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Connective Tissue Growth Factor Is Up-Regulated in the Diabetic Retina: Amelioration by Angiotensin-Converting Enzyme Inhibition

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Connective tissue growth factor (CTGF) has been postulated to have prosclerotic and angiogenic properties. The aim of this present study was to characterize retinal CTGF expression in the absence and presence of diabetes and in the context of treatment with the angiotensin-converting enzyme (ACE) inhibitor, perindopril. Retinas were obtained from control, diabetic, and diabetic plus perindopril-treated (3 mg/d) rats. CTGF gene expression was quantitated by RT-PCR and localized by *in situ* hybridization. CTGF protein expression was analyzed by Western blotting and localized by immunohistochemistry. Diabetes was associated with a greater than 2-fold increase in CTGF mRNA levels, which was attenuated by perindopril treatment. CTGF immunoreactivity was increased almost 2-fold in diabetes and was ameliorated by the ACE inhibitor perindopril. By *in situ* hybridization and immunohistochemistry, the major site of CTGF gene expression in the retina of diabetic rats was the ganglion cell layer. Based on the known *in vivo* effects of CTGF, it is postulated that this growth factor plays a pivotal role in mediating diabetes-associated retinal pathology. Furthermore, the protective effects of ACE inhibitors on retinal pathology may partly be mediated via effects on retinal CTGF expression. (*Endocrinology* 145: 860–866, 2004)

DIABETIC RETINOPATHY is the leading cause of blindness in the working-age population (1). The disease progresses in three stages, ultimately resulting in an advanced stage of proliferative retinopathy with fibrovascular proliferation, neovascularization, and retinal detachment (1). Several growth factors have been implicated in the regulation of angiogenesis in disease states such as diabetes, the most notable being vascular endothelial growth factor (VEGF) (2–4). More recently, the cytokine, connective tissue growth factor (CTGF), has been implicated in the process of angiogenesis (5, 6) in addition to its previously described role in fibroblast proliferation, cell adhesion, and the stimulation of extracellular matrix production (7, 8). Furthermore, VEGF has recently been shown to increase CTGF gene expression in bovine retinal capillary cells (9). However, to date there have been no studies to demonstrate the presence and distribution of CTGF in the normal retina or the effects of diabetes on this growth factor at this site. Therefore, the aim of this study was to investigate the gene and protein expression and distribution of CTGF in diabetic and normal rat retinas using complementary techniques including real time-PCR, *in situ* hybridization, Western blotting, and immunohistochemistry.

Surgical interventions, such as laser photocoagulation for neovascularization and vitrectomy for membrane proliferation and retinal detachment, can often result in the loss of peripheral vision. For this reason the discovery of new noninvasive therapies for the treatment and prevention of diabetic retinal disease is urgently required. Findings from the EUCLID study group have highlighted the importance of the renin-angiotensin system in the pathogenesis of diabetic retinopathy. This multicenter study found that after treatment with the angiotensin-converting enzyme (ACE) inhibitor, lisinopril, patients with type 1 diabetes were less likely to develop proliferative diabetic retinopathy (10). Furthermore, after ACE inhibition, VEGF has been shown to be reduced in the diabetic retina (11, 12). For this reason, a further aim of this study was to examine the effect of the ACE inhibitor, perindopril, on the expression of CTGF in the diabetic retina.

Materials and Methods

Animal model

Diabetes was induced in male Sprague Dawley rats, weighing 200 g, by iv injection of the β-cell toxin streptozotocin (60 mg/kg). Two to 4 U ultralente insulin (Ultratard HM, Novo-Nordisk, Bagsvaerd, Denmark) were administered daily to each diabetic animal to promote well-being and improve survival. Body weight, urinary albumin excretion rate, blood glucose (millimoles per liter), glycated hemoglobin, and blood pressure were measured after 4, 8, and 12 wk of diabetes. Control animals were followed concurrently (sham-injected with citrate buffer alone). A separate group of diabetic animals was treated with the ACE inhibitor, perindopril (Servier, Neuilly, France), at a dose of 3 mg/d in

Abbreviations: ACE, Angiotensin-converting enzyme; Ang II, angiotensin II; C_T, threshold cycle; CTGF, connective tissue growth factor; DTT, dithiothreitol; FAM, 6-carboxy fluorescein; GCL, ganglion cell layer; PKC, protein kinase C; TAMRA, tetramethylrhodamine; TBS/Tween, Tris-buffered saline and 0.1% Tween; VEGF, vascular endothelial growth factor.

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drinking water. All animals were killed at 12 wk. Perindopril was commenced on the first day of insulin therapy in this group of diabetic rats. The dose of perindopril was based on previous studies that have demonstrated the ability of this concentration of perindopril to stimulate plasma renin activity and suppress plasma ACE activity, both markers of inhibition of the renin-angiotensin system (13). Throughout the study animals were given access to food and water *ad libitum*. All animal procedures were in accordance with guidelines set by the Austin and Repatriation Medical Center ethics committee and the National Health and Medical Research Council of Australia.

Isolation of total RNA and synthesis of cDNA

Retinas were first minced and homogenized using an Ultra-Turrax homogenizer (Janke & Kunkel IKA, Labortechnik, Staufen, Germany) in TRIzol (Life Technologies, Inc., Gaithersburg, MD), and total RNA was isolated. cDNA was synthesized with a reverse transcriptase reaction carried out using standard techniques (SuperScript First Strand Synthesis System for RT-PCR, Life Technologies, Inc.) with random hexamers, deoxy-NTPs, and total RNA extracted from control, diabetic, and perindopril-treated diabetic rat retinas. An aliquot of the resulting single-stranded cDNA was used in the real-time PCR experiments as described below. To assess genomic DNA contamination, controls without reverse transcriptase were included.

Real-time PCR

Real-time PCR is a fully quantitative method for the determination of amounts of mRNA. Briefly, gene-specific 5′-oligonucleotide corresponding to the rat CTGF (5′-GAGGAAAACATTAAGAAGGGCAAA), CTGF 3′-oligonucleotide primer (5′-CGGCACAGGTCTTGATGA), and CTGF probe [6-carboxy fluorescein (FAM)-5′-TTTGAGCTTTCTGGCTGCAC-CAGTGT-tetramethylrhodamine (TAMRA)] were designed using the software program Primer Express (PE Applied Biosystems, Foster City, CA). The primers and probes were manufactured by PE Applied Biosystems. The probe is labeled with a FAM reporter dye at the 5′ end and a TAMRA dye as the quencher on the 3′ end. The increase in FAM reporter dye fluorescence emission is recorded during PCR amplification by the PRISM 7700 sequence detector (PE Applied Biosystems). Similarly, the fluorescent dye attached to the 18S ribosomal mRNA was VIC, and dual detection was possible using the multiplexing protocol. The generation of amplicons was defined by the point during cycling when amplification of the PCR product is first detected. The parameter, threshold cycle (C_T), is defined as the fractional cycle number at which fluorescence, generated by cleavage of the probe, passes a fixed threshold above the baseline.

Real-time amplification was measured in a reaction volume of 25 μl. A volume of 0.6 μl of cDNA (60 ng) sample was used for the CTGF reaction and the 18S ribosomal mRNA control reaction. The real-time reaction consisted of a two-step PCR (PRISM 7700, PE Applied Biosystems) with 10 min at 95 C and 40 cycles of 15 sec at 95 C and 1 min at 65 C. The reaction took place with 500 nmol/liter forward and reverse primer and 50 nmol/liter FAM/TAMRA CTGF probe and VIC/TAMRA 18S ribosomal probe, in 1× TaqMan universal PCR master mix (PE Applied Biosystems). Each sample was run and analyzed in triplicate, and the C_T value for the CTGF probe was subtracted from the C_T value of 18S ribosomal mRNA to obtain ΔC_T. The control samples were then used as the calibrator with a given value of 1, and the diabetic groups were compared with this calibrator.

In situ hybridization: CTGF riboprobe

The 1047-bp human CTGF cDNA sequence (coding for the open reading frame) was inserted in the sense direction into the *Bam*HI and *Xho*I sites of the pcDNA3 vector (Promega Corp., Madison, WI). The vector was digested with *Kpn*I and transcribed with SP6 polymerase to provide the antisense CTGF riboprobe. The site-specific expression of CTGF mRNA was determined by *in situ* hybridization, with background hybridization controlled for by the inclusion of a sense riboprobe (14). Antisense and sense riboprobes labeled with [^{35}S]CTP were prepared using the Promega Transcription System. Between 500 ng and 1 μg linearized template cDNA were transcribed in a reaction mix containing 1× transcription buffer; 10 mM dithiothreitol (DTT); 0.6 mM each of ATP, GTP, and UTP; 100 μCi ^{35}S; 0.5 μl RNasin; and 1 μl of the appropriate RNA polymerase. If the probe length was greater than 400 bp 4.25 pM cold CTP was added to the transcription reaction. The reaction was incubated at 37 C for a period of 90 min. After this time 1000 U deoxyribonuclease (ribonuclease-free; Roche, Basel, Switzerland) were added, and the reactions were incubated for a further 15 min at 37 C. The riboprobe was precipitated with ammonium acetate and ethanol using yeast tRNA as carrier, then reconstituted in 10 mM DTT. The length of the purified riboprobe was adjusted to approximately 150 bp using alkaline hydrolysis, followed by further purification with sodium acetate and ethanol, and was resuspended in 10 mM DTT.

Four-micrometer-thick sections were cut onto slides pretreated with aminopropyltriethoxysilane and baked overnight at 37 C. Before hybridization, sections were dewaxed in histolene and then rehydrated in graded ethanols and distilled water. The samples were equilibrated in P Buffer containing 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA (pH 8.0), followed by a 10-min incubation in 125 μg/ml pronase E (Sigma-Aldrich Corp., St. Louis, MO) at 37 C. Samples were then rinsed twice for 2 min each time in 0.1 M sodium phosphate buffer, postfixed in 4% paraformaldehyde for 10 min, and washed twice more in 0.1 M sodium phosphate buffer. The sections were rinsed in distilled water, dehydrated in 70% ethanol, and left to air-dry.

A hybridization buffer containing 2×10^4 cpm/μl ^{35}S-labeled riboprobe, 0.72 mg/ml yeast RNA, 50% deionized formamide, 100 mM DTT, 10% dextran sulfate, 0.3 M NaCl, 10 mM Na_2HPO_4, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 0.02% BSA, 0.02% Ficoll 400, and 0.02% polyvinyl pyrrolidone was prepared. The buffer was vortexed and incubated at 85 C for 5 min before adding 25 μl to each section to be covered by a 22 × 22-mm coverslip. The slides were then hybridized overnight in a light-proof, humidified chamber at 60 C.

Coverslips were removed in a solution containing 50% formamide and 2× standard saline citrate prewarmed to 55 C. This was followed by two 30-min washes in the above solution in a shaking water bath. Sections were rinsed three times in ribonuclease buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), and 0.5 M NaCl, then incubated at 37 C in the same buffer containing 150 μg/ml ribonuclease A (Roche) for 1 h. Samples were washed in 2× standard saline citrate for 1 h at 55 C, followed by dehydration in graded ethanols. After air-drying, the slides were exposed to BioMax MR film (Eastman Kodak Co., Rochester, NY) for 3–5 d.

Slides were coated in LM-1 emulsion (Amersham Pharmacia Biotech, Little Chalfont, UK) in a darkroom, dried for at least 1 h, and then incubated at 4 C in a light-proof container with desiccant for a period of 2–4 wk depending on the autoradiography results. The slides were equilibrated to room temperature, then immersed in Kodak D19 developer (Eastman Kodak Co.) for 4 min, 1% acetic acid for 1 min, and Illford Hypan fixative (Integrated Sciences, Melbourne, Australia) for 4 min, followed by rinsing in distilled water for at least 15 min. The sections were then fixed in 4% formaldehyde and treated with a progressive hematoxylin/eosin stain.

Generation of anti-CTGF polyclonal antisera for immunohistochemistry

A peptide corresponding to residues 247–260 (EENIKKGKKCIRTP) of human CTGF, was generated as four-branched multiple antigenic peptides and were purified by C_{18} reverse phase HPLC (Mimotopes Pty. Ltd., Clayton, Australia). The peptide sequence was chosen because it is not present in other CTGF family members and is conserved for CTGF across murine and human species (15). Each peptide was used to immunize two New Zealand White rabbits. Each rabbit was injected sc with 1 mg peptide in Freund's complete adjuvant, followed 2 wk later by a sc injection of 0.5 mg Freund's incomplete adjuvant, then two sequential im injections of 0.5 mg in PBS at 10-d intervals. The antiserum used was collected as a terminal bleed, in each case 10 d after the final im boost. The rabbit antiserum to peptide 247–260, referred to as 197, was used for the immunohistochemical studies.

Western blotting

Retinas from six control, diabetic, and diabetic plus perindopril-treated animals were pooled, quickly removed, minced with a scalpel blade, resuspended in buffer [10 mM HEPES, 150 mM NaCl, 1 mM EGTA,

5 mM $MgCl_2$, and 0.02% NaN_3, pH 7.4, containing 0.5 μg/ml pepstatin (Sigma-Aldrich Corp.), 0.25 μg/ml leupeptin (Sigma-Aldrich Corp.), 0.1 mg/ml benzamidine (Sigma-Aldrich Corp.), and 0.1 mg/ml bacitracin (Sigma-Aldrich Corp.)], homogenized at 13,000 rpm with the Ultra-Turrax (Janke and Kunkel IKA, Labortechnik), and centrifuged at 1,000 × *g* (4 C) for 30 min. The resultant supernatant was harvested and stored in aliquots at −80 C.

Before each run, the protein concentration of each sample was determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). A mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA) was used with a 10% sodium dodecyl sulfate-denaturing gel system. Samples were prepared after thawing by centrifugation (10,000 × *g*; Eppendorf 5414C) for 5 min. Each sample was adjusted up to a desired protein content of 50 μg, an equal volume of 2× sodium dodecyl sulfate loading buffer (with 200 mM dithiothreitol) was added, and the sample was incubated at 95 C for 5 min. Samples and molecular weight markers (Kaliedoscope, Bio-Rad Laboratories) were loaded onto the gel, and after running the proteins at 120 V through the stacking gel, the voltage was increased to 160 V. Proteins were trans-blotted onto nitrocellulose filters (Hybond P, Amersham-Pharmacia Biotech) using a transfer tank at 15 V for 30 min. At the end of the transfer, the filters were blocked with 10% nonfat skim milk powder in Tris-buffered saline and 0.1% Tween (TBS/Tween) for 1 h at room temperature with gentle rocking. The primary antiserum used was anti-CTGF, specifically directed against amino acids 223–348 (ab6992, abcam, Cambridgeshire, UK; 1:1,000 with 10% skim milk powder in TBS/Tween) and was incubated overnight at room temperature. The following day the membrane was washed thoroughly three times (10-min washes) in wash solution (TBS/Tween). Positive bands were developed using the Western Blotting Analysis system (Amersham Pharmacia Biotech), in which horseradish peroxidase-labeled secondary antirabbit antibody was diluted at 1:2,000 and incubated for 1 h at room temperature. After washing three times, the filter was exposed to x-ray film (Kodak-Biomax) at four different exposure times (1 min and 30, 15, and 5 sec). Exposed Biomax film of bands representing CTGF protein were quantified on an Automated Imaging System (Imaging Research, Inc., St. Catherines, Canada).

Immunohistochemistry

Immunohistochemical staining for CTGF protein was performed as outlined below. Four-micrometer-thick paraffin serial sections were prepared from 4% paraformaldehyde-fixed, paraffin-embedded rat retina. Sections were dewaxed and hydrated, then endogenous peroxidase was quenched for 20 min using 3% (vol/vol) hydrogen peroxide in PBS. Endogenous immunoglobulins were blocked by incubation for 20 min with 10% normal goat serum/PBS. The primary antiserum (197 at a 1:500 titer) was applied at room temperature for 1 h. Specific staining was detected using the standard avidin-biotin complex method (16). Briefly, slides were incubated for 20 min with the secondary antibody (biotin-conjugated goat antirabbit IgG, DAKO, Copenhagen, Denmark) at a concentration of 1:250. The avidin-biotin Vectastain ABC system (Vector Laboratories, Burlingame, CA) was then applied for 20 min. After thorough washing, the final detection step was carried out using 3,3′-diaminobenzidine (Sigma-Aldrich Corp.) as the chromogen. Sections were lightly counterstained with hematoxylin.

Statistics

Data are shown as the mean ± SEM. Comparisons were performed by ANOVA using StatView 5.0 (Brainpower, Calabasas, CA). $P < 0.05$ was viewed as statistically significant. For RT-PCR and Western blotting data, values for control animals were arbitrarily standardized to 1, and data for diabetic animals were expressed relative to this control value.

Results

Clinical data

The diabetic rats demonstrated hyperglycemia, modestly elevated blood pressure, and increased urinary albumin excretion compared with the control group. Perindopril treatment reduced blood pressure and urinary albumin excretion, but had no significant effect on body weight or glycemic control (Table 1).

RT-PCR

Retinal CTGF gene expression was increased more than 2-fold in retinas of diabetic compared with control rats (control, 1.0 ± 0.2 arbitrary units; diabetic, 2.4 ± 0.4; $P < 0.01$). CTGF mRNA levels in perindopril-treated diabetic rats were similar to those in control animals (diabetic plus perindopril, 1.2 ± 0.2) and significantly less than in untreated diabetic rats ($P < 0.01$; Fig. 1).

In situ hybridization

Using *in situ* hybridization, CTGF mRNA was localized to the retinal ganglion cell layer (GCL; Fig. 2A). An increase in CTGF mRNA signal was seen in the GCL of diabetic rats (Fig. 2, B and C). This indicates that the increase in CTGF mRNA seen in whole retinas from diabetic rats (Fig. 1) is primarily localized to the GCL. In the diabetic rats receiving perindopril, the CTGF mRNA was at a level of intensity and cellular distribution similar to those in the nondiabetic controls (Fig. 2D).

Western blotting

Western immunoblotting detected three groups of immunoreactive bands, all different forms of CTGF. There were monomeric forms at approximately 36–38 kDa, homodimeric forms at approximately 70 kDa, and lower molecular mass fragment forms (Fig. 3) as reported by others (15). No such bands were detected when normal rabbit serum was used in place of the polyclonal CTGF antiserum (data not shown). When the monomeric immunoreactive bands were

TABLE 1. Physiological and biochemical parameters

	n	Body weight (g)	SBP (mm Hg)	Blood glucose (mmol/liter)	HbA_{1c} (%)	AER (mg/24 h)
Control	8	605 ± 18	118 ± 4	6.0 ± 0.1	3.5 ± 0.1	0.9 ×/÷ 1.6
Diabetic	11	456 ± 17[a]	140 ± 6[a]	23.7 ± 1.6[a]	9.4 ± 0.5[a]	2.5 ×/÷ 1.4[a]
Diabetic + P	10	419 ± 11	110 ± 7[b]	21.0 ± 1.5[a]	8.5 ± 0.3[a]	0.4 ×/÷ 1.2[b]

P, Perindopril; AER, albumin excretion rate; SBP, systolic blood pressure; HbA_{1c}, glycated hemoglobin. Data are shown at wk 12 unless otherwise specified as the mean ± SEM, except for AER values, which are shown as the geometric mean ×/÷ tolerance factors. Data for blood glucose and HbA_{1c} are shown as the means for wk 4, 8, and 12.

[a] $P < 0.01$ *vs.* control.

[b] $P < 0.01$ *vs.* diabetic.

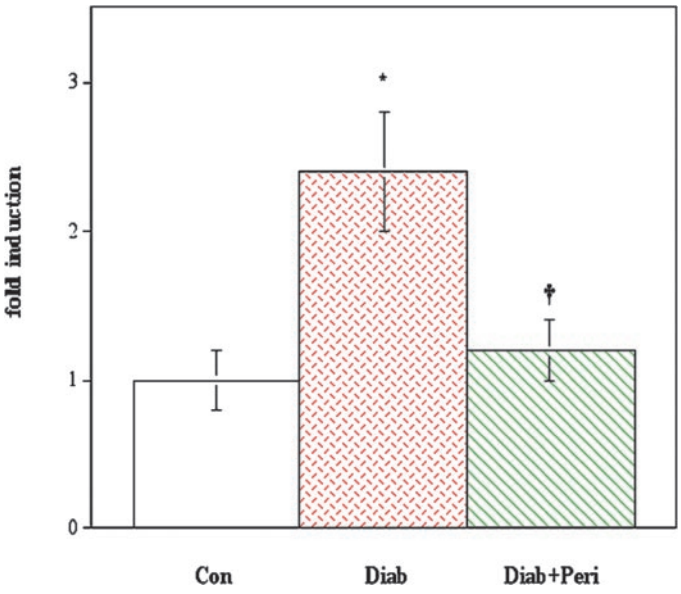

FIG. 1. Quantitation of CTGF mRNA in control (Con; n = 6), diabetic (Diab; n = 6), and diabetic plus perindopril-treated (Diab + Peri; n = 6) rats, measured by real-time RT-PCR. Data are shown as the mean ± SEM. *, $P < 0.01$ *vs.* control; †, $P < 0.01$ *vs.* diabetic plus perindopril.

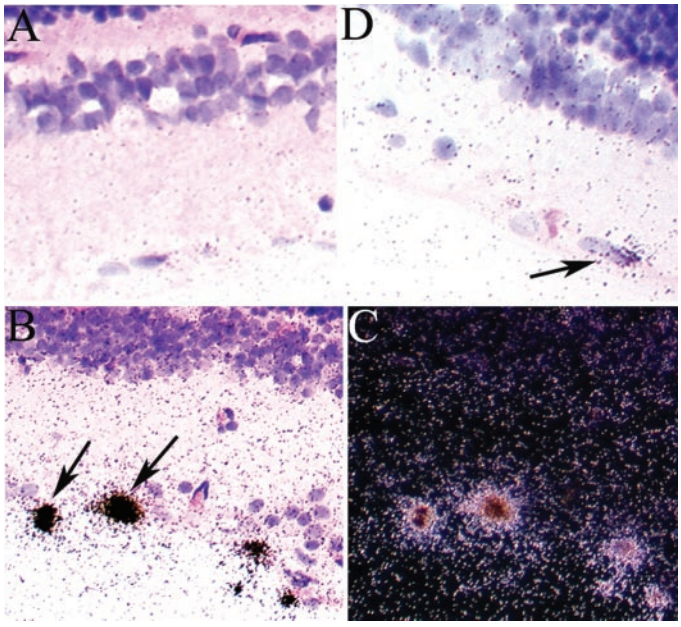

FIG. 2. Localization of CTGF mRNA by *in situ* hybridization to the GCL using a ^{35}S-labeled riboprobe in control (A), diabetic, lightfield (B) and darkfield (C), and diabetic plus perindopril-treated retina (D). Original magnification, ×850.

quantitated using semiquantitative Western analysis, an 85% increase in the level of the monomeric CTGF protein was measured in the retinas of diabetic rats compared with the controls. The higher molecular weight forms of CTGF were also increased in diabetic animals. Compared with the diabetic animals, the increase in monomeric CTGF in diabetic animals treated with perindopril was attenuated by 65% toward a level similar to that seen in control retinas.

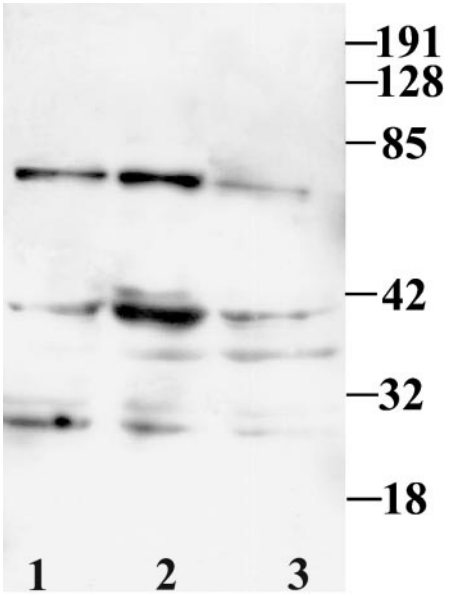

FIG. 3. Western immunoblot of whole retina preparations obtained from samples of control (lane 1), diabetic (lane 2), and diabetic plus perindopril-treated (lane 3) animals run under reducing conditions and after probing with a polyclonal antibody to residues 223–348 of human CTGF. The immunoreactive bands detected were for monomeric CTGF (~36–38 kDa), a dimeric form of CTGF (~70 kDa), and low molecular mass fragment forms of CTGF.

Immunohistochemistry

In retinas of control rats, CTGF immunoreactivity was sparsely distributed within the GCL and inner nuclear layer (Fig. 4A). After 12 wk of diabetes, CTGF immunoreactivity was increased in the GCL, but not in the inner nuclear layer (Fig. 4, B and C), compared with controls (Fig. 4A). These data show that the observed increase in CTGF protein present in whole retinas of diabetic rats (Fig. 3) was localized to the GCL, and it parallels the observed increase in CTGF mRNA in the GCL in diabetic rats (Fig. 2, B and C). Treatment of diabetic rats with the ACE inhibitor perindopril (Fig. 4D) inhibited the up-regulation of CTGF immunostaining in the GCL that was observed in untreated diabetic rats.

Discussion

This study has demonstrated the presence of CTGF mRNA and protein in the normal adult rat retina. Moreover, we have shown that the levels of both CTGF mRNA and protein are increased in the retina after streptozotocin-induced diabetes and that this increase could be attenuated with the ACE inhibitor, perindopril.

Despite the occurrence of apoptosis, fibrovascular proliferation, and neovascularization in diabetic retinopathy, processes that can occur in cells and tissues as a result of CTGF induction, there have been no studies examining the expression of CTGF in any type or grade of diabetic retinopathy. CTGF immunostaining has recently been localized to both cellular and fibrotic regions of epiretinal membrane in nondiabetic proliferative vitreoretinopathy (17). Although the link between hyperglycemia and CTGF up-regulation has not been previously reported in the diabetic retina, other studies have documented both *in vitro* and *in vivo* increases

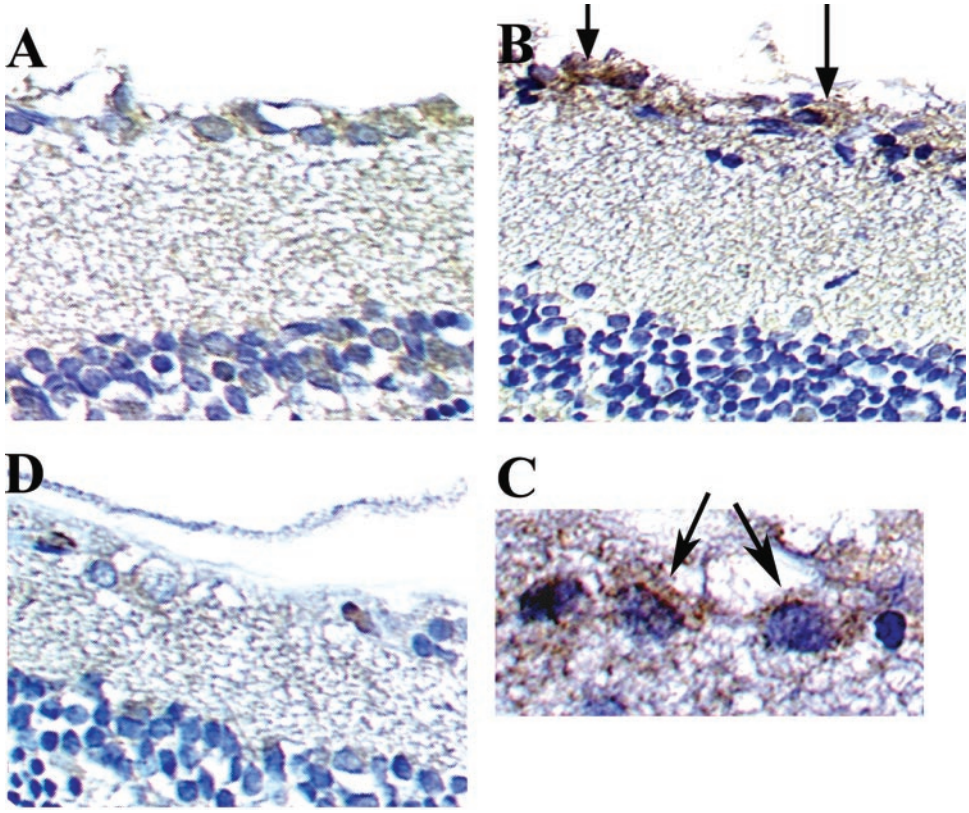

FIG. 4. CTGF immunoreactivity (dilution of 1/500) in control (A), diabetic (B and C), and diabetic plus perindopril-treated (D) retinas, showing localization to the GCL. Original magnification, ×850; except for C, ×1700.

in CTGF in response to hyperglycemia and to the diabetic milieu in a range of nonretinal cell populations (18, 19) and at various sites of diabetic vascular complications, including the kidney (14, 19) and aorta (20).

A variety of cellular mechanisms could mediate CTGF up-regulation in the retina by diabetes. CTGF can be increased by reactive oxygen species (21), advanced glycosylation end products (22), cellular stretch (23), TGFβ (18), protein kinase C (PKC)-dependent pathways (24), and hypoxia (25), albeit in a variety of cell types. Each of these factors is more active in diabetic retinopathy. In addition, CTGF expression is induced in bovine retinal capillary cells by VEGF (9), itself a factor strongly implicated in diabetic retinal complications. That the rodent model of diabetic eye disease presented in this current work is a preproliferative model of diabetic retinopathy that demonstrates early retinal changes of increased permeability and increases in VEGF (11) is consistent with the concept that one of the factors that up-regulates retinal CTGF may be VEGF.

The GCL is the main site of CTGF mRNA and protein up-regulation in the diabetic retina in this study. In addition to retinal ganglion cells, this layer contains astrocytes, and the end-feet of another population of retinal glial cells, Müller cells. Although the precise site of CTGF production cannot be deduced from these experiments alone, we postulate that it is produced by astrocytes and/or Müller cells. Previous studies have detected CTGF in astrocytes, ependymal cells, and tanycytes (26), whereas Müller cells have been reported to synthesize VEGF and its tyrosine kinase receptors (27, 28). After the production of CTGF by these cells, the factor may either remain within the cell or be deposited outside the cell, leading to the accumulation of extracellular matrix.

Whether CTGF acts predominantly in an intracrine, autocrine, or paracrine manner in the retina has yet to be determined, as does the identification of a functional receptor for CTGF. Recently, the integrin $\alpha_v\beta_3$ has been reported to function as a receptor on endothelial cells for CTGF-mediated endothelial cell adhesion, migration, and angiogenesis (5, 6). Furthermore, immunoreactivity for the integrin $\alpha_v\beta_3$/$\alpha_v\beta_5$ has been detected in retinal and intravitreal growing vascular cells, the GCL, and the inner and outer nuclear layers in the retina in a murine model of ischemia-induced retinal neovascularization (29). Thus, integrin $\alpha_v\beta_3$ appears to be a likely candidate as a site of cellular CTGF binding in the retina.

CTGF protein exists in a number of molecular weight forms, including glycosylation variants, proteolysed fragments, and high molecular weight forms (15, 22, 30). In this study we observed that retinal CTGF exists in monomeric, high molecular weight, immunoreactive and fragment forms by Western immunoblot. These immunoreactive forms were specifically detected on Western immunoblot using a number of anti-CTGF antisera, including antisera directed against differing ends of the CTGF molecule. CTGF has a self-dimerization domain, and rodent as well as human CTGF have been shown by others to exist in a molecular weight form consistent with CTGF homodimers (15). Whether dimeric CTGF varies in its bioactivity compared with monomeric CTGF has not been reported.

The exact consequences of up-regulation of CTGF in the GCL in diabetic retina are speculative. In addition to its role

in ECM production, cell adhesion, neovascularization, and fibroblast proliferation (7, 8), CTGF is involved in apoptosis (31, 32). This may in part explain the finding that retinal ganglion cells are significantly reduced in experimental diabetes (33). That CTGF alone induces neovascularization in vascular endothelial cells and yet may bind directly to VEGF and modulate VEGF-mediated vascular neogenesis (34) exemplifies the complex behavior of this growth factor. Clearly, further *in vitro* and *in vivo* studies need to be performed before the role of CTGF in various cell types and stages of diabetic retinopathy is defined. The current studies of CTGF in the diabetic retina together with the growing body of work implicating CTGF as a factor contributing to renal complications in diabetes (19, 35, 36) suggest that CTGF may be a mediator in the development of complications in a number of microvascular tissues in diabetes.

A major finding from this study was that the ACE inhibitor, perindopril, resulted in a significant attenuation of both the CTGF mRNA and protein up-regulation in the diabetic retina. Using immunohistochemistry and *in situ* hybridization, this reduction was localized to the retinal GCL. This result suggests that angiotensin II (Ang II) mediates, at least in part, the overexpression of CTGF in the retina of diabetic rats. Whether the effect of perindopril on CTGF in the current work is through the observed attenuation of blood pressure or through other mechanisms is unclear. Although this is the first study to examine the association between the renin-angiotensin system and CTGF in diabetic retinopathy, a recent study by our group has shown that perindopril treatment inhibits the overexpression of CTGF in the aorta of diabetes-induced apolipoprotein E-deficient mice (20). Furthermore, Ang II has been shown to partly mediate the overexpression of the CTGF gene in arterial smooth muscle cells in an experimental model of cyclosporine-induced myocardial damage (37). More recently, angiotensin II-induced CTGF expression in vascular smooth muscle cells was shown to involve a range of intracellular signals, including PKC and reactive oxygen species (38). Furthermore, it has previously been shown that ACE inhibition reduces retinal PKC expression in experimental diabetes (39).

In addition to a direct effect of Ang II on the regulation of CTGF in the diabetic retina, Ang II may mediate the increased CTGF expression indirectly, via the up-regulation of VEGF. *In vitro* experiments have shown that Ang II directly stimulates the secretion of VEGF in cultured vascular smooth muscle (40) and in cardiac endothelial cells (41, 42). ACE inhibition has also been shown to reduce retinal VEGF expression in streptozotocin-induced diabetic rats (11) as well as in the model of retinopathy of prematurity (43). Together with the findings that CTGF is induced by VEGF via the VEGF receptors, kinase domain receptor and Flt1, in retinal vascular cells (9), the increase in CTGF in the diabetic retina may be a result of the induction of VEGF by Ang II.

In summary, these results have provided evidence that CTGF mRNA and protein are expressed in the GCL and inner nuclear layer in the adult rat retina. More importantly, CTGF mRNA and protein are up-regulated in the GCL after streptozotocin-induced diabetes, an increase that could be attenuated using the ACE inhibitor, perindopril. This study suggests that Ang II-mediated CTGF expression, either directly or indirectly via the up-regulation of VEGF induced by diabetes, may contribute to the pathogenesis of diabetic retinopathy. However, one must be cautious in interpreting these findings, because animal models of diabetic retinopathy do not exhibit advanced retinal lesions such as those seen in man.

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References

1. **Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano1 JD, Ferris III FL, Klein R** 1998 Diabetic retinopathy. Diabetes Care 21:143–156
2. **Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT** 1994 Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118:445–450
3. **Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Hung VN, Aiello LM, Ferrara N, King GL** 1994 Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331:1480–1487
4. **Witmer AN, Blaauwgeers HG, Weich HA, Alitalo K, Vrensen GF, Schlingemann RO** 2002 Altered expression patterns of VEGF receptors in human diabetic retina and in experimental VEGF-induced retinopathy in monkey. Invest Ophthalmol Vis Sci 43:849–857
5. **Babic AM, Chen CC, Lau LF** 1999 Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin $\alpha_v\beta_3$, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol 19:2958–2966
6. **Shimo T, Nakanishi T, Nishida T, Asano M, Kanyama M, Kuboki T, Tamatani T, Tezuka K, Takemura M, Matsumura T, Takigawa M** 1999 Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. J Biochem 126:137–145
7. **Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR** 1996 Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol 107:404–411
8. **Kireeva ML, Latinkic BV, Kolesnikova TV, Chen CC, Yang GP, Abler AS, Lau LF** 1997 Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. Exp Cell Res 233:63–77
9. **Suzuma K, Naruse K, Suzuma I, Takahara N, Ueki K, Aiello LP, King GL** 2000 Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. J Biol Chem 275:40725–40731
10. **Chaturvedi N, Sjolie AK, Stephenson JM, Abrahamian H, Keipes M, Castellarin A, Rogulja-Pepeonik Z, Fuller JH** 1998 Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. The EUCLID Study Group. EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus. Lancet 351:28–31
11. **Gilbert RE, Kelly DJ, Cox AJ, Wilkinson-Berka JL, Rumble JR, Osicka T, Panagiotopoulos S, Lee V, Hendrich EC, Jerums G, Cooper ME** 2000 Angiotensin converting enzyme inhibition reduces retinal overexpression of vascular endothelial growth factor and hyperpermeability in experimental diabetes. Diabetologia 43:1360–1367
12. **Hogeboom van Buggenum IM, Polak BC, Reichert-Thoen JW, de Vries-Knoppert WA, van Hinsbergh VW, Tangelder GJ** 2002 Angiotensin converting enzyme inhibiting therapy is associated with lower vitreous vascular endothelial growth factor concentrations in patients with proliferative diabetic retinopathy. Diabetologia 45:203–209
13. **O'Brien RC, Cooper ME, Jerums G, Doyle AE** 1993 The effects of perindopril and triple therapy in a normotensive model of diabetic nephropathy. Diabetes 42:604–609
14. **Twigg SM, Cao Z, McLennan SV, Burns WC, Brammar G, Forbes JM, Cooper ME** 2002 Renal connective tissue growth factor induction in experimental diabetes is prevented by aminoguanidine. Endocrinology 143:4907–4915
15. **Steffen CL, Ball-Mirth DK, Harding PA, Bhattacharyya N, Pillai S, Brigstock DR** 1998 Characterization of cell-associated and soluble forms of connective

tissue growth factor (CTGF) produced by fibroblast cells in vitro. Growth Factors 15:119–213
16. **Hsu SM, Raine L, Fanger H** 1981 Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 29:577–580
17. **Hinton DR, He S, Jin ML, Barron E, Ryan SJ** 2002 Novel growth factors involved in the pathogenesis of proliferative vitreoretinopathy. Eye 16:422–428
18. **Murphy M, Godson C, Cannon S, Kato S, Mackenzie HS, Martin F, Brady HR** 1999 Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells. J Biol Chem 274:5830–5834
19. **Riser BL, Denichilo M, Cortes P, Baker C, Grondin JM, Yee J, Narins RG** 2000 Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. J Am Soc Nephrol 11:25–38
20. **Candido R, Jandeleit-Dahm KA, Cao Z, Nesteroff SP, Burns WC, Twigg SM, Dilley RJ, Cooper ME, Allen TJ** 2002 Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. Circulation 106:246–253
21. **Park SK, Kim J, Seomun Y, Choi J, Kim DH, Han IO, Lee EH, Chung SK, Joo CK** 2001 Hydrogen peroxide is a novel inducer of connective tissue growth factor. Biochem Biophys Res Commun 284:966–971
22. **Twigg SM, Chen MM, Joly AH, Chakrapani SD, Tsubaki J, Kim HS, Oh Y, Rosenfeld RG** 2001 Advanced glycosylation end products up-regulate connective tissue growth factor (insulin-like growth factor-binding protein-related protein 2) in human fibroblasts: a potential mechanism for expansion of extracellular matrix in diabetes mellitus. Endocrinology 142:1760–1769
23. **Hishikawa K, Oemar BS, Nakaki T** 2001 Static pressure regulates connective tissue growth factor expression in human mesangial cells. J Biol Chem 16797–16803
24. **Way KJ, Isshiki K, Suzuma K, Yokota T, Zvagelsky D, Schoen FJ, Sandusky GE, Pechous PA, Vlahos CJ, Wakasaki H, King GL** 2002 Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase C β2 activation and diabetes. Diabetes 51:2709–2718
25. **Shimo T, Kubota S, Kondo S, Nakanishi T, Sasaki A, Mese H, Matsumura T, Takigawa M** 2001 Connective tissue growth factor as a major angiogenic agent that is induced by hypoxia in a human breast cancer cell line. Cancer Lett 174:57–64
26. **Kondo S, Kubota S, Shimo T, Nishida T, Yosimichi G, Eguchi T, Sugahara T, Takigawa M** 2002 Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. Carcinogenesis 23:769–776
27. **Gilbert RE, Vranes D, Berka JL, Kelly DJ, Cox A, Wu LL, Stacker SA, Cooper ME** 1998 Vascular endothelial growth factor and its receptors in control and diabetic rat eyes. Lab Invest 78:1017–1027
28. **Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G** 1998 Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. Diabetes 47:401–406
29. **Takagi H, Suzuma K, Otani A, Oh H, Koyama S, Ohashi H, Watanabe D, Ojima T, Suganami E, Honda Y** 2002 Role of vitronectin receptor-type integrins and osteopontin in ischemia-induced retinal neovascularization. Jpn J Ophthalmol 46:270–278
30. **Yang DH, Kim HS, Wilson EM, Rosenfeld RG, Oh Y** 1998 Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF-β in Hs578T human breast cancer cells. J Clin Endocrinol Metab 2593–2596
31. **Hishikawa K, Nakaki T, Fujii T** 1999 Transforming growth factor-β_1 induces apoptosis via connective tissue growth factor in human aortic smooth muscle cells. Eur J Pharmacol 385:287–290
32. **Hishikawa K, Nakaki T, Fujii T** 2000 Connective tissue growth factor induces apoptosis via caspase 3 in cultured human aortic smooth muscle cells. Eur J Pharmacol 392:19–22
33. **Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW** 1998 Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 102:783–791
34. **Hashimoto G, Inoki I, Fujii Y, Aoki T, Ikeda E, Okada Y** 2002 Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. J Biol Chem 277: 36288–36295
35. **Ito Y, Aten J, Bende RJ, Oemar BS, Rabelink TJ, Weening JJ, Goldschmeding R** 1998 Expression of connective tissue growth factor in human renal fibrosis. Kidney Int 53:853–861
36. **Wang S, Denichilo M, Brubaker C, Hirschberg R** 2001 Connective tissue growth factor in tubulointerstitial injury of diabetic nephropathy. Kidney Int 60:96–105
37. **Finckenberg P, Lassila M, Inkinen K, Pere AK, Krogerus L, Lindgren L, Mervaala E, Vapaatalo H, Nurminen ML, Ahonen J** 2001 Cyclosporine induces myocardial connective tissue growth factor in spontaneously hypertensive rats on high-sodium diet. Transplantation 71:951–958
38. **Ruperez M, Lorenzo O, Blanco-Colio L M, Esteban V, Egido J, Ruiz-Ortega M** 2003 Connective tissue growth factor is a mediator of angiotensin ii-induced fibrosis. Circulation 108:1499–1505
39. **Osicka TM, Yu Y, Lee V, Panagiotopoulos S, Kemp BE, Jerums G** 2001 Aminoguanidine and ramipril prevent diabetes-induced increases in protein kinase C activity in glomeruli, retina and mesenteric artery. Clin Sci 100: 249–257
40. **Williams B, Baker AQ, Gallacher B, Lodwick D** 1995 Angiotensin II increases vascular permeability factor gene expression by human vascular smooth muscle cells. Hypertension 25:913–917
41. **Chua CC, Hamdy RC, Chua BH** 1998 Upregulation of vascular endothelial growth factor by angiotensin II in rat heart endothelial cells. Biochim Biophys Acta 1401:187–194
42. **Otani A, Takagi H, Suzuma K, Honda Y** 1998 Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. Circ Res 82:619–628
43. **Moravski CJ, Kelly DJ, Cooper ME, Gilbert RE, Bertram JF, Shahinfar S, Skinner SL, Wilkinson-Berka JL** 2000 Retinal neovascularization is prevented by blockade of the renin-angiotensin system. Hypertension 36:1099–1104

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