Insulin-Induced Vascular Endothelial Growth Factor Expression in Retina

Ming Lu,1,2 Sibo Amano,1,2,5 Kazuaki Miyamoto,1,2 Rebecca Garland,1 Karen Keough,1 Wenyiing Qin,1,2 and Anthony P. Adamis1,2

PURPOSE. Clinical studies have demonstrated that intensive insulin therapy causes a transient worsening of retinopathy. The mechanisms underlying the initial insulin-induced deterioration of retinal status in patients with diabetes remain unknown. Vascular endothelial growth factor (VEGF) is known to be operative in the pathogenesis of diabetic retinopathy. The current study was conducted to characterize the effect of insulin on retinal VEGF gene expression in vitro and in vivo.

METHODS. The effect of insulin on VEGF expression in vivo was examined by in situ hybridization studies of rat retinal VEGF transcripts. To examine the mechanisms by which insulin regulates VEGF expression, human retinal pigment epithelial (RPE) cells were exposed to insulin, and VEGF mRNA levels were quantified with RNase protection assays (RPAs). Conditioned media from insulin-treated RPE cells were assayed for VEGF protein and capillary endothelial cell proliferation. The capacity of insulin to stimulate the VEGF promoter linked to a luciferase reporter gene was characterized in transient transfection assays.

RESULTS. Insulin increased VEGF mRNA levels in the ganglion, inner nuclear, and RPE cell layers. In vitro, insulin increased VEGF mRNA levels in human RPE cells and enhanced VEGF promoter activity without affecting transcript stability. Insulin treatment also increased VEGF protein levels in conditioned RPE cell media in a dose-dependent manner with a median effective concentration of 5 nM. The insulin-conditioned RPE cell media stimulated capillary endothelial cell proliferation, an effect that was completely blocked by anti-VEGF neutralizing antibody.


Clinical trials1–10 have investigated whether intensive insulin therapy decreases the frequency and severity of diabetic retinopathy. A consistent finding has been that intensive insulin therapy causes a transient worsening of retinopathy in the first 2 years. Continued intensive therapy, however, leads to a marked reduction in the risk of progression beyond the third year.1,10,11 The effect of insulin therapy on the progression of retinopathy in type 2 diabetes has also been examined.12–15 Short-term studies of 1 to 3 years have shown a marked increase in the risk of retinopathy progression with insulin therapy when compared with oral hypoglycemic therapy. The risk of progression was related to the degree of glycemic control by insulin.13,15 Although hyperglycemia was identified as a risk factor for the progression of retinopathy in all patients, change of treatment from oral drugs to insulin was associated with a twofold increased risk of retinopathy progression and a threefold increased risk of visual loss.15 Progression was seen at all levels of retinopathy.15,15 As in type 1 diabetes, long-term intensive treatment with insulin (more than 6 years) reduced the risk for the development and progression of retinopathy in patients with type 2 diabetes.16,17 The mechanisms underlying the initial deterioration of retinopathy in diabetes by intensive insulin therapy remain unknown.

Vascular endothelial growth factor (VEGF) refers to a family of angiogenic and permeability-enhancing peptides derived from alternatively spliced mRNAs.18,19 The isoforms differ in their affinity for heparin. Smaller isoforms (e.g., VEGF$_{121}$ and VEGF$_{165}$) are diffusible and can be found in conditioned media and biologic fluids. By comparison, larger isoforms (e.g., VEGF$_{165}$ and VEGF$_{206}$) are bound to heparin-like molecules in extracellular matrix and on the surface of cell membranes. VEGF is an endothelial-selective mitogen that binds to high-affinity receptors on retinal endothelial cells.20 In addition to stimulating neovascularization, VEGF, also known as vascular permeability factor, increases vascular leakage 50,000 times more potently than does histamine.18

Recent evidence has identified VEGF as a major mediator of retinal ischemia-associated neovascularization (for review, see Ref. 21). VEGF has also been causally linked to many of the other changes observed in diabetic retinopathy, including reti-
inal edema, ischemia, hemorrhage, and microaneurysm formation.\textsuperscript{22–29} Intraocular VEGF levels are increased in patients with diabetes\textsuperscript{27–29} and correlate with the development of neovascularization.\textsuperscript{27–30,32} Further, the specific inhibition of VEGF bioactivity prevents neovascularization in animal models.\textsuperscript{22,25} Retinal VEGF mRNA and protein levels are also increased in rats with background diabetic retinopathy and correlate with the breakdown of the blood-retinal barrier.\textsuperscript{53,52} Injection of VEGF into normal nonhuman primate eyes induces retinal edema, hemorrhage, intraretinal microvascular abnormalities (IRMA), ischemia, microaneurysms, and intraretinal neovascularization.\textsuperscript{24,25} Thus, it appears that VEGF may participate in the pathogenesis of both background and proliferative diabetic retinopathy. Because the initial worsening of retinopathy correlates with increased insulin doses, we hypothesized that insulin indirectly worsens diabetic retinopathy through increases in VEGF gene expression.

\section*{METHODS}

\section*{Intraocular Injections of Insulin}

The animal experiments were approved by the Animal Care Committee of the Children’s Hospital. All animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague–Dawley rats (Charles River, MA) weighing 200 to 250 g were anesthetized with 40 mg/kg ketamine and 10 mg/kg xylazine. The eyes were injected topically with 10\textsuperscript{-4} mol/L tetracaine hydrochloride eye drops. Insulin (500 nmol) in a total volume of 10\textsuperscript{-2} L sterile phosphate-buffered saline (PBS) was injected through the pars plana into the vitreous with a 30-gauge needle. The estimated volume of the rat vitreous was 10\textsuperscript{-3} L. Thus, the final concentration of insulin in the rat vitreous was 50 nmol. The contralateral control eyes received 10\textsuperscript{-4} mol/L PBS. The eyes were enucleated 2 hours later and placed in RNase-free paraformaldehyde at 25°C for the in situ hybridization study. The rats were killed with 75 mg/kg intraperitoneal pentobarbital and CO\textsubscript{2} gavage. All injections were performed using a surgical microscope. Any eyes that exhibited damage to the lens or retina were discarded and not used for analyses.

\section*{In Situ Hybridization}

The in situ hybridization protocol and the preparation of \textsuperscript{35}S-labeled VEGF riboprobes have been previously described.\textsuperscript{53} Briefly, the antisense probe was prepared by cutting with EcoRII and transcribed with T7, which generated a probe of 650 nucleotides (nt). The antisense probe hybridizes with a region of VEGF mRNA coding sequence common to all known splice variants of VEGF. Deparaffinized sections of the rat retina were hydrated, treated to digest the tissue so that mRNA transcripts were more accessible to hydration,\textsuperscript{33} and hybridized with the labeled probes overnight. The hybridized sections were washed, dried, dipped in photographic emulsion, and stained for microscopic examination.

\section*{RNA Isolation and RNase Protection Assay}

Total RNA was isolated from cultured cells by the method of Chomczynski and Sacchi.\textsuperscript{54} The VEGF riboprobe was produced by subcloning the coding sequence of the human VEGF\textsubscript{121} cDNA into the Smal site of the Bluescript vector (Stratagene, La Jolla, CA). Transcription by T7 RNA polymerase after linearization by NcoI resulted in a probe of 496 nt. This probe protects a 416-nt fragment of VEGF\textsubscript{121} and a 338-nt fragment of VEGF\textsubscript{165}, VEGF\textsubscript{189}, and VEGF\textsubscript{206}. The human β-actin probe was produced by transcribing the human β-actin cDNA template pRI-β-actin-h (Ambion, Austin, TX) by using T3 RNA polymerase and was labeled 1/20th as radioactive as the VEGF probe, because of the relative abundance of β-actin mRNA compared with the VEGF mRNA. Full-length protection of this probe results in an 80-nt fragment. The assay was performed as previously described.\textsuperscript{55} Ten micrograms of total cellular RNA was hybridized with \textsuperscript{32}P-labeled antisense VEGF (200,000 cpm) and β-actin (20,000 cpm) riboprophes overnight at 42°C in 30\textsuperscript{-1} M hybridization buffer. Hybridized RNA was digested with nuclease P1 (20 \textmu M) and RNase T1 (2 \textmu M) for 1 hour at 25°C in 300 \textmu L digestion buffer. Digestions were terminated by addition of 20 \textmu M 10% sodium dodecyl sulfate (SDS) and 50 \textmu g proteinase K for 15 minutes at 37°C. After phenol-chloroform extraction and ethanol precipitation, the protected fragments were resolved on 6% polyacrylamide 7-M urea gels and visualized with autoradiography. Densitometry was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

\section*{Cell Culture}

Human retinal pigment epithelial (RPE) cells were immortalized through the stable integration of a simian virus (SV)-40 large T-antigen expression cassette and cultured on noncoated plates, as previously described.\textsuperscript{56} The cells contain some pigment, grow in a monolayer, and increase VEGF mRNA during hypoxia in a manner identical with the parent cell line.\textsuperscript{56} The RPE cell line was maintained in Dulbecco’s modified essential medium (DMEM; Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT) and 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were plated into six-well plastic dishes and used for experiments when they reached 80% to 100% confluence. Fresh serum-free media were placed on the cells 12 hours before experiments. All reagents were added directly to the wells in a volume of 100 \textmu L DMEM. When actinomycin D was used, it was added to the medium 1 hour before insulin treatment to the final concentration of 5 \textmu g/ml. Each condition was prepared in triplicate, and the experiments were performed at least three times with reproducible results. Representative experiments are shown in the figures.

\section*{Conditioned-Media VEGF Measurements}

Conditioned-media VEGF levels were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Cells were trypsinized and counted on a Coulter counter at the end of the experiment to assure there was no difference among the different treatment groups. VEGF protein levels were normalized to cell counts.

\section*{Endothelial Cell Proliferation Assays}

Bovine capillary endothelial cells (BCE) were plated in 96-well plates with 750 cells/well in DMEM with 10% fetal calf serum for 24 hours. The medium was changed to DMEM with 2% fetal calf serum and 4 \textmu L conditioned media were added for 72 hours. A previously characterized anti-VEGF antibody\textsuperscript{22} or a
Insulin-Induced Retinal Cell VEGF Expression

Figure 1. VEGF in situ hybridization of rat retina 2 hours after injection of insulin or vehicle solution. Rat retina VEGF mRNA levels were analyzed with a mouse VEGF antisense probe in PBS-injected (A) and insulin-injected (B) eyes. The final concentration of insulin in the rat vitreous was 50 nM, based on a volume for the rat vitreous of 100 μl. The retinal cross-section shows the ganglion cell layer (1), the inner nuclear layer (2), the outer nuclear layer (3), the proximal photoreceptor layer (4), the RPE (5), and the choroid (6).

The cells were washed with PBS, fixed with 100% ethanol for 5 minutes, washed with borate buffer (0.1 M; pH 8.5), and stained with methylene blue (1% in borate buffer) for 10 minutes and rinsed with tap water. After 30 minutes of color extraction with 0.1 N HCl, the cell density was quantified with an ELISA reader at 600 nm.

Statistics
Significance testing was performed using the paired Student’s t-test. P < 0.05 was deemed significant.

Results
To examine whether insulin increases VEGF mRNA in vivo, 10 μl insulin (final concentration 50 nM) was injected into the vitreous of rats. We used 50 nM for this study because this concentration of insulin gave a maximal response (see Figs. 3, 5). Using in situ hybridization, VEGF mRNA levels were seen to be elevated in the insulin-injected eyes compared with contralateral eyes receiving 10 μl vehicle alone (PBS). VEGF mRNA levels were increased in the ganglion, inner nuclear, and RPE cell layers (Fig. 1). The insulin-induced increases in VEGF mRNA levels were characterized in human RPE cells in vitro. The RNase protection assay was performed using a riboprobe corresponding to VEGF121 (416-nt protected fragment). This probe also detects three other isoforms of VEGF as a single protected fragment (338 nt). At 2 hours there was an increase of 5.2 ± 0.4-fold (n = 3) in the level of VEGF121 mRNA in 50 nM insulin-treated RPE cells (Fig. 2). The band corresponding to VEGF165, VEGF189, and VEGF206 was also significantly increased (4.2 ± 0.3-fold; n = 3).

The ability of insulin to stimulate the release of VEGF protein into the conditioned media of RPE cells was tested. Human RPE cells exposed to insulin for 24 hours increased VEGF protein levels in conditioned media in a dose-dependent manner, with a median effective dose (EC50) of 5 nM (Fig. 3). The bioactivity of the RPE-conditioned media was determined using BCE cell proliferation assays. Conditioned media from 50 nM insulin-treated RPE cells increased BCE cell density 1.65 ± 0.23-fold, compared with the untreated RPE cell media (n = 3; P < 0.05; Fig. 4), an effect that was completely blocked by the anti-VEGF neutralizing antibody, but not the isotype control anti-gp120 antibody (Fig. 4).

To examine whether the increase in VEGF mRNA levels by insulin was due to an enhancement of VEGF transcription, the effect of insulin on VEGF promoter activity was examined in transient transfection assays. Insulin (5–50 nM) stimulated VEGF promoter activity in a dose-dependent manner (EC50, 9 nM; n = 3), with the maximal effect at 50 nM insulin (2.8 ± 0.2-fold; n = 3; P < 0.01). In the control, 50 nM insulin did not affect the activity of the 81-bp thymidine kinase promoter (1.0 ± 0.1-fold; n = 3; Fig. 5).

The RNase protection assay showed that the addition of actinomycin D to RPE cells 1 hour before insulin treatment abrogated the insulin-induced increases in VEGF expression (Fig. 6A), suggesting that insulin increases VEGF expression mainly through enhanced transcription. To examine whether insulin also alters VEGF transcript stability, the RNase protection assay was performed to measure the half-life of VEGF transcripts in RPE cells after insulin treatment. The addition of 50 nM insulin did not significantly enhance the VEGF121 transcript half-life in RPE cells (1.10 ± 0.13 hours versus 0.98 ± 0.09 hours; n = 3; P > 0.05; Fig. 6B).

Discussion
In this study, we found that insulin increased VEGF mRNA levels in the ganglion, inner nuclear, and RPE cell layers of the rat retina. In vitro, insulin increased VEGF mRNA and secreted protein levels in human RPE cells. The conditioned media of insulin-treated retinal cells stimulated capillary endothelial cell proliferation, a response that was blocked with an anti-VEGF mAb.

C Ins

VEGF121

VEGF165+189+206

Actin

Figure 2. VEGF isoforms were coordinately increased by insulin. RNase protection assay 2 hours after insulin treatment of RPE cells showed protected fragments of expected size. The human β-actin mRNA was used to normalize the samples for quantification of isoform expression. C, control; Ins, 50 nM insulin.
antibody. The insulin-induced increases in VEGF expression were inhibited by actinomycin D, and insulin enhanced VEGF promoter activity without altering VEGF mRNA stability. Insulin also increased the VEGF protein levels in the conditioned media of human RPE cells in a dose-dependent fashion (EC$_{50}$, 5 nM). Serum concentrations of 5 nM are often attained in vivo and may be sufficient to stimulate retinal VEGF expression in vivo, especially in patients with preexisting diabetic retinopathy and blood-retinal barrier compromise. The early worsening of diabetic retinopathy with intensive insulin therapy mainly occurs in patients with preexisting retinopathy.$^1$ The 50 nM insulin concentration was chosen for some experiments, because it maximally increased VEGF promoter activity and VEGF protein secretion in the human RPE cells. Taken together, these data indicate that insulin can enhance VEGF gene expression in vivo and in vitro. This result is consistent with our hypothesis that intensified insulin therapy may worsen diabetic retinopathy partially through increases in retinal VEGF gene expression.

Hypoglycemia induced by intensive insulin therapy may also stimulate VEGF expression.$^{38}$ Continued insulin therapy, however, may reduce the risk of subsequent progression by normalizing blood glucose and decreasing the glucose-dependent production of advanced glycation end products (AGEs). Both glucose and AGEs are stimuli for VEGF expression.$^{35,39-41}$ Hyperglycemia can also activate protein kinase C$^{42}$ and increase intracellular sorbitol production,$^{43}$ pathways known to stimulate VEGF expression.$^{44-46}$ The decreases in glucose, sorbitol, AGE, and PKC activity by long-term intensive insulin therapy may compensate for the short-term deleterious effects of insulin on VEGF production.

Several theories have been proposed to explain the insulin paradox. It was once argued that it was due more to severe disease at an earlier stage than to the treatment or the rapidity of decrease in blood glucose. The consistent and documented finding from randomized clinical trials of transient worsening of retinopathy with insulin therapy$^{1,10}$ suggests this hypothesis is unlikely to be true. Another hypothesis is that insulin decreases retinal blood flow, leading to retinal hypoxia and pre-capillary vasodilatation and thus to increased permeability and edema.$^{13}$ However, there are no direct data on how insulin affects retinal vascular hemodynamics. Finally, it was suggested that insulin is mitogenic, atherogenic, and thrombogenic to the retinal vasculature, causing the transient deterioration of retinopathy with insulin therapy.$^{14}$ If so, the long-term beneficial
glucose reduction cause pericyte apoptosis.\textsuperscript{47} Pericyte loss due to hyperglycemia–hypoglycemia fluctuation may lead to the transient worsening of retinopathy at the beginning of intensive insulin therapy. To prove a role for VEGF or pericyte loss in the initial deterioration of retinopathy after insulin therapy, an examination of the effect of systemic insulin therapy on retinal VEGF expression, pericyte loss, and other aspects of retinal disease is required in diabetic animal models. These studies are under way.

The molecular mechanisms underlying the effect of insulin on VEGF expression have been further uncovered by a recent publication.\textsuperscript{46} It was found that insulin shares with hypoxia the ability to induce the hypoxia-inducible factor-1α (HIF-1α)-aryl hydrocarbon nuclear translocator (ARNT) basic helix–loop–helix–PAS transcription complex. Insulin induces genes containing the hypoxia response element through formation of the HIF-1α/ARNT complex. The observation that insulin stimulates VEGF expression is also supported by the fact that insulin-like growth factor-1 (IGF-1) enhances VEGF expression in a variety of tissues including retinal cells.\textsuperscript{37,49,50} Insulin and IGF-1 share many signaling components, and the induction of VEGF expression is no exception. It is unlikely that the observed response in RPE cells and rat retina is mediated through the IGF-1 receptor. The EC\textsubscript{50} for insulin-induced VEGF protein secretion in RPE is 5 nM. This is comparable to the EC\textsubscript{50} for IGF-1, which is 7 nM.\textsuperscript{57} Because insulin binds to the IGF-1 receptor with 100-fold lower affinity than IGF-1, the comparable EC\textsubscript{50} values for insulin (5 nM) and IGF-1 (7 nM) virtually exclude the possibility of cross-binding to the IGF-1 receptor. The EC\textsubscript{50} of insulin on VEGF promoter activity (9 nM) is also comparable to that of IGF-1 on RPE cells (6 nM, data not shown). Therefore, the observed response in RPE cells and rat retina is probably mediated by the insulin receptor. The observation that insulin enhances VEGF expression may lead to future avoidance of the transient worsening of retinopathy in patients receiving insulin therapy by inhibiting VEGF.

References

9. Moskalets E, Galstyan G, Starostina E, Antsiferov M, Chantelau E. Association of blindness to intensification of glycemic control in...