Role of Connective Tissue Growth Factor in the Retinal Vasculature during Development and Ischemia

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PURPOSE. To investigate the function of connective tissue growth factor (CTGF), a matricellular protein of the CCN (Cyr61/CTGF/Nov) family, in retinal vasculature during development and ischemia.

METHODS. CTGF expression was determined using RT-PCR, immunohistochemistry, and transgenic mice carrying *CTGF promoter-driven-GFP*. CTGF antibody was intraocularly injected into neonates at postnatal day (P)2, and its effect on retinal angiogenesis was analyzed at P4. Transgenic animals expressing GFP regulated by the glial fibrillary acidic protein promoter were used for astrocyte visualization. Retinal vascular occlusion was introduced by rose Bengal and laser photocoagulation on chimeric mice that were reconstituted with GFP⁺ bone marrow cells. Vascular repair in response to VEGF-A and CTGF was analyzed.

RESULTS. A temporal increase in CTGF at both mRNA and protein levels was observed in the ganglion cell layer and inner nuclear layer during development. Endothelial cells and pericytes were identified as the main cellular sources of CTGF during retinal angiogenesis. CTGF stimulated the migration of astrocytes, retinal endothelial cells, and pericytes in vitro. Inhibition of CTGF by specific antibody affected vascular filopodial extension, growth of the superficial vascular plexus, and astrocyte remodeling. In adult mice, CTGF was prominently expressed in the perivascular cells of arteries. CTGF activated bone marrow-derived perivascular cells and promoted fibrovascular membrane formation in the laser-induced adult retinopathy model.

CONCLUSIONS. CTGF is expressed in vascular beds and acts on multiple cell types. It is important for vessel growth during early retinal development and promotes the fibrovascular reaction in murine retinal ischemia after laser injury. (*Invest*

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The retina is a highly vascularized neural tissue in mammals. It is believed that the retinal vasculature is formed in response to “physiological hypoxia” caused by the increased oxygen and nutrient demands of neural cells for differentiation during development.¹ The retinal vasculature becomes quiescent and maintains homeostasis in adults. However, ischemia caused by stimuli such as inflammation or metabolic insults in certain diseases could activate quiescent blood vessels to proliferate.² Newly formed vessels are often immature and prone to leakage. The bleeding complication of neovessels may stimulate scar formation as part of a wound healing response. The aberrant neovascularization and concomitant fibrosis disrupt the local tissue architecture, resulting in catastrophic vision loss in patients with diseases such as proliferative diabetic retinopathy (PDR).³ Understanding the molecular mechanism governing retinal neovessel formation and fibrotic responses during physiological and pathologic conditions is necessary for potential therapeutic intervention.

In mice, retinal angiogenesis occurs postnatally and has been widely used as an excellent model with which to study the molecular mechanisms of angiogenesis.^{4,5} This developmental process involves the proliferation, migration, and differentiation of endothelial cells, pericytes, astrocytes, microglia, and neurons. Astrocytes in front of growing blood vessels secrete the angiogenic regulator vascular endothelial growth factor (VEGF)-A. VEGF-A controls vascular sprouting in the early postnatal retina by guiding filopodial extension from specialized endothelial tip cells.⁶ This protein is also necessary and sufficient for promoting ischemia-induced retinal neovascularization.^{7,8} Exogenous VEGF-A in the retina is able to recruit circulating endothelial progenitor cells (EPCs) of bone marrow origin at neovascularization sites for vascular repair.^{9,10}

CTGF is the prototype member of the CCN (Cyr61/CTGF/Nov) protein family. It is capable of promoting extracellular matrix (ECM) protein production and regulates cell adhesion, migration, and even apoptosis in a variety of biological processes.^{11,12} This protein is involved in basal membrane thickening in PDR and other ocular fibrosis because of its fibrogenic activity.^{13–15} In addition, CTGF is upregulated in various ischemic retinopathies of experimental models and human diseases.^{16,17} CTGF has also been shown to form a complex with VEGF-A and to inhibit VEGF-induced angiogenesis.¹⁸ However, the role of CTGF in angiogenesis is controversial and not fully understood.^{19–21} In this study, we sought to investigate the role of CTGF in neovascularization using two murine models: neonatal retinal angiogenesis during development and adult ischemic retinopathy after laser photocoagulation. Our results from the neonatal retinal angiogenesis model demonstrated that CTGF was specifically expressed in vascular beds of the retina and promoted the migration of primary astrocytes, reti-

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nal endothelial cells, and pericytes in vitro. Inhibition of CTGF affected retinal angiogenesis and astrocyte remodeling. Unlike VEGF-A, which is exclusively known to have endothelial-specific action, CTGF activated bone marrow-derived perivascular cells and promoted fibrovascular membrane formation during retinal vascular repair in adult mice.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the University of Florida Animal Care and Use Committee and were conducted following animal guidelines according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. *GFAP-GFP (FVB/N-TgN(GFAPGFP)14mes/J)* or *CTGFp-GFP (Stock Tg(CTGF-EGFP)156Gsat)* transgenic mice that expressed green fluorescent protein under the control of the glial fibrillary acidic protein (GFAP) promoter or *CTGF* promoter were obtained from the Jackson Laboratory (Bar Harbor, ME) or Mutant Mouse Regional Resource Centers (University of California at Davis). In addition, *UBC-GFP (C57BL/6-Tg(UBC-GFP)30Scha/J)* transgenic mice that expressed GFP under the direction of the human ubiquitin C promoter (Jackson Laboratory) were used for bone marrow transplantation in the laser induced ischemic retinopathy model. Males carrying the transgenes were mated with wild-type C57BL6 females to eliminate any phenotype from FVB or Swiss backgrounds. Progenies were confirmed by PCR analysis using primers specific for GFP.

Semiquantitative RT-PCR and Real-Time PCR

Eyes were harvested at the indicated time points and stored in solution (RNAlater; Qiagen Valencia, CA). Retinas were collected under a dissection microscope and used for RT-PCR analysis. Total RNA was extracted from isolated retinal tissues using an RNA kit (Qiagen) and was treated with an endonuclease (DNase I; Ambion, Austin, TX) to remove genomic DNA. Reverse transcription was performed to synthesize cDNA using the first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Amplification conditions for semiquantitative RT-PCR were as follows: 2 minutes at 94°C, followed by 25 to 28 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C in the presence of 500 nM forward and reverse primers, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-buffer (pH 8.3), 200 μM dNTP, and 1 U *Taq* DNA polymerase. The following primers were used: *CTGF* forward primer 5' GTCTTCACACTGGTGCAGCC 3' and reverse primer 5' ACTGGAA-GACACATTGGCC 3'; *Actin* forward primer 5' TCCTGCTTGCTGATC-CACAT 3' and reverse primer 5' TCCTCCCTGGAGAAGAGCTA 3'. Real-time quantitative PCR analysis was carried out using the above *CTGF* primers (10 μM), supermix (SsoFast EvaGreen; Bio-Rad, Hercules, CA), ~100 ng cDNA, and real-time PCR system (CFX96; Bio-Rad) with the following parameters: 95°C, 30 seconds for one cycle, followed by 40 cycles of 95°C, 5 seconds for denaturation, and 60°C, 10 seconds for annealing and extension. Melt curve was at 65°C to 95°C for at 5 seconds/step for one cycle. The $\Delta\Delta C_t$ method was used to quantify fold increases of *CTGF* mRNAs at various time points. The level of *CTGF* mRNA at P2 was assigned as arbitrary one.

Immunofluorescent Staining and Confocal Microscopy Analysis

Eyes from animals at various ages were fixed in 4% paraformaldehyde. The immunofluorescent staining for CTGF was performed on 6-μm paraffin sections with a rabbit antibody (Abcam, Cambridge, MA) and Alexa Fluor 488-conjugated donkey secondary antibody (Invitrogen). Alexa Fluor 594-conjugated isolectin IB4 (40 μg/mL; Invitrogen) was used to stain endothelial cells in retinal flatmounts. GFAP antibody (Thermo Scientific, Rockford, IL) and Alexa Fluor 350-conjugated anti-rabbit secondary antibody (Invitrogen) were used to stain astrocytes in retinal flatmounts. Visualization was carried out under the same microscopy settings at room temperature using laser spinning

confocal microscopy (TCS SP2; Leica, Wetzlar, Germany) with the accompanying software (Slidebook, Irving, TX).

Isolation and Culturing of Retinal Endothelial Cells, Pericytes, and Astrocytes

Retinas were dissected from neonatal mice at P4 to P5 under a microscope and minced into small pieces, followed by digestion with collagenase type I (1 mg/mL; Worthington, Lakewood, NJ). Primary retinal endothelial cell isolation was performed according to the method described by Su et al.²² with some modifications. Digestion was terminated by the addition of fetal bovine serum (FBS) to a final concentration of 10%. Cell mixtures were passed into sterile membranes with 40-μm pore size (BD Biosciences, San Jose, CA) and were stained with phycoerythrin (PE)-conjugated CD31 antibody (BD Biosciences). Anti-PE magnetic beads (Miltenyi Biotec, Auburn, CA) and a cell isolation kit (EasySep Magnet; StemCell Technologies, Inc, Vancouver, BC) were used to isolate CD31⁺ retinal endothelial cells according to the manufacturer's instructions. Primary endothelial cells were cultured in complete medium (Dulbecco's modified Eagle's medium [DMEM], 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM Hepes, 1% nonessential amino acids, 55 U/mL heparin), and endothelial growth supplement.

Pericytes were separated from the CD31⁻ population after digestion of P4 to P5 retinas with collagenase type II (1 mg/mL; Worthington) and were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, and recombinant interferon-γ (R&D Systems, Minneapolis, MN) at 44 U/mL, as described by Scheef et al.²³

Murine astrocytes were obtained from Tong Zheng (University of Florida, Gainesville, FL) and were cultured in DMEM/F12 medium containing 5% FBS, 20 μg/mL pituitary extract, 100 μM putrescine, 5 μg/mL insulin, 20 μM progesterone, 25 μg/mL transferrin, 30 nM sodium selenite, 20 ng/mL epidermal growth factor, and 10 ng/mL fibroblast growth factor, as described previously.²⁴

Migration Assay

Astrocytes, retinal endothelial cells, and pericytes between passages 3 to 6 were harvested for migration assays. Cells were resuspended in DMEM with 0.5% BSA; 5×10^4 cells were added to fibronectin-coated Transwells with 8.0-μm pore sizes of 24-well companion plates (Corning Inc., Lowell, MA). Mock or recombinant CTGF protein was added in the lower chamber. In some assays, the CTGF protein was preincubated with the rabbit polyclonal CTGF antibody or IgG overnight at 4°C before it was added to the lower chamber. In addition, 0.5 μg/mL CTGF protein or 1% FBS was placed in the upper or lower chamber, or both, to test the chemokinetic activities of these proteins. Migration assay conditions were 6 hours at 37°C in 5% CO₂. After mechanical removal of the cells remaining on the upper surface of the filter, cells that migrated to the undersurface were stained in 0.1% crystal violet and counted in 10 random fields at 100× magnification, as described previously.¹²

Neonatal Retinal Angiogenesis Model

CTGF antibody or rabbit IgG control was injected into the intraocular region between the equator and the corneal limbus of the right eyes of P2 litters using 33-gauge needles. Each eye received 0.5 μg protein per injection and was enucleated at P4; this was followed by fixing in 4% paraformaldehyde and staining with isolectin IB4. Untreated rabbit IgG or CTGF antibody-injected retinal flatmounts were visualized under laser spinning confocal microscopy with 100× magnification using the same setting. The surface areas covered by isolectin IB4-stained vessels were measured as a percentage of the entire retinal areas. For uniformity, all vascularity measurements were further normalized with the untreated collateral eyes.

Images of tip cells with filopodial extension at vascular fronts were taken at 600× magnification. Projected images were produced from a set of Z-series images and converted to grayscale to facilitate filopodia visibility. The outline of the vessels at vascular fronts was measured,

and filopodia numbers per 100 μm -membrane length were counted using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Laser-Induced Ischemic Retinopathy Model

GFP⁺ C57BL6 chimeric mice ($n = 40$) were generated according to the previously described method.²⁵ In brief, 6-week-old C57BL6 recipient mice were irradiated with 9.50 Gy and transplanted with GFP⁺ whole bone marrow cells through retro-orbital injection. GFP⁺ bone marrow cells were isolated from the tibias and femurs of *UBC-GFP* transgenic mice, and 1×10^5 cells in 100 μL PBS were injected. Flow cytometry was performed to analyze hematopoietic reconstitution 3 months later. Chimeric mice with engraftment levels greater than 80% of donor-derived peripheral blood monocytes were used for laser injury. Retinal vessel photocoagulation was performed on half the optic disc of each right eye with an argon laser. Venous occlusions were accomplished using laser parameters of 1-second duration, 50- μm spot size, and 100-mW intensity, in accordance with a previous publication.²⁵ Intravenous infusion of the photosensitizing rose bengal dye (Sigma, St. Louis, MO; 20 mg/kg body weight) through tail veins was performed right before laser photocoagulation to induce specific vascular thrombosis. Recombinant CTGF protein tagged with 3 $\times$ FLAG epitopes was purified from Chinese hamster ovary cells using M2-conjugated beads (Sigma) in accordance with a previous study.¹² VEGF-A (100 ng; R&D Systems, Minneapolis, MN), 1 μg CTGF protein, or CTGF antibody were intravitreally injected immediately after laser photocoagulation. In some experiments, CTGF protein was preincubated with its specific antibody (1 μg) at room temperature for 30 minutes before the intravitreal injection. In brief, mice were anesthetized by intraperitoneal injection of tribromoethanol (Avertin; 200 mg/kg) diluted in PBS. Pupils were dilated with 1% tropicamide (Tropicacyl) and 1% atropine sulfate ophthalmic solution. Intravitreal injections were made using a trinocular stereo dissecting microscope. A small pilot hole 0.5 mm behind the limbus of each right eye of the GFP⁺ C57BL6 chimeric mice was created with a 30-gauge needle. Samples in 2- μL volume were delivered through the pilot hole into the intravitreal regions of right eyes with a 30-gauge steel needle with a conical style noncoring point that was fixed on a 10- μL Hamilton microsyringe (Hamilton Company, Reno, NV). Left eyes served as untreated controls. Retinal flatmounts

were used to visualize the extent of vascular repair at 3 weeks after laser treatment.

Statistical Analysis

Each experiment was repeated at least three times with similar results. All statistical analyses were conducted using Student's *t*-test and one-way analysis of variance. Values were considered statistically significant when $P < 0.05$.

RESULTS

Postnatal Induction of CTGF during Retinal Development

Murine postnatal retinal development involves extensive neovascularization resulting in the formation of three layers of retinal vasculature within 3 weeks after birth.⁵ The superficial vascular plexus grows radially in the ganglion cell layer (GCL) and covers the periphery of the retina within the first 10 postnatal days (P0–P10). Subsequent sprouting during P7 to P15 results in the formation of the deep plexus in the inner nuclear layer (INL). The third retinal vascular layer, termed the intermediate plexus, grows between these two layers of the vascular plexuses around P15 to P21. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) showed a sustained induction of *CTGF* mRNA in postnatal developing retinas (Fig. 1A). Real-time quantitative PCR analysis confirmed that there were 3.50-, 5.49-, 9.66-, and 10.64-fold increases of *CTGF* mRNA at P10, P15, P21, and adult stages compared with that at P2. Further immunofluorescent staining of retinal cross-sections detected no CTGF protein at P0, whereas accumulating CTGF protein was found in the GCL and INL at various stages of developing retinas compared with IgG control (Fig. 1B–F). Nonetheless, no perfect correlation was seen between CTGF staining (Fig. 1E) and the high level of *CTGF* mRNA at the adult stage (Fig. 1A).

To determine the cellular sources of *CTGF* in GCL, we used transgenic mice expressing GFP under the control of the *CTGF* promoter (*CTGFp-GFP*) and carried out laser scanning confocal microscopy analysis of retinal flatmounts. *Griffonia simplicifolia*

FIGURE 1. Induction of *CTGF* at the mRNA and protein levels during postnatal retinal development in mice. (A) Semiquantitative RT-PCR analysis of *CTGF* mRNA in retinas from P0 to adult stage. Fold increase of *CTGF* mRNA at various time points was also analyzed in real-time quantitative PCR by comparison with that of P2. β -Actin was used for a loading or normalization control. * $P < 0.01$. (B–E) Immunofluorescent staining of retinal cross-sections from P0 to the adult stage shows an accumulation of CTGF protein on the GCL and INL of developing retinas. CTGF immunoreactive signals were also found in the choroid (CH), a vascular layer adjacent to the retina during development. (F) IgG control staining. (blue) Nuclei are labeled with DAPI (4', 6-diamidino-2-phenylindole). Scale bars, 20 μm .

folia-derived isolectin B4 (GSI), which specifically labels the basement membrane of endothelial cells,²⁶ was used to visualize blood vessels. As early as P0.5, GFP fluorescence as an indicator of *CTGF* expression was observed in cells of the growing GSI⁺ superficial vascular plexus around the optic nerve head (ONH) (Fig. 2A). GSI⁺ perivascular cells and GSI⁺ endothelial cells, even the tip cells with filopodia at vascular fronts, highly expressed *CTGF* (Fig. 2B). It has been known that arteries and veins often alternate in a strict radial pattern around ONH. Arterial vessels are often associated with large capillary-free zones, whereas veins are connected with many small capillaries. We observed that *CTGF* expression was similar in veins and arteries when they were morphologically distinguishable at P6 (Fig. 2C). A relatively high level of *CTGF* expression was found in cells situated outside the vessel trunks of the growing P6 primitive plexus (Fig. 2D). In contrast, astrocytes that were stained with cell-specific marker GFAP were not cellular sources of *CTGF* (Fig. 2E). Such vascular-specific expression of *CTGF* was also observed in the intermediate and deep plexuses within INL during postnatal retinal development (data not shown).

Stimulation of the Migration of Primary Retinal Endothelial Cells, Pericytes, and Astrocytes by CTGF Protein In Vitro

The vascular-specific expression of *CTGF* led us hypothesize that this protein might act on astrocytes, retinal endothelial cells, and pericytes. To test this hypothesis, we used primary murine astrocytes, CD31⁺ retinal endothelial cells, and CD31⁺ pericytes. The effects of CTGF on the migration of these cells were tested by in vitro transwell assays. As shown in Figure 3A, recombinant CTGF protein stimulated a nearly 2.68-fold increase of astrocyte migration, a 2.39-fold increase of retinal endothelial cell migration, and a 3.17-fold increase of pericyte migration. The polyclonal rabbit antibody that stained CTGF protein specifically (Figs. 1B–F) recognized the C terminus of the CTGF protein, which contains the thrombospondin type I repeat and cystine knot motif, the two regions that are important for integrin-mediated cell adhesion.²⁷ Preincubation with this antibody almost abolished these CTGF-mediated activities, whereas preincubation with IgG control did not change cell migration

FIGURE 2. Expression of *CTGF* gene in the superficial vascular plexus of murine retinas during early postnatal development. Retinal flat-mounts from transgenic mice carrying *CTGFp-GFP* were visualized for GFP as an indicator for *CTGF* expression, GSI-stained endothelial cells (red), and merged images. (A, C) Superficial plexus that grew radially from ONH as a monolayer toward the periphery of P0.5 (A) and P6 (C) retinas. (B, D) Higher magnifications of boxed areas in A and C. PC, pericytes; F, filopodia of tip cells at vascular front; v, vein; a, artery. (E) Little colocalization was observed between GFP and the astrocyte marker GFAP (blue), indicating that *CTGF* was not expressed by astrocytes. Scale bars: 50 μ m (A, C); 25 μ m (B, D, E).

FIGURE 3. Stimulation of the migration of primary astrocytes, retinal endothelial cells, and pericytes by CTGF protein in vitro. **(A)** Each cell type was incubated with or without 500 ng/mL recombinant CTGF protein in transwell assays. The CTGF-stimulated migration of primary astrocytes, retinal endothelial cells, and pericytes was abolished by preincubation of CTGF protein with 25 μ g/mL CTGF antibody, whereas preincubation with rabbit IgG control (25 μ g/mL) had little effect on the CTGF-mediated migration. RECs, retinal endothelial cells; Ab, CTGF antibody. The relative chemotactic index was calculated in comparison with Mock controls. **(B)** CTGF was chemokinetic to mediate astrocyte migration. CTGF protein or serum was added in the upper, lower, or both chambers of Transwells in the checkerboard method. Migrated cells per field were counted under a microscope. Each value is the mean $\pm$ SD from triplicate measurements. * $P < 0.01$; ** $P < 0.05$; N.S.: not significant.

very much. These results indicate that CTGF stimulates the migration of astrocytes, retinal endothelial cells, and pericytes. The polyclonal rabbit antibody could functionally block CTGF-mediated migration in vitro.

Chemokinesis is a cellular response associated with the speeding up of random movement and is gradient independent.²⁸ We examined the chemokinetic activity of CTGF on astrocyte migration using the checkerboard method. It was found that CTGF was capable of stimulating astrocyte migration to the lower chamber even when it was placed with cells in the upper chamber (Fig. 3B). Similar activity was observed when CTGF was placed in either the upper or the lower chambers. The highest cell migration was found when CTGF was present in both chambers. In contrast, few astrocytes migrated when serum was in the lower or both chambers. These results demonstrated a chemokinetic response of CTGF to promote astrocyte migration.

Inhibition of Retinal Angiogenesis by Intraocular Injection of CTGF Antibody

The superficial vascular plexus has been known to grow along preexisting astrocyte networks that not only serve as scaffold but also produce guidance cues such as VEGF-A to guide vascular sprouting. The formation of the superficial vascular plexus is a good experimental system with which to quantitatively examine the activity of proangiogenic or antiangiogenic factors. A pharmacologic approach of blocking CTGF activity was used to test the importance of CTGF in the growth of the superficial vascular plexus at an early stage of retinal angiogenesis. Blocking of CTGF function was attained by injection of the rabbit polyclonal antibody with blocking activity into the right eyes of P2 mice expressing GFP under the control of *GFAP* promoter (*GFAPp-GFP*). As shown in Figures 4A to 4E, CTGF antibody treatment decreased the areas of vascular and astrocyte networks at P4 by $57.0\% \pm 9.0\%$ and $72.6\% \pm 4.8\%$, respectively, compared with the corresponding untreated collateral eyes. In contrast, IgG treatment did not influence the growth of the superficial plexus and astrocyte networks and showed $92.5\% \pm 8.8\%$ vascularization and $100.3\% \pm 2.1\%$ dense astrocyte networks compared with the untreated collateral eyes. These results indicate that CTGF is important for the growth of the superficial plexus and astrocyte networks.

Filopodia extension is a dynamic process by which tip cells at vascular fronts sense guidance cues during vessel growth. CTGF antibody treatment seemed to disturb filopodial formation (Fig. 4F). Quantification analysis showed that the filopodial number per 100- μ m membrane was 19.8 ± 0.93 in IgG-treated retinas and 7.98 ± 0.94 in CTGF antibody-treated retinas (Fig. 4G). This significant decrease in filopodial number further supports our observations that CTGF inhibition affects vascular sprouting of the superficial plexus during early stages of retinal development.

CTGF Expression in Perivascular Cells of Arterial Trunks of Adult Retinas

The expression pattern of the *CTGF* gene was also examined in the superficial vascular plexus at the adult stage using *CTGFp-GFP* transgenic mice. Confocal immunofluorescence microscopy analysis in retinal flatmounts revealed a differential pattern of *CTGF* expression between arteries and veins that showed distinct morphology in adult retinas.²⁹ Veins tend to be wider and more tortuous than arteries, which have a strong muscular wall. As shown in Figures 5A to 5C, histologic analysis of the GFP expression pattern in *CTGFp-GFP* mice showed that arterial trunks highly expressed *CTGF*, particularly in smooth muscle-like cells that were made up of outer layers of blood vessel walls. Only a few pericytes with typical processes situated on the outside surface of venules were GFP⁺. In the arterial trunks, endothelial cells inside the blood vessel walls were GFP⁻ and did not express *CTGF* (Fig. 5D). These results indicated a unique pattern of *CTGF* expression in perivascular cells of mature retinal vasculatures. This pattern was different from the patterns we observed in the early stages of retinal development (Figs. 2A–D) and might reflect different cellular functions of CTGF between retinal angiogenesis and homeostasis.

Promotion of Fibrovascular Proliferation by CTGF in the Retinal Ischemia Model

We used a murine ischemic retinopathy model that mimics diabetic retinopathy in human patients to test whether CTGF contributes to vascular repair. This model has been shown to induce neovessel formation in response to exogenous VEGF-A administration combined with laser photoco-

FIGURE 4. Inhibition of retinal angiogenesis during early postnatal development by intravitreal injection of CTGF antibody. Transgenic mice carrying *GFAPp-GFP* at P2 were treated with IgG control or CTGF antibody (Ab). Astrocytes were visualized directly by GFP, and blood vessels were stained with GSI in red. Vascularized areas (A, E), underlying dense astrocyte networks (C, E), and filopodial extension (F) were analyzed in retinal flatmounts of P4 neonates. (white lines) Borders of growing blood vessels and dense astrocyte networks underlying nascent blood vessels. Scale bars: 100 μ m (A, C); 50 μ m (E); 20 μ m (F). In quantitative analysis, total areas of GSI-stained vessels (B) and the dense *GFAPp-GFP*⁺ astrocyte networks underlying blood vessels (D) in IgG- or Ab-treated eyes were expressed as a percentage of the corresponding contralateral eyes (100%). Data were obtained from 15 IgG-injected retinas and 15 Ab-injected retinas from three independent experiments. Each value is mean $\pm$ SEM. (G) Quantitative analysis of filopodial number per 100- μ m membrane length. Data are mean $\pm$ SEM from five random images in sprouting edges per retina ($n = 6$). * $P < 0.01$.

agulation.^{9,25} Chimeric mice that had been transplanted with GFP⁺ bone marrow cells were used to facilitate the visualization of neovascularization sites by incorporating GFP⁺ cells. Intravenous injection of the photosensitizing dye rose bengal was used to induce specific vascular damage. As shown in Figure 6A, retinal flatmounts showed that VEGF-A primarily recruited GFP⁺GSI⁺ endothelial cells that were incorporated into damaged vessels after treatment by laser photocoagulation and rose bengal. This result was consistent with our previous observations in this ischemia retinopathy model in which EPCs are recruited by VEGF-A to mediate neovascularization.⁹ Interestingly, the addition of CTGF after rose bengal/laser injury significantly induced GFP⁺GSI⁺ perivascular cell recruitment around repairing vessels (Fig. 6B). Preincubation of CTGF protein with its specific antibody lost its activity in recruiting GFP⁺GSI⁺ perivascular cells (Fig. 6C). These results indicate that CTGF activates perivascular cells of bone marrow origin, whereas VEGF-A only gives an endothelial-specific reaction during retinal ischemia after laser injury.

Retinal ischemia in advance stages of diabetic retinopathy in human patients is capable of inducing the formation of scar-like fibrocellular membranes, which occur on the surface of the vitreous in the periretinal space and are rich in ECM molecules such as collagen fibers. In the laser-induced retinopathy model, 2 of 5 mice that received both CTGF and VEGF-A developed fibrovascular membranes detected by hematoxylin and eosin (H&E) staining (Figs. 7A–D). Further, Van Gieson's trichrome staining confirmed that abundant collagen fibers were heavily deposited in the fibrovascular membranes from CTGF- and VEGF-A-treated eyes compared with control eyes in the same animals (Figs. 7E, 7F). Such fibrovascular membranes were not observed in mice that received VEGF-A. These results indicate that CTGF promotes fibrovascular proliferation during retina ischemia after laser injury.

DISCUSSION

Murine postnatal retinal development involves several processes. The migration, proliferation, and differentiation of neu-

FIGURE 5. Differential expression of *CTGF* in perivascular cells between the artery and vein of the superficial plexus at adult stage. (A) Adult retinal flatmounts showing strong expression of *CTGF* as indicated by GFP in arteries. a, artery; v, vein. The most prominent expression was found in first-order arterioles (B) and decreased subsequently in second-order arterioles and third-order arterioles (C). (D) *CTGF* was specifically expressed in GSI^{-} perivascular cells (white arrows) but not in GSI^{+} endothelial cells (white arrowheads) inside the blood vessel walls. (blue) Nuclei were stained with DAPI. Scale bars: (A) 50 μm ; (B, C) 25 μm ; (D) 20 μm .

ronal precursors are initiated at embryonic day 12. Neuronal differentiation often ends at P8. The onset of vision occurs at P14 when the mice open their eyes. Vascularization is another key physiological process to supply oxygen and nutrients for neuronal differentiation and visual cascade. The mouse retina is avascular at birth.⁵ Three layers of retinal vasculatures are formed in the GCL and INL within 3 weeks. We found minimal *CTGF* at the mRNA and protein levels at P0 with a constant increase during development, which is consistent with previous expression patterns seen in whole embryos³⁰ and in developing retinas that have been reported in a database for global gene expression analysis of the developing mouse retina.³¹ Furthermore, a 2.8-fold increase of *CTGF* expression has been reported in P18 retinas of neonatal mice exposed to >75% oxygen in the oxygen-induced retinopathy model.³² In this study, we performed a comprehensive study to identify the specific cell sources of *CTGF* expression using a *CTGFp-GFP* reporter mouse. *CTGF* expression was found in perivascular cells, endothelial cells, and tip cells of the superficial vascular plexus but not in astrocytes underlying the nascent blood vessels. The *CTGF* antibody used in this study specifically stained *CTGF* in retinas and blocked the *CTGF*-stimulated migration of astrocytes, retinal endothelial cells, and pericytes in vitro. Intravitreal injection of this antibody blocked the growth of the superficial vascular plexus and filopodial burst during angiogenic sprouting. Smaller areas of underlying astrocyte networks were associated with the disturbed retinal angiogenesis.

These observations led us to conclude there is a primary role for *CTGF* in vascularization during retinal development.

In this study, a high level of *CTGF* mRNA was detected by semiquantitative RT-PCR analysis at the adult stage. Retinal flatmounts on *CTGFp-GFP* transgenic mice showed that *CTGF* was highly expressed in perivascular cells, particularly the cells situated outside arterial trunks of the superficial plexus in GCL at the adult stage. Vascular-specific *CTGF* expression in deep and intermediate plexuses of the INL was also observed (data not shown). However, the immunofluorescent staining of *CTGF* only detected faint spots in the GCL and INL of adult retinas, whereas strong *CTGF* immunoreactive signals were found in the GCL and INL of P10 and P21 retinas. This discrepancy between the high level of *CTGF* expression and the faint staining of *CTGF* protein might be due to rapid turnover of *CTGF* protein with unknown mechanism in adult retinas.

Fischer et al.³³ showed a specific pattern of contractile pericytes in arterial walls of adult retinas using transgenic mice carrying GFP under the control of α -smooth muscle actin (SMA). In adult retinal flatmounts, GFP signal as an indicator for SMA is most prominent in first-order arterioles and decreases subsequently in second-order arterioles and third-order arterioles. Few GFP⁺ SMA cells have been found in retinal venules.³³ This pattern was similar to that of the *CTGF* expression we observed in adult retinas of *CTGFp-GFP* transgenic mice, supporting pericytes in the artery as a major cellular source of *CTGF* in mature vessels. However, *CTGF* expression spread all over vessel trunks and small capillaries in the growing super-

FIGURE 6. Activation of perivascular cells of bone marrow origin by CTGF in the laser-induced ischemic retinopathy model. Neovascularization and vascular repair were indicated by the incorporation of bone marrow-derived GFP cells around GSI-stained vessels. (A) VEGF-A injection specifically recruited GSI⁺GFP⁺ endothelial cells. (B) Exogenous CTGF protein activated a large number of GSI⁺GFP⁺ perivascular cells during vascular repair. (C) Preincubation of CTGF protein with its specific antibody lost its activity in activating GSI⁺GFP⁺ perivascular cells during vascular repair. Ab, CTGF antibody. (blue) Nuclei were stained with DAPI. Scale bars, 25 μ m.

ficial vascular plexus at P0.5 and P6. This is in a contrast to the differential expression of SMA among artery, vein, and capillary in the early growth of the superficial plexus during retinal development.⁵⁴ Thus, *CTGF*-expressing cells in developing retinas must be heterogeneous populations. Some of them should be mesenchymal progenitor cells that are eventually differentiated into SMA⁺ pericytes after being exposed to hemodynamic factors such as the hydrostatic pressure gradient during the establishment of blood flow.

Astrocytes are important cell types in retinas. They not only form preexisting networks to guide the growth of the superficial vascular plexus during development but also play an important role in the function of the blood barrier.^{5,55} Although CTGF was not normally expressed in GFAP⁺ astrocytes during development, CTGF antibody treatment significantly decreased areas of dense astrocyte networks underlying nascent blood vessels. Furthermore, recombinant CTGF protein stimulated astrocyte migration in vitro. CTGF was chemokinetic and stimulated astrocyte migration through a gradient-independent manner. Thus, CTGF that is produced from perivascular cells in retinas likely acts in a paracrine manner and regulates astrocyte function. Given that astrocytes represent one potential source of adult stem cells in the central nervous system (CNS) and often become active in the repair of CNS injuries such as gliosis,³⁶ CTGF may act on astrocytes and participate in wound healing of the CNS.

Wound healing involves a tightly coordinated series of events, including recruitment, activation, proliferation and migration of inflammatory and resident cells, release of cytokines and growth factors, and secretion of ECM during tissue repair. CTGF has been known to be an active player during tissue repair in various models.^{37,38} The upregulation of *CTGF* in diabetic retinas and the colocalization of CTGF proteins with VEGF-A at neovascularization sites have been reported in human patients and experimental animal models.^{13,39–41} An amino-terminal fragment of CTGF is accumulated in the vitreous of patients with PDR.⁴² Using the laser-induced ischemic retinopathy mouse model that mimics PDR in human patients, we showed that CTGF promoted a distinct cellular response in

comparison to VEGF-A. VEGF-A primarily induced endothelial cells of bone marrow origin that were directly incorporated into damaged blood vessels. In contrast, CTGF recruited a large number of perivascular cells of bone marrow origin to the sites of vascular injury. These cells may be contractile cells, such as myofibroblasts that are derived from activated pericytes responsible for tissue repair. CTGF promoted the formation of fibrovascular membranes in mice that were treated with VEGF-A, rose bengal, and laser injury. These results are consistent with the current notion that CTGF and VEGF-A regulate an angiofibrotic switch in PDR.⁴³ CTGF appears to have a dual role in angiogenesis and wound repair/fibrosis. A physiological level of CTGF in retinal vascular beds is required for its proper function during retinal angiogenesis and homeostasis, whereas an aberrant upregulation of CTGF seen in many ocular diseases promotes fibrosis, at least in part, by activating perivascular cells that are highly contractile and causes scar formation as part of uncontrolled tissue repair. Targeting to CTGF may be important for therapeutic intervention against PDR and other eye diseases related to ocular angiogenesis and injury repair.

The ability to remodel the vascular system in response to growth or injury is a requirement for life as a complex organism. A variety of cells, from bone marrow-derived endothelial progenitor cells to peripheral circulating endothelial cells, serve as remote progenitors that can participate with locally proliferating cells in the formation of new blood vessels in response to a wide array of proangiogenic factors. Ischemia/hypoxia is a primary inducer of many of these factors, as are other stress and injury response pathways. CTGF is a hypoxia-inducible protein^{44,45} with multiple cellular targets in ocular repair. The broad cellular effect of CTGF is likely due to the lack of its specific cognate receptor. CTGF is able to bind to many subtypes of integrins on the cell surface and exhibits contextual effects in different cell types. This protein is also capable of interacting with growth factors, ECM protein, and many other cell surface receptors. Future studies about understanding the diverse function of CTGF at the molecular and cellular levels may help in the development of therapeutic

FIGURE 7. CTGF and VEGF-A promoted the formation of thick fibrovascular membranes in the laser-induced ischemic retinopathy model. (A, B) H&E staining of retinal cross-sections from mice that received CTGF and VEGF-A after laser injury. (B) H&E staining of untreated control eyes. (A, B) 100 $\times$ magnification observed in bright-field and phase-contrast microscopy. (C, D) Taken from 400 $\times$ magnification of boxed areas in A and B. (blue) Nuclei. (pink) Cytoplasm. (A, C) Notably, subretinal fibrovascular membranes (FVM) were observed. (E, F) The Van Gieson's trichrome staining for collagen fibers using the same retinal cross-sections as in A and B. (E, F) Images were taken with 400 $\times$ magnification. (dark brown) Nuclei. (pink) Cytoplasm. (blue) Collagen fibers in FVMs.

strategies against aberrant neovascularization and fibrosis pathways in the eye and other organs.

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