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Research Article

Advanced glycation end products (AGEs) are linked with the development of diabetic retinopathy; however, the pathogenic mechanisms are poorly defined. Vascular endothelial growth factor (VEGF) levels are increased in ischemic and nonischemic diabetic retina, and VEGF is required for the development of retinal and iris neovascularization. Moreover, VEGF alone can induce much of the concomitant pathology of diabetic retinopathy. In this study, we found that AGEs increased VEGF mRNA levels in the ganglion, inner nuclear, and retinal pigment epithelial (RPE) cell layers of the rat retina. In vitro, AGEs increased VEGF mRNA and secreted protein in human RPE and bovine vascular smooth muscle cells. The AGE-induced increases in VEGF expression were dose- and time-dependent, inhibited by antioxidants, and additive with hypoxia. Use of an anti-VEGF antibody blocked the capillary endothelial cell proliferation induced by the conditioned media of AGE-treated cells. AGEs may participate in the pathogenesis of diabetic retinopathy through their ability to increase retinal VEGF gene expression.

Advanced Glycation End Products Increase Retinal Vascular Endothelial Growth Factor Expression

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Abstract

Advanced glycation end products (AGEs) are linked with the development of diabetic retinopathy; however, the pathogenic mechanisms are poorly defined. Vascular endothelial growth factor (VEGF) levels are increased in ischemic and nonischemic diabetic retina, and VEGF is required for the development of retinal and iris neovascularization. Moreover, VEGF alone can induce much of the concomitant pathology of diabetic retinopathy. In this study, we found that AGEs increased VEGF mRNA levels in the ganglion, inner nuclear, and retinal pigment epithelial (RPE) cell layers of the rat retina. In vitro, AGEs increased VEGF mRNA and secreted protein in human RPE and bovine vascular smooth muscle cells. The AGE-induced increases in VEGF expression were dose- and time-dependent, inhibited by antioxidants, and additive with hypoxia. Use of an anti-VEGF antibody blocked the capillary endothelial cell proliferation induced by the conditioned media of AGE-treated cells. AGEs may participate in the pathogenesis of diabetic retinopathy through their ability to increase retinal VEGF gene expression. (*J. Clin. Invest.* 1998. 101:1219–1224.) Key words: advanced glycation end products • diabetic retinopathy • vascular endothelial growth factor • gene expression • retina

Introduction

Retinal edema, hemorrhage, ischemia, microaneurysms, and neovascularization characterize diabetic retinopathy. Vascular endothelial growth factor (VEGF)[1] has been causally linked to many of these changes (1–3). Intraocular VEGF levels are increased in diabetic patients (4–6) and are correlated with the development of edema and neovascularization (4, 5, 7, 8). Further, the specific inhibition of VEGF prevents ocular neovascularization in animal models (1, 2), and the injection of VEGF into normal nonhuman primate eyes induces retinal edema, hemorrhage, ischemia, microaneurysms, and intraretinal neovascularization (3).

The Diabetes Control and Complications Trial identified hyperglycemia as a major risk factor for the development and progression of retinopathy (9). Considerable evidence now supports a causal role for advanced glycation end products (AGEs) in the development of this complication. AGEs represent an integrated measure of glucose exposure over time (10), are increased in diabetic retina, and correlate with the onset and severity of retinopathy (11). Specific high affinity receptors bind AGEs (12) and lead to the downstream production of reactive oxygen intermediates (ROI) (13). The latter are correlated with retinopathy (14) and increase retinal VEGF expression (15). Exogenously administered AGEs induce vascular hyperpermeability (16) in normal animals. The inhibition of endogenous AGEs in diabetic animals prevents vascular leakage (17) and the development of acellular capillaries and microaneurysms in retina (18). Based on these data, we examined the role of AGEs in the induction of retinal VEGF gene expression in vivo and in vitro.

Methods

Preparation of AGEs. BSA (Fraction V) (Sigma Chemical Co., St. Louis, MO) was glycated by incubation with glucose-6-phosphate (Sigma Chemical Co.) (0.5 M) in PBS for 6 wk at 37°C, as described previously (19). Dialyzed glycated protein was characterized based on fluorescence at 450 nm upon excitation at 390 nm using a fluorescence spectrometer (model LS-3B; Perkin-Elmer Corp., Norwalk, CT). Endotoxin content in each sample was measured by the Limulus amebocyte lysate assay (E-Toxate; Sigma Chemical Co.) and found to be below detectable levels (< 0.2 ng/ml). Control, nonglycated albumin consisted of the same initial preparations of albumin incubated at 37°C in the same manner, except that no sugar was present. Two different preparations of AGEs yielded similar results.

M. Lu and M. Kuroki contributed equally to this work.

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1. *Abbreviations used in this paper:* AGE, advanced glycation end product; BCE, bovine capillary endothelial; DMTU, *N*,*N*′-dimethylthiourea; NAC, *N*-acetylcysteine; nt, nucleotide(s); ROI, reactive oxygen intermediate(s); RPE, retinal pigment epithelial; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

Intraocular injections of AGEs. The rabbit and rat experiments were approved by the Animal Care Committees of the Massachusetts Eye and Ear Infirmary and Children's Hospital, respectively. All animal experiments conformed to the Association for Research in Vision and Ophthalmology guidelines for animal experimentation. Male Sprague-Dawley rats weighing 200–250 g were anesthetized with 40 mg/kg ketamine and 10 mg/kg xylazine. New Zealand albino rabbits weighing 1.2–1.8 kg were anesthetized with an intramuscular injection of 50 mg/kg ketamine and 10 mg/kg xylazine. Both species received topical instillation of tetracaine hydrochloride. For the rats, AGE-BSA (10 μg) in a total volume of 10 μl PBS was injected through the pars plana into the vitreous with a 30-gauge needle. The contralateral control eyes received 10-μl injections of nonglycated BSA (10 μg). The eyes were enucleated 4 h later and placed in RNase-free paraformaldehyde at 25°C for the in situ hybridization assays. The rats were killed with 75 mg/kg intraperitoneal pentobarbital and cervical dislocation. All injections were done under direct observation using a surgical microscope. Any eyes that had damage to the lens or retina were discarded and not used for analyses. For the rabbits, 100 μg AGEs were injected into the vitreous in a total volume of 100 μl PBS. 4 h later, the eyes were enucleated and bisected at the equator. In preparation for Northern assay, the rabbit retinas were gently dissected free and cut at the disc. The tissue was placed in a 50-ml conical plastic tube, snap-frozen in liquid nitrogen, and stored at −80°C. The animals were killed with 50 mg/kg intravenous pentobarbital. The retinas were homogenized in 2 ml RNAzol (Biotecx Laboratories, Houston, TX) at 25°C and prepared for Northern blotting.

In situ hybridization. The in situ hybridization protocol and the preparation of ^{35}S-labeled VEGF riboprobes have been described previously (20). Briefly, the sense probe was prepared from a mouse plasmid DNA construct by cutting with BamHI and transcribed with T3, generating a riboprobe with a length of 650 nucleotides (nt). The antisense probe was prepared by cutting with EcoRI and transcribed with T7, which generated a probe of 650 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 (20), and then hybridized with the labeled probes overnight. The hybridized sections were washed, dried, dipped in photographic emulsion, and stained for microscopic examination.

RNA isolation and Northern blot analysis. Total RNA was isolated from cultured cells and rabbit retinas by the method of Chomczynski and Sacchi (21). RNA (15 μg) was electrophoresed through a 1% agarose/formaldehyde gel and transferred to nylon filters (GeneScreen Plus; New England Nuclear, Boston, MA). The filters were prehybridized in buffer containing 50% deionized formamide, 5× SSPE (sodium chloride sodium phosphate), 5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml) and hybridized at 42°C in fresh buffer without salmon sperm DNA. The hybridization buffer contained either a 520-bp NcoI/BglII 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′ untranslated region of the human β-actin cDNA. The cDNA probes were labeled with a random-primed DNA labeling kit using [α-^{32}P]deoxy-CTP (Boehringer Mannheim Biochemicals, Indianapolis, IN). Filters were washed twice in 2–0.5× SSPE, 0.1% SDS 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 X-Omat AR x-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C for 12–72 h. Densitometry was performed on all blots and normalized to the corresponding actin signal for each lane using an IS-1000 digital imaging system with version 1.97 software (Alpha Innotech Corp., Torrance, CA).

RNase protection assay. The VEGF riboprobe was produced by subcloning the coding sequence of the human VEGF121 cDNA into the SmaI site of the Bluescript vector (Stratagene Inc., 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 VEGF121 and a 338-nt fragment of VEGF165, VEGF189, and VEGF206. The human β-actin probe was produced by transcribing the human β-actin cDNA template pTRI-β-actin-h (Ambion Inc., 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 described previously (22). 10 μg of total cellular RNA was hybridized with ^{32}P-labeled antisense VEGF and actin riboprobes (200,000 cpm of each) 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 the addition of 20 μl of 10% SDS 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 M urea gels and visualized with autoradiography. Densitometry was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell culture. Human retinal pigment epithelial (RPE) cells were immortalized through the stable integration of a cytomegalovirus-driven SV-40 large T-antigen expression cassette and cultured on noncoated plates as described previously (23). The cells contain pigment, grow in a monolayer, and increase VEGF mRNA during hypoxia in a manner identical to the parent cell line (23). Previously characterized bovine vascular smooth muscle cells (SMCs) derived from bovine aorta explants (24) were obtained from Dr. Patricia D'Amore (Children's Hospital, Harvard Medical School). The RPE and bovine smooth muscle cell lines were maintained until experimentation in DME (Sigma Chemical Co.) containing 10% heat-inactivated FCS (Hyclone Labs, Logan, UT) and 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine. Cells were plated into six-well plastic dishes and used for experiments when they reached 80–100% confluence. Fresh serum-free media were placed on the cells 12 h before experiments. All reagents were added directly to the wells in a volume of 100 μl DME. For the experiments in hypoxia, cells were placed in a sealed chamber (Billups Rothenberg, Del Mar, CA) 12 h after changing media and exposed to continuous hypoxia (5% CO_2/3% O_2/92% N_2) or normoxia (5% CO_2/21% O_2/74% N_2) for 8 h. For the antioxidant experiments, *N*,*N*′-dimethylthiourea (DMTU) and *N*-acetylcysteine (NAC) (Sigma Chemical Co.) were added directly to the wells in a volume of 100 μl DME to achieve a 10 mM final concentration. Each condition was prepared in triplicate, and the experiments were carried out at least three times with reproducible results. Representative experiments are shown in the figures.

Conditioned media VEGF measurements. Conditioned media VEGF levels were determined using a sandwich ELISA assay according to the manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN). Cells were trypsinized and counted on a counter (Coulter Corp., Hialeah, FL) 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.

Endothelial cell proliferation and DNA synthesis assays. For the proliferation assays, bovine capillary endothelial (BCE) cells were plated in 96-well plates with 750 cells/well in DME with 10% FCS for 24 h. The medium was changed to 2% FCS, and 4 μl of conditioned media was added for 72 h. The cells were washed with PBS, fixed with 100% ethanol for 5 min, then washed with borate buffer (0.1 M, pH 8.5), stained with methylene blue (1% in borate buffer) for 10 min, and rinsed with tap water. After 30-min color extraction with 0.1 N HCl, the cell density was quantified with an ELISA reader at 600 nm. For the DNA synthesis assays, conditioned media were collected at the indicated times and incubated with heparin sepharose for 16 h. The heparin sepharose was washed twice with 0.5 ml of 0.5 M NaCl and eluted with 50 μl of 1.5 M NaCl. BCE cells were seeded in 96-well plates (400 cells/well/200 μl) and grown in DME supplemented with 2% bovine calf serum. 3-μl samples of concentrated conditioned media were added to the cells. A previously characterized anti-VEGF

mAb (1) was added in some wells, and a previously characterized anti-gp120 mAb (1) was added to other wells as a control. After 48 h, 10 μl (0.2 μCi) of [^{3}H]thymidine (specific activity 27 mCi/mg) was added for 6 h, and [^{3}H]thymidine incorporation into DNA was determined by liquid scintillation counting. Values were normalized to cell number.

Statistics. Significance testing was done using the paired Student's *t* test. *P* values < 0.05 were deemed significant.

Results

To examine whether AGEs increase retinal VEGF mRNA levels in vivo, AGEs were injected into the vitreous of rat and rabbit eyes, and in situ hybridization studies and Northern blot analyses were completed. 4 h after the injection of nonglycated BSA or AGE-BSA (final vitreous concentration 100 μg/ml), rat retinal VEGF mRNA levels were increased in the ganglion, inner nuclear, proximal photoreceptor, RPE, and choroidal layers of the AGE-injected rat eyes (Fig. 1, *A–C*). Northern blot analyses of rabbit neurosensory retina identified a 4.8-fold increase in VEGF mRNA levels in the AGE-injected eyes (Fig. 1 *D*).

Human RPE and bovine smooth muscle cell lines were used to define the mechanisms by which AGEs stimulate VEGF gene expression. AGEs (100 μg/ml) increased RPE VEGF mRNA to peak levels within 4 h, and the increases were sustained for at least 20 h (data not shown). VEGF mRNA levels were increased by as little as 1 μg/ml AGE, peaked with 100 μg/ml, and had an ED_{50} of 35 μg/ml. AGE levels have been reported to be between 25–80 μg/ml in the serum of human diabetic patients (12). A recent report demonstrated similar in vitro AGE-induced VEGF increases, which could be blocked with an anti-AGE antibody (25).

Up to four VEGF isoforms are produced by a single gene (26, 27). RNase protection assays showed that VEGF121 increased 4.9±0.4-fold after exposure to 100 μg/ml AGEs for 8 h and represented 54% of the VEGF mRNA pool (Fig. 2 *A*, $n = 4$, $P < 0.01$). The band representing VEGF165, 189, and 206 was increased to a similar degree (4.2±0.3-fold, $n = 4$, $P < 0.01$). Since ischemic hypoxia is a pathophysiologically relevant stimulus for retinal VEGF gene expression (1), the effect of AGEs and hypoxia on VEGF gene expression was examined. Human RPE cells were exposed to hypoxia (3% O_2) or normoxia (21% O_2) for 6 h, with or without 100 μg/ml AGEs. Hypoxia and AGEs alone increased VEGF mRNA 4.5- and 5.0-fold, respectively. The combination of hypoxia and AGEs increased VEGF mRNA levels 10.8-fold (Fig. 2 *B*). Since it has been shown that AGE-induced increases in gene expression can occur through ROI (13), the ability of AGEs to stimulate VEGF gene expression by similar mechanisms was examined. The antioxidants DMTU and NAC blocked completely the AGE-induced increases in VEGF mRNA in bovine SMCs and RPE cells (Fig. 2, *C* and *D*), implicating ROI as mediators of the VEGF response.

Finally, the AGE-associated increases in VEGF mRNA levels were translated into secreted bioactive VEGF protein. After 24 h of incubation, AGEs (100 μg/ml) increased VEGF protein levels in the conditioned media of RPE cells by 1.7±0.1-fold ($n = 6$, $P < 0.001$) (Fig. 3 *A*). The increases occurred in a time-dependent manner from 8 to 24 h (data not shown). The bioactivity of the secreted VEGF protein was examined with BCE DNA synthesis assays. Conditioned media from AGE-treated RPE cells increased significantly capillary endothelial DNA synthesis 2.0±0.2-fold ($n = 3$, $P < 0.01$) (Fig. 3 *B*). The identity of the endothelial cell mitogen in the conditioned media was examined using a previously characterized

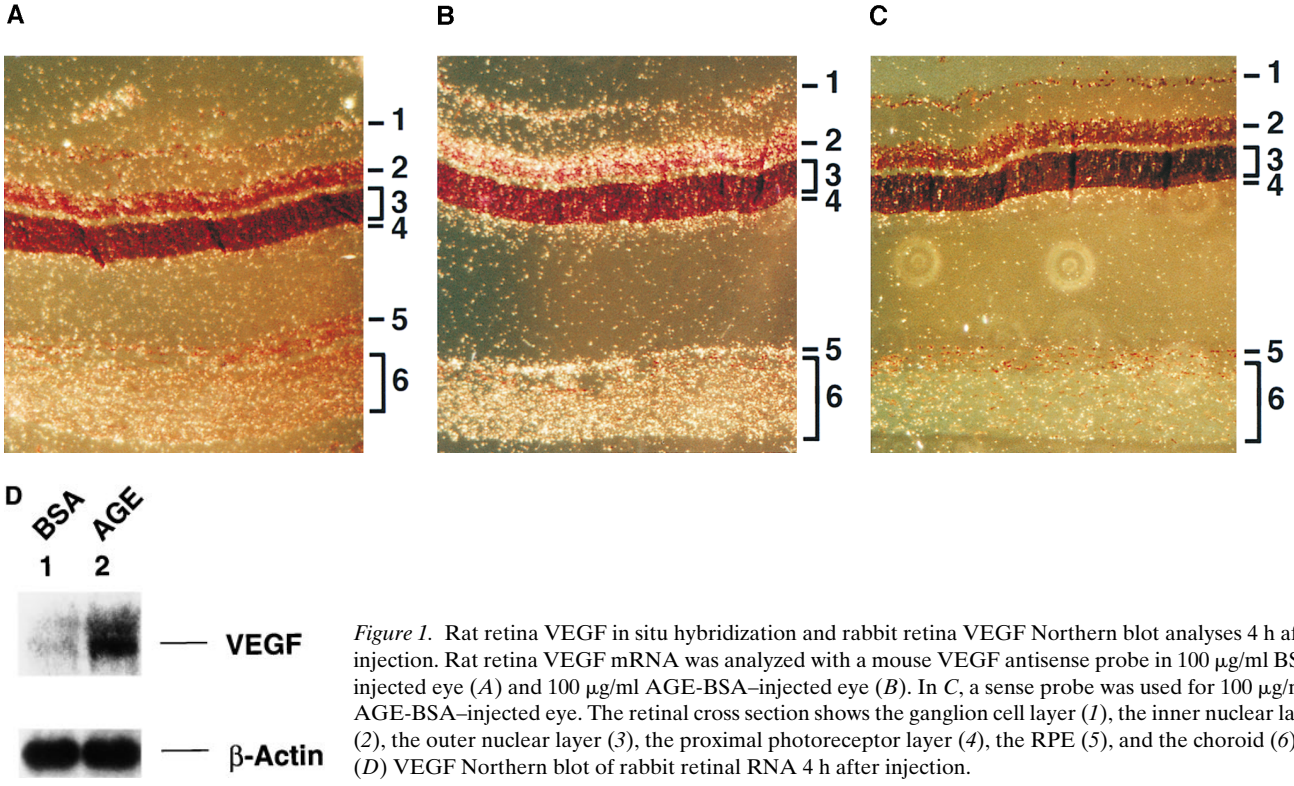

Figure 1. Rat retina VEGF in situ hybridization and rabbit retina VEGF Northern blot analyses 4 h after injection. Rat retina VEGF mRNA was analyzed with a mouse VEGF antisense probe in 100 μg/ml BSA–injected eye (*A*) and 100 μg/ml AGE-BSA–injected eye (*B*). In *C*, a sense probe was used for 100 μg/ml AGE-BSA–injected eye. 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*). (*D*) VEGF Northern blot of rabbit retinal RNA 4 h after injection.

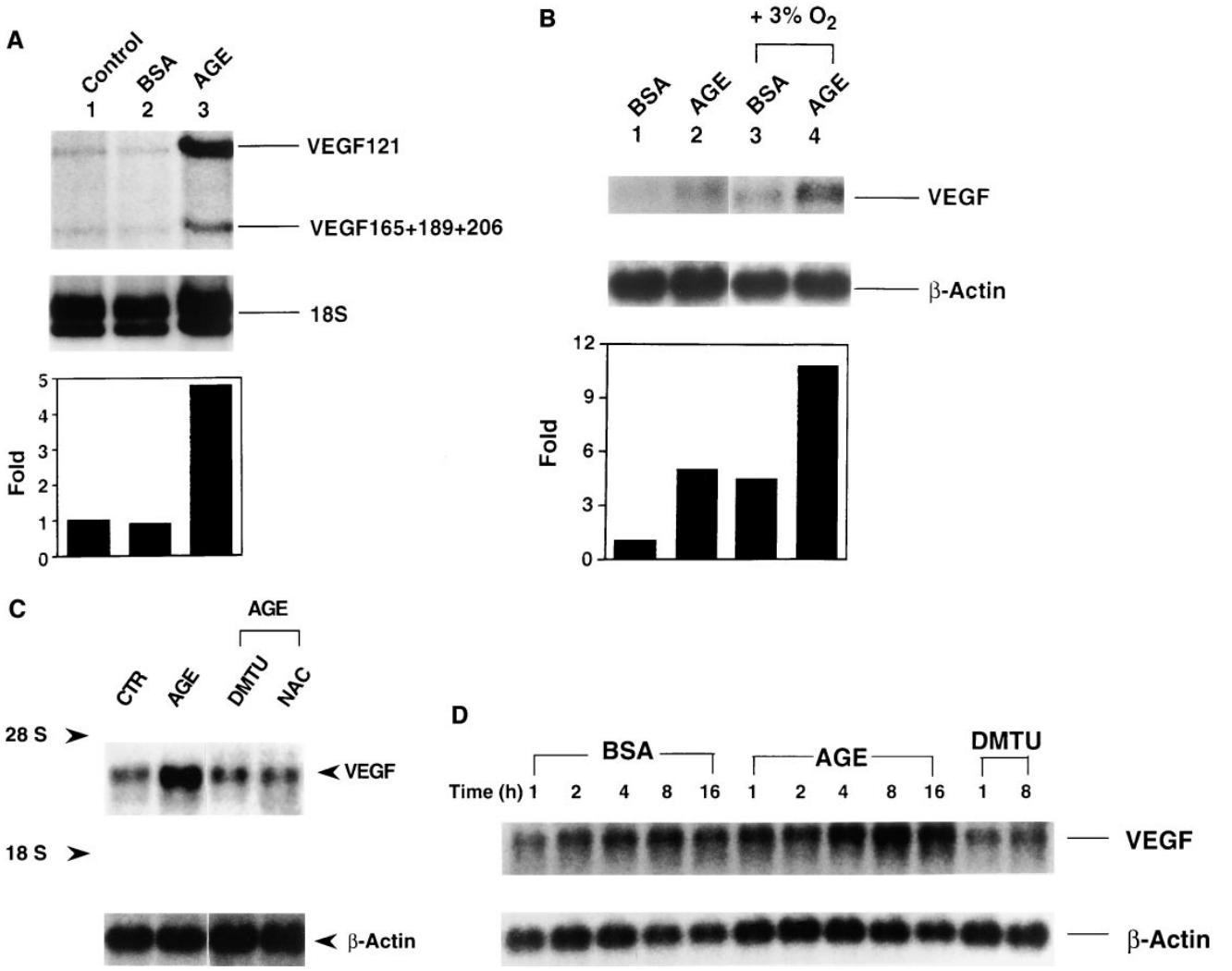

Figure 2. RNase protection (*A*) and Northern blot analyses (*B–D*) for VEGF mRNA in human RPE cells (*A*, *B*, and *D*) and bovine SMCs (*C*) after exposure to 100 μg/ml BSA (labeled *CTR* in *C*) or 100 μg/ml AGE for 8 h.

anti-VEGF neutralizing mAb (Fig. 3 *C*) (1). Conditioned media from AGE-treated RPE cells enhanced significantly the proliferation of BCE cells ($n = 3$, $P < 0.05$), and the increases were blocked completely with the anti-VEGF neutralizing mAb ($n = 3$, $P < 0.05$).

Discussion

It is well established that hyperglycemia is a major risk factor for the development and progression of retinopathy (9). A central question in the understanding of diabetic retinopathy has been how hyperglycemia can produce the vascular pathology of diabetic retinopathy.

This study demonstrates that AGEs can stimulate the expression of VEGF in rat and rabbit retina. The ganglion and inner nuclear layers, cell layers that express VEGF in diabetic retinopathy (28), also express VEGF after acute exposure to exogenous AGEs. In vitro, the increases in VEGF expression are time- and dose-dependent, with pathophysiologically relevant AGE levels triggering the response. The increases are also additive with hypoxia, another well-characterized and operative stimulus for VEGF expression in diabetic retinopathy (29–31). Antioxidants block the AGE-induced VEGF increases, implicating ROI as the downstream mediators of the response. These data are relevant in light of the known ROI increases in diabetic retina and vitreous (14, 32) and the ability of ROI to induce VEGF expression in retina (15). Finally, the AGE-induced increases in endothelial cell proliferation are due specifically to VEGF. Use of an anti-VEGF neutralizing antibody blocked completely the increased endothelial cell proliferation induced by the conditioned media of AGE-treated retinal cells.

Taken together with our previous observation that VEGF can induce the microvascular pathology of diabetic retinopathy (3), these data provide a potential mechanistic link between hyperglycemia, VEGF, and diabetic retinopathy. Such a link is further supported by two recent reports showing that antioxidants can reduce retinopathy (33), and that aminoguanidine can suppress retinal VEGF (34). However, not all data support our hypothesis. Direct proof can be obtained only

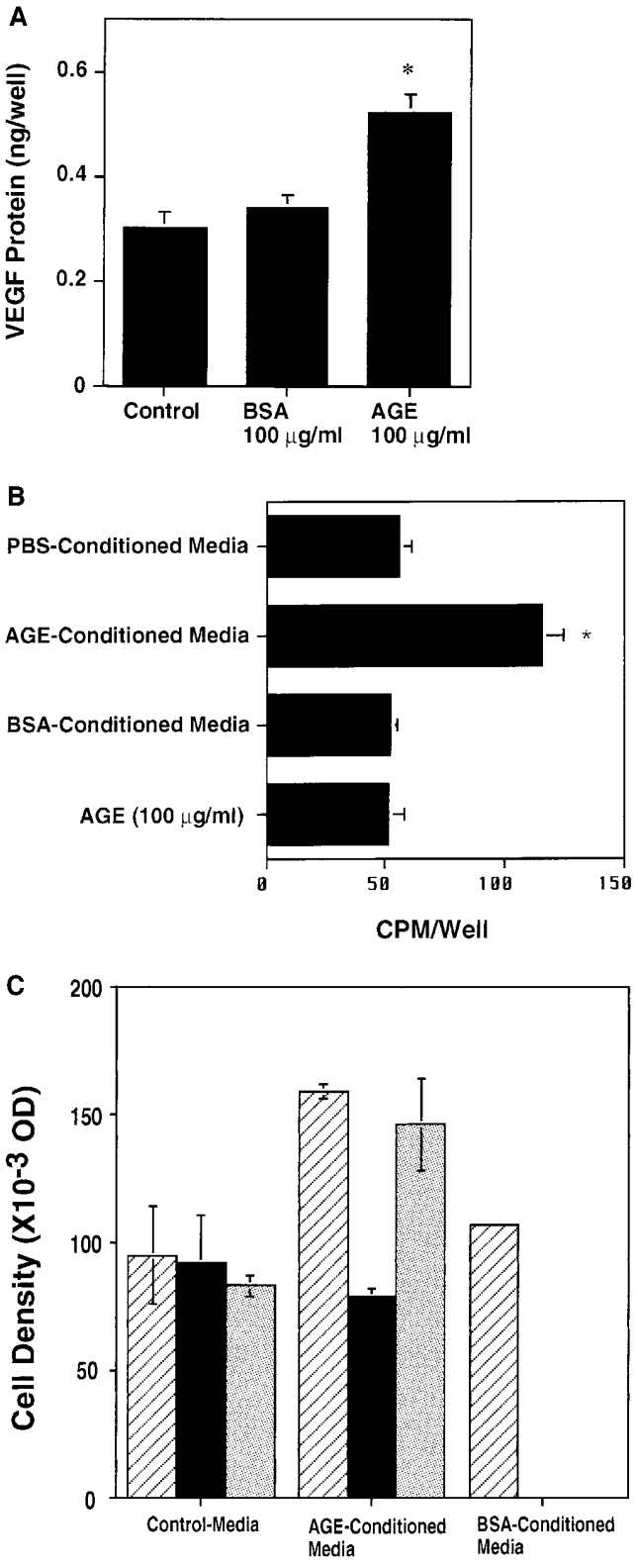

Figure 3. AGEs increase bioactive VEGF protein levels in conditioned media of RPE cells. VEGF protein levels of RPE cell–conditioned media were quantified by ELISA after a 24-h incubation with BSA or AGE (both at 100 μg/ml) (*A*). Values were normalized to the protein concentrations of each well. Each value represents mean±SD for a triplicate experiment that was repeated three to six times. *Significantly different from control. Conditioned media from *A* were placed on BCE cells and assayed for DNA synthesis (*B*) and cell proliferation (*C*). When the antibodies were used, 1.6 μg/ml of either anti-VEGF mAb or anti-gp120 antibody were added 30 min before the addition of 4 μl concentrated conditioned media. In *C*, *striped bars*, no antibody; *black bars*, anti-VEGF mAb; *dotted bars*, anti-gp120 antibody.

when highly specific AGE and VEGF inhibitors are shown to suppress retinal VEGF expression and diabetic retinopathy, respectively. These studies are under way.

If the effect of AGEs on VEGF gene expression can be shown to be causative for the vascular pathology of background diabetic retinopathy, then targeting VEGF may prove useful as a therapeutic strategy for the treatment of early diabetic retinopathy. With an ever-expanding list of molecular targets, it may be possible in the near future to prevent or forestall the ocular complications of diabetes with specific pharmacological agents, obviating the need for ablative pan-retinal photocoagulation.

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