model for VEGF gene regulation in the retina (20,37,38). In vivo, IGF-I may act on other retinal cell types demonstrated to make VEGF in disease, such as Muller and ganglion cells (19,25). VEGF mRNA and secreted protein levels increase substantially and in a sustained fashion when exposed to IGF-I in low nanomolar concentrations. Further, the increases are mediated through the IGF-I receptor and are additive with hypoxia, a functionally relevant stimulus for retinal VEGF gene expression in vivo.

Even though these in vitro data support a role for IGF-I in VEGF-driven ocular angiogenesis, direct confirmation requires

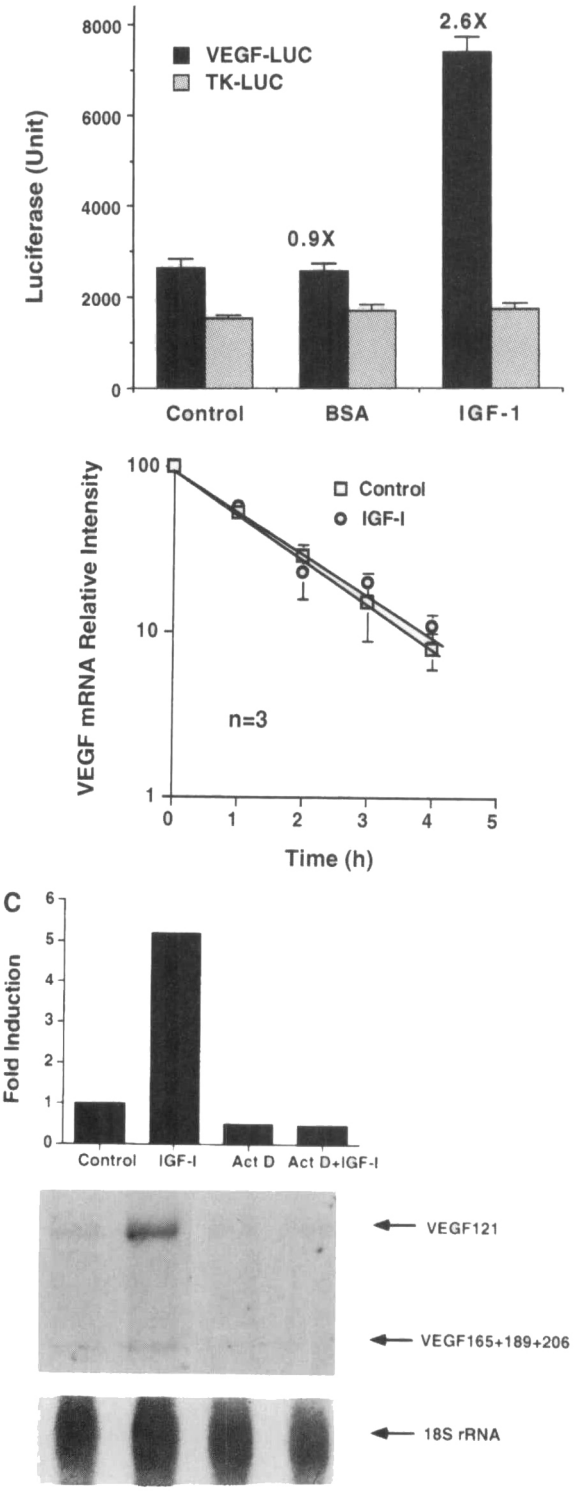

FIG. 8. IGF-I enhances VEGF promoter activity in RPE cells but not transcript stability. *A*: human RPE cells were transfected with luciferase expression vectors containing a 1.7-kb fragment of the rat VEGF gene 5′ flanking region (from approximately –1700 to +21) and exposed to IGF-I for 24 h. The data represent three experiments. Ther relative luciferase activity of each sample was normalized against the protein concentration. rVEGF= rat VEGF promoter-luciferase reporter construct in pXP2 plasmid; TK = 81 bp of the thymidine kinase promoter-luciferase construct in pXP2 plasmid. *B*: normalized densitometry readings of $VEGF_{121}$ RNA from RPE cells exposed to 50 nmol/l IGF-I and actinomycin D for the indicated times. The addition of IGF-I did not significantly enhance the $VEGF_{121}$ transcript half-life in the RPE cells. *C*: RNase protection assay showing that addition of actinomycin D to RPE cells 1 h before IGF-I treatment for 2 h abrogates the IGF-I–induced increases in VEGF expression.

the measurement of retinal VEGF gene expression in vivo *1*) in the retinas of normal eyes injected with IGF-I and *2*) following the inhibition of IGF-I in models of VEGF-driven retinal neovascularization. A related experiment has been reported. Transgenic mice overexpressing growth hormone had significantly less retinal neovascularization when administered a growth hormone antagonist (48). Further, the neovascularization in this model was reduced with anti-VEGF soluble receptors (15). However, the direct effect of growth hormone antagonists on retinal VEGF gene expression was not examined.

$VEGF_{121}$, a secreted form of VEGF, was identified as the predominant isoform induced by IGF-I. This is consistent with the in vivo situation where $VEGF_{121}$ is a major retinal isoform (25). Further, the increased conditioned media VEGF in response to IGF-I was associated with an increase in capillary endothelial cell proliferation, suggesting that IGF-induced VEGF is bioactive and freely soluble, as occurs in vivo. This is also consistent with freely soluble $VEGF_{121}$ being a major mediator of the angiogenic effect. Although IGF-I did not stimulate endothelial proliferation as reported by Grant et al. (9) in our control experiments, this negative result may be due to differences in the species and tissues from which the cells were isolated and/or the lack of required progression factors such as basic fibroblast growth factor.

IGF-I and hypoxia were additive in their ability to stimulate VEGF, and the increases were largely mediated through increases in VEGF transcription. IGF-I did not affect the RPE cell VEGF mRNA half-life after the inhibition of transcription with actinomycin D. In a separate report, it was recently found that IGF-I enhances VEGF gene expression in SaOS-2 osteoblast-like cells (49), but that the increases did not occur through increases in VEGF mRNA stability (49). In contrast, Warren et al. (50) showed that IGF-I–mediated increases in VEGF in colorectal cancer cells occurred, in part, through enhanced VEGF transcript stability. These data point out that the mechanism(s) of the IGF-I-induced VEGF increases may vary with cell type, transformation state, and other variables. Similar disparate observations have been made concerning hypoxia-induced VEGF mRNA increases (39,45,51–54).

In summary, our data support the hypothesis that IGF-I acts as an indirect ocular angiogenesis factor. Thus, IGF-I may serve, like VEGF, as a potential therapeutic target for the treatment of intraocular neovascularization.

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