

Connective Tissue Growth Factor as a Mediator of Intraocular Fibrosis

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PURPOSE. To investigate the role of connective tissue growth factor (CTGF) in the pathogenesis of proliferative vitreoretinopathy (PVR).

METHODS. Expression of CTGF was evaluated immunohistochemically in human PVR membranes, and the accumulation of CTGF in the vitreous was evaluated by ELISA. The effects of CTGF on type I collagen mRNA and protein expression in RPE were assayed by real-time PCR and ELISA, and migration was assayed with a Boyden chamber assay. Experimental PVR was induced in rabbits with vitreous injection of RPE cells plus rhCTGF; injection of RPE cells plus platelet derived-growth factor, with or without rhCTGF, or by injection of RPE cells infected with an adenoviral vector that expressed CTGF.

RESULTS. CTGF was highly expressed in human PVR membranes and partially colocalized with cytokeratin-positive RPE cells. Treatment of RPE with rhCTGF stimulated migration with a peak response at 50 ng/mL ($P < 0.05$) and increased expression of type I collagen ($P < 0.05$). There was a prominent accumulation of the N-terminal half of CTGF in the vitreous of patients with PVR. Intravitreal injection of rhCTGF alone did not produce PVR, whereas such injections into rabbits with mild, nonfibrotic PVR promoted the development of dense, fibrotic epiretinal membranes. Similarly, intravitreal injection of RPE cells infected with adenoviral vectors that overexpress CTGF induced fibrotic PVR. Experimental PVR was associated with increased CTGF mRNA in PVR membranes and accumulation of CTGF half fragments in the vitreous.

CONCLUSIONS. The results identify CTGF as a major mediator of retinal fibrosis and potentially an effective therapeutic target for PVR. (*Invest Ophthalmol Vis Sci.* 2008;49:4078–4088) DOI:10.1167/iovs.07-1302

Fibrosis plays an important role in the pathogenesis of several common blinding disorders, including proliferative diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration, and PVR^{1–4}; however, much remains to

be learned about the basic pathophysiology of fibrosis in the intraocular environment.¹ PVR may be viewed as a prototypical example of a protracted intraocular wound-healing response that occurs when traction-generating cellular membranes develop in the vitreous and on the inner or outer surfaces of the retina after rhegmatogenous retinal detachment or major ocular trauma.^{5–7} RPE cells play a critical role in this epiretinal membrane formation.^{8,9} These cells proliferate and migrate from the RPE monolayer to form sheets of dedifferentiated cells within a provisional extracellular matrix (ECM) containing fibronectin and thrombospondin.^{9–11} The protracted wound-healing response causes the cellular membrane to become progressively more paucicellular and fibrotic.¹¹ Experimental models of PVR have been developed to evaluate intraocular proliferation^{12–16}; however, these models typically exhibit cellular fibrinous strands without prominent fibrotic responses. Studies evaluating the role of those factors that elicit this fibrotic response are of particular interest.

Normal ocular wound healing involves a tightly coordinated series of events: recruitment and activation of inflammatory cells; release of cytokines and growth factors; activation, proliferation, and migration of ocular cells; secretion of ECM; and tissue remodeling and repair.^{1,17} CTGF is an important stimulant of fibrosis,¹⁸ but its role in intraocular wound healing or PVR has not been studied in detail.

CTGF is a secreted, cysteine-rich, heparin-binding polypeptide growth factor^{19,20} that is rapidly upregulated after stimulation with serum or transforming growth factor (TGF)- β . Various CTGF fragments have been shown to accumulate in tissue culture or body fluids while retaining their biological activity.^{20–22} CTGF functions as a downstream mediator of TGF- β action on fibroblasts; it stimulates cell proliferation and cell matrix deposition (collagen 1 and fibronectin),^{18,20,23} and it may induce apoptosis.^{24,25} In addition to its action as a growth factor, CTGF has been implicated as an adhesive substrate in fibroblasts, mediated through $\alpha 6 \beta 1$ integrin.²⁶ Of importance, CTGF is coordinately expressed with TGF- β , and it demonstrates increased expression in numerous fibrotic disorders, including systemic sclerosis,^{27,28} pulmonary, renal, and myocardial fibrosis,^{29–32} and atherosclerosis.³³ In the present study, we examine the process by which CTGF mediates the transformation of activated RPE into a fibrotic epiretinal membrane. Our results identify CTGF as a major mediator of retinal fibrosis and potentially an effective therapeutic target.

MATERIALS AND METHODS

The institutional review board (IRB) of the University of Southern California approved our use of cultured human RPE cells, human PVR specimens, and human vitreous samples. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from all participants.

RPE Cultures

Human RPE cells were isolated from fetal human eyes of >22 weeks' gestation (Advanced Bioscience Resources, Inc., Alameda, CA). The cells were cultured in DMEM (Fisher Scientific, Pittsburgh, PA) with 2

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mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA). The culture method used, a standard practice in our laboratory for more than 10 years, regularly yields >95% cytokeratin-positive RPE cells.³⁴ The cells used were from passages 2 to 4.

Rabbit RPE cell cultures were obtained from pigmented adult rabbits. Briefly, the globes were opened and the cornea, lens, and vitreous humor were removed by a circumferential cut just posterior to the ora serrata. The neural retina was carefully washed out with the RPE medium. The eye cups were washed with Hanks' balanced salt solution and digested with 0.012% (wt/vol) trypsin (Sigma-Aldrich) in 0.005% (wt/vol) EDTA (Sigma) for 1 hour at 37°C. The trypsinization was stopped by adding excess DMEM. The dissociated RPE cells were carefully washed out without disturbing the underlying choroid. The RPE cells were first cultured with the medium in 12-well plates to near confluence and then passaged to 25-cm² flasks.

Growth Factor and Vectors

rhCTGF, rabbit anti-CTGF polyclonal antibody, domain-specific anti-CTGF monoclonal antibodies for enzyme-linked immunosorbent assay (ELISA), and a recombinant adenovirus vector that expresses human CTGF were all gifts from FibroGen, Inc. (South San Francisco, CA).

Human Vitreous Samples

Vitreous samples were obtained at the time of pars plana vitrectomy by participating ophthalmologists from patients with PVR+retinal detachment ($n = 6$), patients with uncomplicated retinal detachment ($n = 5$), and control patients without proliferative retinal disease (macular hole, macular pucker, or epiretinal membrane; $n = 12$). Undiluted samples were placed on ice. Samples were promptly centrifuged at 36,000 rpm at 4°C, and the supernatants were frozen at -80°C until assayed.

Immunohistochemical Staining of PVR Membranes

Epiretinal membranes were surgically excised from 10 patients with PVR (men 3, women 7; age range: 48-78 years). Tissues were snap frozen and 6-µm sections were cut with the cryostat. Thawed tissue sections were air dried, fixed in 4% paraformaldehyde (10 minutes), washed with phosphate-buffered saline (PBS; pH 7.4), and blocked with 5% normal goat serum for 15 minutes. Anti-CTGF polyclonal antibody (1:400; FibroGen, Inc.) was applied to the tissue sections and then with secondary biotinylated anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA) for 30 minutes and sites of immunostaining revealed with the ABC staining methods (Vector Laboratories). For double staining, the sections were incubated with anti-CTGF antibody for 60 minutes and with rhodamine secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 30 minutes; mouse anti-human pancytokeratin (Sigma-Aldrich) was added to cover the tissue, and the slides were incubated for 1 hour at room temperature to label the cytokeratin-positive cells. Secondary fluorescein isothiocyanate-conjugated anti-mouse antibody (Vector Laboratories) was then applied for another 30 minutes. After each step of the incubation, sections were washed with PBS three times for 5 minutes each. Finally, the samples were examined with a confocal laser scanning microscope (LSM510; Carl Zeiss Meditec, Inc., Thornwood, NY).

Type 1 collagen expression in rabbit PVR membranes was analyzed by the application of goat anti-type 1 collagen antibody (Chemicon International, Inc., Temecula, CA). After incubation for 60 minutes with primary antibodies, biotinylated secondary anti-goat antibody (1:200; Vector Laboratories) and streptavidin peroxidase (Vector Laboratories) were then applied to the sections sequentially. Between each step, the sections were washed three times with PBS. Immunoreactivity was visualized using the peroxidase substrate amino ethyl carbazole (AEC kit; Zymed Laboratories, Inc. South San Francisco, CA). Slides were rinsed with tap water, counterstained with hematoxylin, and mounted with glycerin-gelatin medium.

For each of the immunostain procedures, negative controls included omission of primary antibody and use of an irrelevant polyclonal or isotype-matched monoclonal primary antibody; in all cases negative controls showed only faint, insignificant staining.

Migration Assay

Migration was measured with a modified Boyden chamber assay, as previously described.³⁵ Briefly, 5×10^4 human fetal RPE cells (passage 2 to 4) were seeded in the upper part of a Boyden chamber in 24-well plates, with inserts coated with fibronectin (2 µg/cm²). The lower chamber was filled with 0.4% FBS-DMEM containing 1 to 100 ng/mL rhCTGF. After a 5-hour incubation, the inserts were washed three times with PBS, fixed with cold methanol (4°C) for 10 minutes, and counterstained with hematoxylin for 20 minutes. The number of migrated cells was counted by phase-contrast microscopy ($\times 320$). Four randomly chosen fields were counted per insert. The experiment was repeated three times.

Real-Time Polymerase Chain Reaction for Analysis of Collagen 1 Gene Expression

The RPE cells were treated with rhCTGF 30 ng/mL in the presence of 1% FBS for 24 to 96 hours. Total RNA was extracted from RPE cells (TriZol reagent; Invitrogen, Carlsbad, CA). The primer sequence for type I collagen was CCGCGGTGACCCCACTCA (forward) and CGC-CATACTCGAACTGGAATC (reverse). Each polymerase chain reaction (PCR) contained equivalent amounts of total RNA. Real-time PCR was performed in duplicate with a kit used according to the manufacturer's recommendation (Roche Diagnostics, Indianapolis, IN). The quantity of mRNA was calculated by normalizing the threshold cycle value of type I collagen to the threshold cycle value of the housekeeping gene β -actin of the same RNA sample, according to the published formula.³⁶ Experiments were repeated three times.

Reverse Transcriptase PCR for Analysis of CTGF Gene Expression

Semiquantitative RT-PCR was used to measure expression of CTGF in retinal tissues microdissected from control rabbits, in retinal tissues+adherent membranes from rabbits with experimental PVR induced by intravitreal injection of RPE cells+PDGF+rhCTGF (see Rabbit PVR Models section), and in the human RPE cell line ARPE-19. Total RNA was isolated as described earlier. A set of oligo deoxynucleotide primers corresponding to sequences in the first and fifth exons of both human and rabbit CTGF genes was designed and synthesized for the amplification of full-length CTGF cDNA. After cDNA synthesis, CTGF specific primers (forward: GTC GCC TTC GTG GTC CTC CT; reverse: GCC GTC AGG GCA CTT GAA CT) were used for PCR amplification with a PCR kit (Qiagen, Valencia, CA). Since the complete rabbit CTGF sequence was not available, human CTGF primers were used to amplify rabbit CTGF cDNA. The rabbit CTGF amplicons were of the same size as those from human cDNA and DNA sequencing confirmed that the amplified rabbit CTGF cDNA matched the online partial rabbit CTGF sequence (GenBank accession number: AB217855; <http://www.ncbi.nlm.nih.gov/Genbank>); provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). After 35 cycles of PCR, products were resolved on 1.2% agarose gel and stained with ethidium bromide. The gel was photographed under ultraviolet illumination, and the appropriate product size was determined by using a 100-bp DNA ladder (Invitrogen-Gibco, Grand Island, NY) as a molecular weight marker. CTGF expression levels were normalized using β -actin cDNA as an internal loading control.

Rabbit PVR Models

Forty-one adult pigmented rabbits, 2.5 to 3.5 kg each, were used. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All proce-

dures were approved by Keck School of Medicine Institutional Animal Care and Use Committee.

RPE Injection with Platelet-Derived Growth Factor and rhCTGF. Subconfluent rabbit RPE cells (passage 2–3) were used for the injection. After trypsinization and two PBS washes, the cells were resuspended in PBS and kept on ice.

Before the RPE cell injection, 0.2 mL of vitreous was removed from each rabbit eye, using a 25-gauge needle. For each RPE cell injection, a 27-gauge needle was attached to a tuberculin syringe. The syringe was loaded with 100 μ L RPE cells (15×10^4) and 50 ng platelet-derived growth factor (PDGF-BB; R&D Systems, Inc., Minneapolis, MN). The needle was inserted through the sclera under indirect ophthalmoscopic control, 3 mm posterior to the limbus. The RPE/PDGF-BB was then deposited just over the optic disc. The second group of rabbits received the same RPE cells +PDGF injection with an additional injection of 200 ng rhCTGF 1 week after the first surgery. The same technique was used, but the injection was given at a different entrance site. On day 14, transpupillary optical coherence tomography (Humphrey Instruments, San Leandro, CA) was performed in randomly selected rabbits from each group to confirm the clinical observations. Classification of PVR was based on clinical findings according to Fastenberg et al.³⁷ Stage 0: normal retina; stage 1: intravitreal membrane; stage 2: focal traction, localized vascular changes, hyperemia, engorgement dilation; and blood vessel elevation; stage 3: localized detachment of medullary rays; stage 4: extensive retinal detachment, total medullary ray detachment, and peripapillary retinal detachment; and stage 5: total retinal detachment, retinal folds, and macular holes.

Recombinant Adenovirus CTGF-Infected RPE Cell Injection. Rabbit RPE cells were isolated and washed three times with serum-free DMEM. After the cells reached confluence, 1 mL DMEM containing 1% FBS and 1×10^7 E1/E3 region-deleted recombinant adenoviruses encoding CTGF (Ad CMV, CTGF) or green fluorescence protein (Ad CMV, GFP) (FibroGen, Inc.) was added to a monolayer of cultured RPE cells. After incubation for 60 minutes at 37°C, the medium was replaced with fresh MEM (containing 2% FBS) and incubated for an additional 48 hours. Western blot revealed a weak CTGF immunoreactive band in Ad CMV, GFP supernatants, whereas Ad CMV, CTGF supernatants contained a prominent CTGF immunoreactive band (>10 -fold increase on densitometry; results not shown). The cells were collected by using the conventional method of trypsin digestion, washed twice with PBS, and then resuspended (1×10^7 /mL) in PBS.

To establish the rabbit PVR model using CTGF adenovirus-infected RPE cell injection, the pigmented rabbits were divided into three groups: RPE cells only, Ad CMV, CTGF, and Ad CMV, GFP. Using the injection technique described in PVR model A, we injected the eyes of the first group of rabbits with 0.1 mL PBS with 10^5 normal rabbit RPE cells, the second group with 10^5 rabbit RPE cells infected with Ad CMV, CTGF, and the third group with 10^5 rabbit RPE cells infected with Ad CMV, GFP.

In all rabbits in PVR models A and B, fundus photographs were taken 1 and 2 weeks after surgery. The eyes were enucleated at 2 weeks for histologic analysis.

Western Blot Assay

Vitreous samples were obtained from rabbits with PVR at the time of death. The vitreous samples were resolved on Tris hydrochloride 10% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Hercules, CA) at 120 V, with 10 μ g of protein added to each lane. The proteins were transferred to a polyvinylidene fluoride blotting membrane (Millipore, Billerica, MA). The membranes were probed, first with polyclonal anti-CTGF antibody (FibroGen, Inc.) and then with horseradish peroxidase-conjugated goat anti-rabbit antibody (Vector Laboratories) for 30 minutes at room temperature. Images were developed with chemiluminescence detection solution (GE Healthcare, Piscataway, NJ).

ELISA Methods

CTGF ELISA. Because CTGF can undergo proteolysis and some bioactive fragments have been reported,²¹ assessing both CTGF content and form is important for understanding a potential role of CTGF in PVR. CTGF sandwich ELISAs (FibroGen, Inc.) were performed to determine the content of CTGF and CTGF fragments in vitreous humor. Pairs of CTGF-specific monoclonal antibodies were selected for capture and detection of full-length CTGF (W ELISA), full-length CTGF+CTGF NH₂-terminal half fragments (N+W ELISA), and full-length CTGF+CTGF COOH-terminal half fragments (C+W ELISA). Microtiter plates were coated overnight at 4°C with capture antibody (10 μ g/mL) in 100 μ L of coating buffer (0.05 M sodium bicarbonate, pH 9.6). The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and washed with PBS/0.05% Tween. Vitreous samples were diluted 1:15 with 0.05 M sodium carbonate, and a 50- μ L sample was added to each well, together with 50 μ L of biotinylated monoclonal anti-human CTGF detection antibody diluted in assay buffer (0.05 M Tris [pH 7.8], 0.1% BSA, 4 mM MgCl₂, 0.2 M ZnCl₂, 0.1% sodium azide, 50 mg/L sodium heparin, and 0.1% Triton X-100). The plates were incubated for 2 hours at 37°C, washed with the Tris assay buffer, and incubated with 100 μ L of streptavidin-conjugated alkaline phosphatase for 1 hour at room temperature. The ELISA was developed with *p*-nitrophenyl phosphate (1.5 mg/mL; Sigma-Aldrich) in diethanolamine buffer (1 M diethanolamine, 0.5 mM MgCl₂, 0.02% sodium azide) and read at an optical density of 405 nm. Purified rhCTGF was used as the standard. Sensitivities of these assays as determined by buffer standards were approximately 1.0 ng/mL. We did not find evidence of a substantial presence of CTGF COOH-terminal half fragments and therefore only report results for whole CTGF and CTGF NH₂-terminal half fragments. Because the ELISAs do not have identical efficiencies, CTGF values are expressed as whole CTGF and whole CTGF+CTGF NH₂-terminal half fragments.

Determination of rabbit vitreous CTGF used a separate N+W ELISA that is capable of detecting both rabbit and human CTGFs. Affinity-purified goat anti-CTGF NH₂-terminal half fragment was used as capture antibody, and detection was with a monoclonal antibody that also reacts with CTGF NH₂-terminal half fragment. Interference of the capture and detecting antibodies was tested and found to be negligible. NH₂-terminal half fragments of CTGF were generated by proteolytic cleavage of rhCTGF and purified by affinity chromatography. This material was used to affinity purify the goat polyclonal antibody. The standard used in this ELISA was rhCTGF. For these determinations, total vitreous CTGF content is reported (N+W CTGF) in nanograms per milliliter.

Detailed information about the capture and detection antibodies used in each of the ELISA assays is summarized in Table 1.

Type I Collagen ELISA Assay. Human fetal RPE cell cultures (passages 2–4) were grown to subconfluence in normal DMEM medium and incubated in serum-free medium for 24 hours. Cells were treated with TGF- β 2 (R&D Systems) or with full-length rhCTGF in 0.4% FBS for 24–96 hours. To determine collagen content, a human type I collagen detection kit (Chondrex, Redmond, WA) was used. Collagen in the tested samples was first solubilized with pepsin under acidic conditions and then further digested with pancreatic elastase at neutral pH to convert polymeric collagen to monomeric collagen. Supernatants were collected after brief centrifugation and tested directly in the ELISA kit. The experiment was repeated three times.

Statistics

The data were analyzed with Student's *t*-test. $P < 0.05$ was accepted as significant.

RESULTS

Expression of CTGF in Human PVR Membranes

Each of the 10 human PVR membranes we examined showed extensive immunoreactivity for CTGF. CTGF staining was

TABLE 1. Characteristics of Anti-CTGF Antibodies Used in ELISA Assays

ELISA	ELISA Detection	Capture Antibodies			Detection Antibodies				
		Epitope Location	Antibody Type	Antibody Species	Epitope Location	Antibody Type	Antibody Species	Antibody Tag	Standard Used
Human W CTGF	Human full-length CTGF	Domain 3	mAb	Human	Domain 1*	mAb	Human	AP	rhCTGF
Human N+W CTGF	Human full-length CTGF and human N-half CTGF	Domain 2	mAb	Human	Domain 1*	mAb	Human	AP	rhCTGF
Human C+W CTGF	Human full-length CTGF and human C-half CTGF	Domain 3	mAb	Human	Domain 3†	mAb	Human	AP	rhCTGF
Cross Species N+W CTGF	Human and rabbit full-length CTGFs and human and rabbit N-half CTGFs	CTGF N-half	Affinity-purified polyclonal	Goat	CTGF domain 2	mAb	Human	AP	rhCTGF

The epitope location, antibody type and antibody species of each of the capture and detection antibodies utilized in the 4 ELISA assays are listed. W, whole CTGF; N, N-terminal CTGF; C, C-terminal CTGF; mAb, monoclonal antibody.

* This mAb is specific for human CTGF.

† Different mAb than that used for capture in the human C+W ELISA (the capture and detection mAbs used in this C+W ELISA recognize separate, noninterfering epitopes in domain 3).

found in fibrotic regions of membranes; but was most predominantly localized in the stromal cells (Fig. 1). Many of the CTGF-positive cells were also cytokeratin positive, indicating that the cells were derived from RPE cells (Fig. 1). A smaller fraction of CTGF-positive cells showed colocalization with glial fibrillary acidic protein, indicating derivation from retinal glial cells (results not shown).

CTGF Levels in Human Vitreous

Vitreous samples were obtained from the eyes of patients with retinal detachment (RD) complicated by PVR (RD+PVR) and patients with RD but no PVR, as well as from control eyes without proliferative retinal disease (macular hole, macular

pucker, and epiretinal membrane). Levels of whole CTGF did not show an increase in the vitreous of patients with PVR or in the vitreous samples of patients with RD alone (Fig. 2a); however, there was a prominent increase in whole CTGF+CTGF NH₂-terminal half fragments in the vitreous of patients with PVR compared with the other samples ($P < 0.01$). RD without PVR was associated with a moderate increase in the level of whole CTGF+CTGF NH₂-terminal half fragments compared with the control ($P < 0.05$). Since the efficiencies in detecting CTGF differs in the two ELISA assays, the level of N-terminal half fragment CTGF cannot be determined by subtracting whole CTGF from whole CTGF+CTGF NH₂-terminal half fragments; however, it can be implied that the increase in whole

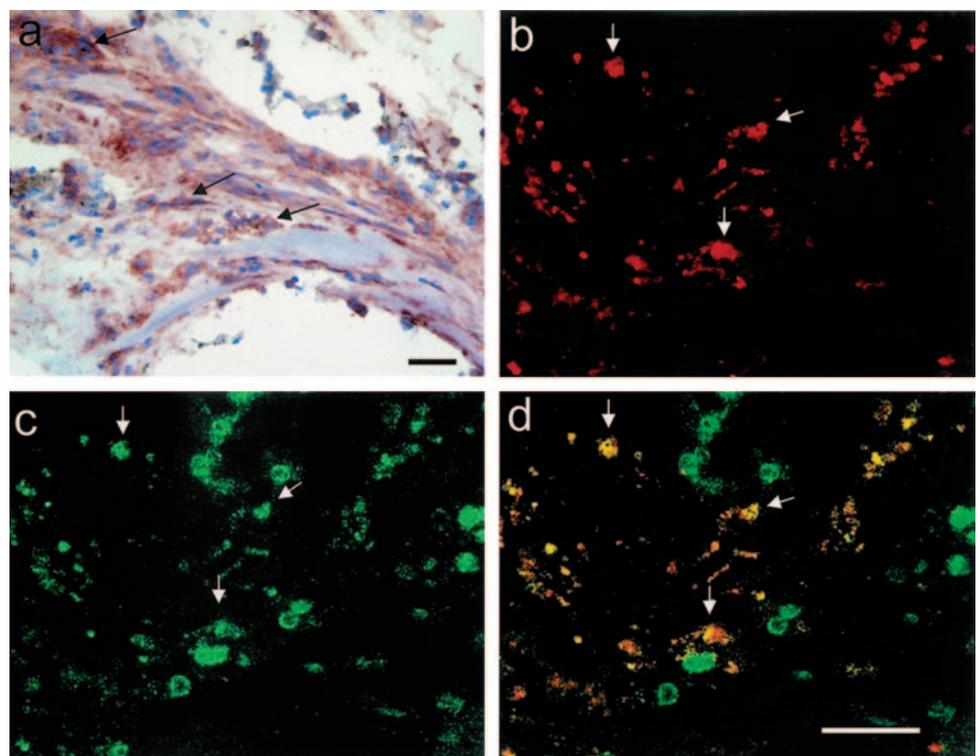


FIGURE 1. Photomicrograph of surgically excised human proliferative vitreoretinopathy (PVR) membranes. (a) Immunohistochemical staining for CTGF (red, arrows) revealed positive staining in the stromal cells of PVR membranes (hematoxylin counterstain). (b-d) Confocal double-stained immunofluorescent images of human PVR membrane showing stromal cells positive for CTGF (b, red, arrows) and cytokeratin (c, green, arrows). (d) Overlay of (b) and (c) revealed that many of the stromal cells were positive for both CTGF and cytokeratin (yellow, arrows). Bar, 50 μ m

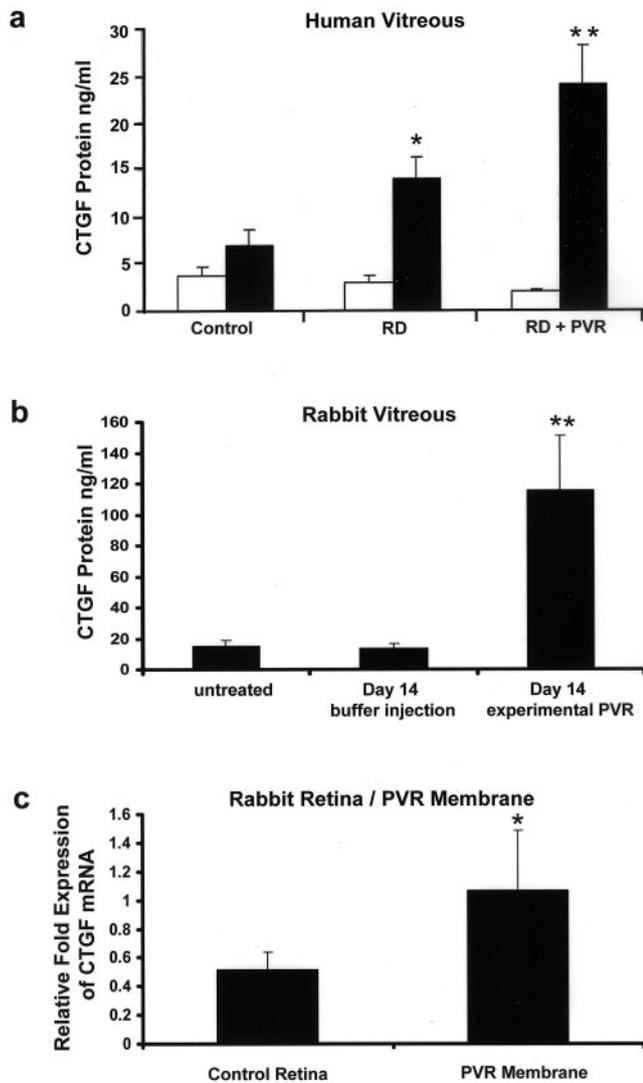


FIGURE 2. CTGF Expression in human and experimental PVR. (a) Accumulation of CTGF fragments in the vitreous of patients with RD ($n = 5$) or RD complicated by PVR ($n = 6$) or of control patients with macular hole or epiretinal membrane ($n = 12$). The level of CTGF was determined by ELISA of vitreous samples. (□) Whole CTGF; (■) represents total (whole+NH₂-terminal half fragment) CTGF. C-terminal half fragment of CTGF was below limits of detection. * $P < 0.05$ compared with control samples; ** $P < 0.01$ compared with control samples. (b) Accumulation of total (whole+NH₂-terminal) CTGF in rabbit vitreous in experimental PVR, as assayed by ELISA. Vitreous samples were obtained from rabbits before injection at d 0 (untreated; $n = 5$), from rabbits at d 14 after injection of buffer only ($n = 12$), and from rabbits with experimental PVR at d 14 injected with PDGF and rhCTGF ($n = 5$). ** $P < 0.01$ compared with buffer treated control samples. (c) Relative expression of CTGF mRNA as evaluated by semiquantitative RT-PCR in normal rabbit retina ($n = 4$) and PVR membrane+retina of rabbits with experimental PVR injected with PDGF and rhCTGF at d 14 ($n = 5$). * $P < 0.05$ compared with control retina.

CTGF+CTGF NH₂-terminal half fragments is a result of an increase in the N-terminal half fragments. Assay for C-terminal CTGF was near or below the limits of detection in all samples; therefore (whole+N-terminal half fragment CTGF) is the best representation of total CTGF in the samples.

CTGF-Induced Fibrosis in Experimental Rabbit PVR

The development of PVR in eight experimental conditions is summarized in Table 2. Intravitreal injection of rhCTGF (200 ng)

alone, PDGF (50 ng) alone, or RPE cells alone into the rabbit eye did not induce detectable PVR in the model. Injection of cultured rabbit RPE cells along with CTGF (200 ng) induced a mild PVR (Figs. 3c, 3d). Injection of cultured rabbit RPE along with PDGF resulted in PVR in 90% (10/11) of rabbits within 2 weeks. The PVR was mild, and the membranes were paucicellular and nonfibrotic (Fig. 3e). Optical coherence tomography revealed slight traction in the area with an epiretinal membrane (Fig. 4a). Histologic examination revealed thin epiretinal membranes with low cellularity and no significant fibrosis (Fig. 4c). However, injection of rhCTGF (200 ng) 1 week after injection of RPE+PDGF BB (50 ng) produced a thick fibrotic membrane with focal traction RD after the second injection (Fig. 3f), and PVR developed in 100% of the rabbits in the group (10/10). Optical coherence tomography showed that the combined injection of PDGF and CTGF resulted in traction from the overlying fibrotic membrane associated with RD (Fig. 4b). Histologic analysis showed a densely fibrotic epiretinal membrane attached to the retina with associated RD (Fig. 4d). We have previously shown that subretinal injection of Ad CMV, CTGF or Ad CMV, GFP results in preferential infection of a normal RPE monolayer and does not result in RD or PVR.³⁸ Similarly, when RPE cells infected with Ad CMV, GFP were injected into the vitreous cavity, there was no evidence of PVR (Fig. 3g). However, when RPE cells infected with Ad CMV, CTGF were injected into the vitreous, a highly fibrotic PVR with extensive RD was induced (Fig. 3h). PVR was more advanced in the AdCTGF induced PVR than the PVR induced by rhCTGF (Table 2). It is likely that this was a result of increased CTGF dose in the AdCTGF experiments. RPE cells transduced with AdCTGF would show continuous, high production of CTGF, as opposed to the single pulse of CTGF provided in the CTGF injection model.

CTGF Levels in Rabbit Vitreous

Western blot analysis of normal rabbit vitreous demonstrated that the vitreous contained a low level of whole CTGF (Fig. 5). However, when PVR was induced by injection of either RPE cells infected with adenovirus expressing full-length human CTGF or RPE cells with PDGF and rhCTGF, there was a modest increase in whole CTGF, and a much more prominent accumulation of an 18-kDa CTGF fragment in the vitreous at day (d) 14 (Fig. 5).

CTGF was also measured at d 14 in the vitreous of animals with experimental PVR injected with RPE+PDGF+rhCTGF by ELISA (Fig. 2b). ELISA specific to human+rodent CTGF revealed a CTGF (N+whole) concentration of 15.15 ± 3.06 in

TABLE 2. PVR Grade in Eight Experimental Models

Vitreous Injection	PVR Formation (Stage 0–5)					
	0	1	2	3	4	5
RPE cells ($n = 3$)	3					
RPE+CTGF ($n = 5$)		5				
CTGF ($n = 2$)	2					
PDGF ($n = 2$)	2					
RPE+PDGF ($n = 11$)	1	8	2			
RPE+PDGF+CTGF ($n = 10$)		1	1	6	2	
Ad CMV, CTGF ($n = 5$)				1	2	2
Ad CMV, GFP ($n = 3$)	3					

Classification of PVR was based on clinical findings according to Fastenberg et al.³⁷ Stage 0: normal retina; stage 1: intravitreal membrane; stage 2: focal traction, localized vascular changes, hyperemia, engorgement dilation, and blood vessel elevation; stage 3: localized detachment of medullary ray (vascularized portion of the retina); stage 4: extensive retinal detachment; total medullary ray detachment; peripapillary retinal detachment; stage 5: total retinal detachment, retinal folds, and macular holes.

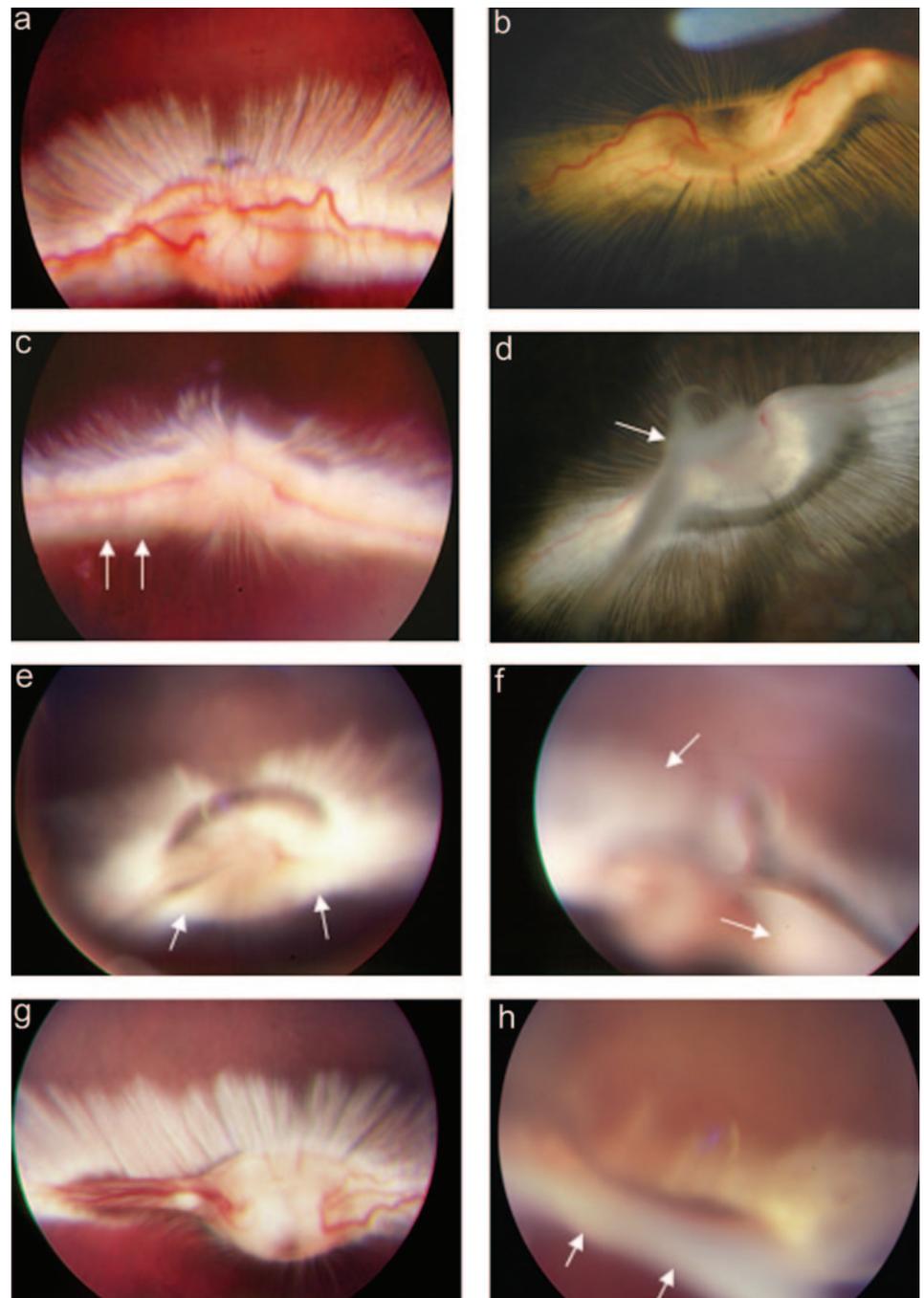


FIGURE 3. Experimental PVR in rabbits: ocular fundus photographs at the 2-week time point. Photograph of normal fundus in vivo (a) and from a normal dissected, enucleated eye (b). That there was a vascularized band of myelinated axons (medullary ray) on both sides of the optic disc. Injection of RPE cells+CTGF resulted in mild PVR with focal membrane formation (c, arrows); the thin, nonfibrotic membrane was clearly visible adjacent to the optic disc after dissection of the enucleated eye (d, arrow). Injection of RPE cells+PDGF resulted in a mild, nonfibrotic PVR with focal RD (e, arrows). Injection of RPE cells+PDGF+CTGF resulted in severe, fibrotic PVR with RD (f, arrows). Intravitreal injection of RPE cells infected with Ad, CMV, GFP did not result in PVR (g); however, injection of RPE cells infected with Ad, CMV, CTGF resulted in thick fibrotic PVR membranes with extensive RD (h, arrows).

untreated controls ($n = 5$) and 13.3 ± 3.55 in buffer-injected control samples ($n = 12$), which increased to 115.5 ± 35.21 ($n = 5$; $P < 0.01$) in animals with experimental PVR. Human-specific ELISA showed that in d 14 vitreous, most of the increase in CTGF level was rabbit in origin; however, the exact relative contribution was difficult to determine because of differences in antibody detection efficiencies. Plasma samples from these animals were also evaluated for CTGF (N+whole) and all samples were below the level of detection (results not shown), suggesting that there was not a systemic accumulation of CTGF in these animals.

To confirm the local intraocular synthesis of CTGF in experimental rabbit PVR, we semiquantitatively measured levels of CTGF mRNA in normal control retina and in retina and attached PVR membranes from rabbits with PVR induced by injection of RPE+PDGF+rhCTGF at d 14. RT-PCR revealed that

there was a twofold increased expression of CTGF mRNA in PVR retina/membrane samples compared with normal control retinas ($P < 0.05$; Fig. 2c). To evaluate the possibility that the accumulation of CTGF fragments in PVR is due to alternative exon splicing, oligo deoxynucleotide primers corresponding to sequences in the first and fifth exons of both human and rabbit CTGF genes were used to amplify full-length CTGF cDNA. RNA isolated from normal rabbit retina, rabbit PVR retina/membranes, and a human RPE cell line (ARPE-19) were evaluated, and in all cases only a single 1-kb cDNA band was amplified, indicating that there were no alternative splice products of the CTGF gene (results not shown).

CTGF Stimulates Chemotactic Migration of RPE

Migration of human RPE in the modified Boyden chamber assay was stimulated by recombinant CTGF. The chemotactic re-

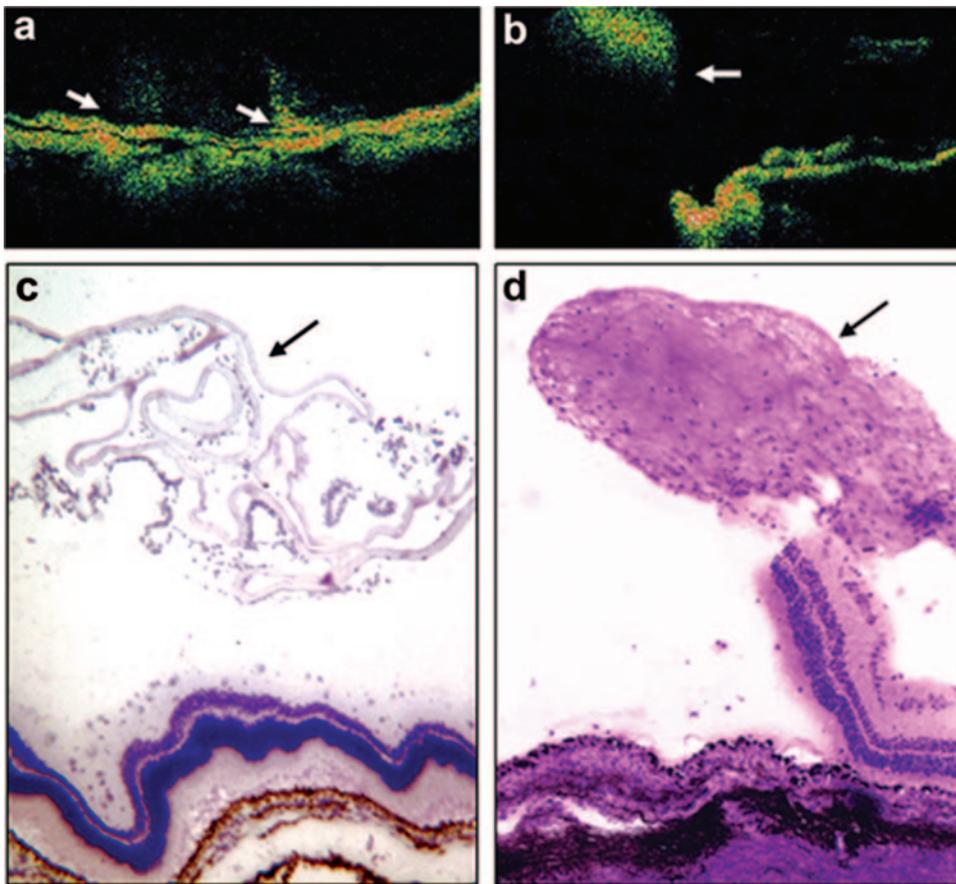


FIGURE 4. Experimental PVR in rabbits: optical coherence tomography (OCT) and histology at the 2-week time point. PVR induced by injection of rabbit RPE cells+PDGF results in a thin epiretinal membrane on OCT (**a**, arrows) and on histologic analysis (**c**, arrow, H&E). PVR induced by injection of rabbit RPE cells+PDGF+CTGF results in a thick, optically dense epiretinal membrane on OCT (**b**, arrow); histology shows a fibrotic, cellular membrane attached to the retinal surface (**d**, arrow, H&E).

sponse of RPE to whole molecular CTGF was dose-dependent over a range of 1 to 100 ng/mL (Fig. 6). The migration response to full-length CTGF was similar to that seen for PDGF-BB (20 ng/mL). The addition of up to 10 ng/mL CTGF significantly increased cell migration, compared with that in control cells ($P < 0.01$).

Type I Collagen Expression in Rabbit PVR Membrane and CTGF-Stimulated Expression of Type I Collagen mRNA and Protein in RPE Cells

Type 1 collagen expression was predominantly revealed in fibrotic rabbit PVR membranes by immunohistochemical staining (Fig. 7a). A 3.5-fold increase in the expression of type I collagen mRNA was detected in CTGF-stimulated RPE cells, by

quantitative real-time PCR (Fig. 7b). The CTGF-induced upregulation of type I collagen mRNA in RPE cells was much higher than in controls at 48 hours ($P < 0.01$). The highest response of type I collagen protein production (2.81 ng/mL) was seen after stimulation with rhCTGF for 72 hours (compared with TGF- β 1.51 ng/mL and control 0.53 ng/mL; $P < 0.05$).

DISCUSSION

RPE cells play a prominent role in the pathogenesis of PVR.^{4,8,9} In PVR, proliferating RPE cells transdifferentiate into myofibro-

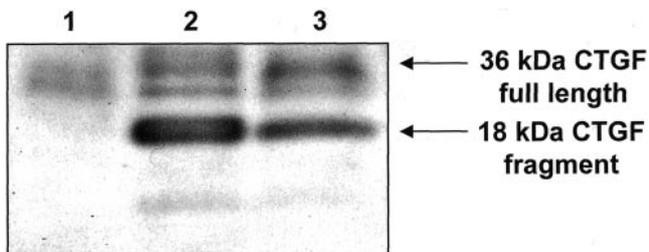


FIGURE 5. CTGF expression in experimental PVR in rabbit: Western blot of vitreous for CTGF at the 2-week time point. *Lane 1*: intravitreal injection of saline; *lane 2*, injection of rabbit RPE infected with Ad CMV, CTGF; *lane 3*: injection of rabbit RPE+PDGF+CTGF. Control rabbit vitreous (*lane 1*) contained a low level of full-length CTGF but no CTGF fragments. Vitreous from animals with PVR (*lanes 2, 3*) showed increased levels of full-length CTGF and prominent expression of the 18-kDa CTGF fragment.

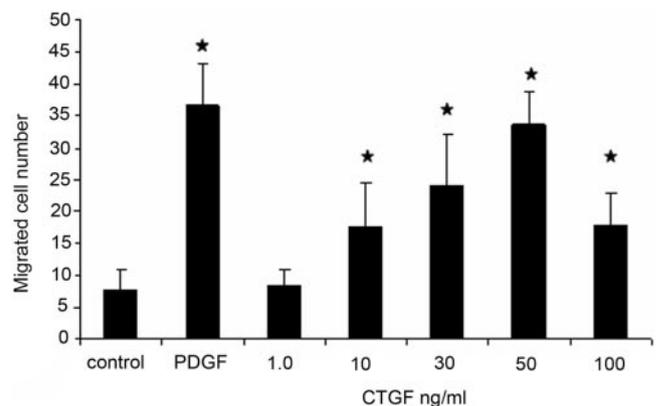


FIGURE 6. CTGF stimulated RPE cell migration. A dose-dependent increase in migrating human RPE cells was found in a modified Boyden chamber assay with a maximum effect at 50 ng/mL (* $P < 0.05$). The maximum stimulation was similar to that found in response to PDGF-BB 20 ng/mL.

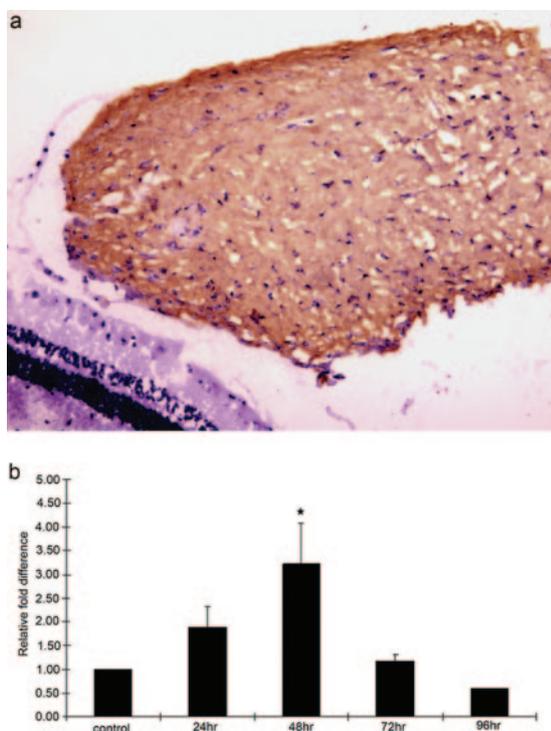


FIGURE 7. Effect of CTGF on expression of type 1 collagen. (a) Immunohistochemical staining for type 1 collagen (red chromogen, hematoxylin counterstain) in PVR membrane induced by intravitreal injection of RPE cells+PDGF+CTGF. (b) Induction of type 1 collagen mRNA in cultured RPE stimulated with CTGF, shown by quantitative real-time PCR. Increased levels of type 1 collagen mRNA were seen after CTGF stimulation at 24 hours, reaching a peak at 48 hours. (* $P < 0.01$, compared with control).

blasts or mesenchymal-like cells to form epiretinal membranes.^{4,8,37} These membranes exert a contractile force on the attached underlying retina, leading to detachment.^{4,8,37} Fibrosis of epiretinal membranes involves increased ECM production and accumulation in the RPE. Herein, we provide the first description of the effect of CTGF on experimental PVR. We used human PVR specimens and a rabbit PVR model to gain further insight into the potential role of CTGF in the fibrogenesis of PVR.

We and others have shown that CTGF is prominently expressed in human PVR membranes.^{39,40} In the present study, CTGF expression was most prominent in stromal cells within the membrane. Our finding that much of the CTGF expression is localized in cells that stain positively for pancytokeratin, a RPE specific marker in the retina,³⁸ further supports the idea that RPE play a central role in the pathogenesis of PVR.

We also found that CTGF accumulated in the vitreous of patients with PVR. When compared to vitreous of patients with nonproliferative control disorders (macular hole, macular pucker, and epiretinal membrane), vitreous of patients with RD with PVR showed high levels of CTGF ($P < 0.01$), whereas vitreous of patients with RD without PVR showed only a moderate increase ($P < 0.05$).⁴¹ Of interest, it appeared that in PVR the increase in total vitreal CTGF is due to accumulation of the N-terminal half fragment of CTGF.

To evaluate these findings in more detail, the accumulation of CTGF in the vitreous was studied in two models of experimental rabbit PVR. Aspirates of vitreous from the PVR rabbit models were analyzed by CTGF immunoblot and ELISA to determine whether full-length CTGF or CTGF half fragments accumulate. Consistent with the human studies, we found that

experimental PVR was associated with a predominant accumulation of CTGF 18-kDa half fragments.

Together, these results suggest that the N-terminal half fragments of CTGF may play an important role in fibrosis formation in vivo. These results are consistent with the findings of Grotendorst and Duncan,⁴² who demonstrated that myofibroblast differentiation and collagen synthesis were preferentially induced by the N-terminal domain of CTGF; therefore, the N-terminal half fragment of CTGF may represent a biomarker for fibrotic disease.⁴³ C-terminal fragments were extremely rare in all our samples, suggesting that C-terminal CTGF may be rapidly degraded or bind to target cells and the ECM, where it may be sequestered and serve as a persistence stimulator in fibrotic tissue. Ball et al.²² presented evidence that 16-, 18- and 20-kDa CTGF are intermediate forms produced by proteolysis of the 38-kDa CTGF. Steffen et al.²¹ indicated that those soluble forms of CTGF can stimulate mitosis in fibroblasts. Further support for the contention that full-length CTGF is locally expressed in experimental eyes with PVR, we evaluated expression of CTGF mRNA and found a twofold increase in full-length CTGF mRNA expression in PVR retina/membranes when compared with normal rabbit retina. Furthermore, these RT-PCR experiments showed no evidence of alternative splicing of the CTGF gene. Overall, our experiments are consistent with the idea that the accumulation of CTGF half fragments in the PVR vitreous is due to proteolysis of locally synthesized whole CTGF.

The overall tendency of vitreous CTGF levels to be higher in human eyes with RD+PVR than in those with RD alone, together with the high CTGF levels in the vitreous of rabbits with PVR, suggests that CTGF plays a critical role in the development of retinal fibrosis. We have shown that expression of CTGF in RPE cells in vitro is stimulated by TGF- β ,³⁹ suggesting that the synthesis and secretion of CTGF depends on stimulation from other growth factors or cytokines that are present in PVR. Experiments have shown that RPE cell transdifferentiation is modulated by soluble growth factors and cytokines.^{8,44} Fibroblast growth factor, tumor necrosis factor- α , interleukin-1, interleukin-6, interferon- γ , TGF- β , PDGF, and insulin-like growth factor, among others, are elevated in PVR.^{4,5,8,16} These polypeptides are capable of activating RPE. The RPE may then respond to CTGF stimulation to induce RPE behavior changes, including proliferation, migration, matrix synthesis, enzyme production, and contraction. In the present study, although we injected human CTGF into the rabbit vitreous, an increased amount of rabbit CTGF was also found in the vitreous, suggesting that on stimulation by exogenous CTGF, the host cell itself is able to produce CTGF, thereby enhancing fibrosis. The induction of rabbit CTGF may also be due to the exposure of host RPE to TGF- β . The TGF- β may be released from the vitreous into the retina, where it may stimulate the RPE to produce more rabbit CTGF.

Fibrosis is characterized by ECM deposition. In the present study, we investigated the effect of rhCTGF on the expression of type I collagen mRNA and protein in RPE cells and the expression of type I collagen in experimental rabbit PVR membrane as demonstrated by immunostaining. Our findings showed that the type I collagen was expressed strongly throughout the extracellular matrices of the rabbit PVR membrane. This result correlates with collagen type I expression in vitro, which showed a significant upregulation in type I collagen mRNA and protein expression in RPE cells after exposure to CTGF. Delayed collagen mRNA expression after CTGF stimulation is in accord with the results of previous experiments.⁴⁵ Pretreatment of RPE cells with TGF- β enhanced the expression of type I collagen stimu-

lated by CTGF. The enhanced expression of interstitial type I collagen correlated with an increased accumulation of CTGF in specimens from patients with PVR, suggesting a close relationship between CTGF upregulation and ECM production. Besides CTGF, the increased ECM deposition may relate to TGF- β upregulation in PVR because it was found that high concentration of TGF- β in subretinal fluid was associated with RD in the complication of retinal strands.⁴⁶

It is well accepted that TGF- β is a potent inducer of fibrosis and that it regulates CTGF expression. We have found that TGF- β promotes CTGF protein expression in RPE,⁴⁷ whereas Kanemoto et al.⁴⁸ recently reported that the blockade of endogenous CTGF with an antisense oligodeoxynucleotide significantly attenuated TGF- β -induced ECM synthesis in kidney cells. Of interest, bleb failure after glaucoma filtration surgery is believed to be related to high ECM synthesis stimulated by CTGF.⁴⁹ Our present study shows that type I collagen production is highly stimulated by CTGF alone without TGF- β addition. These findings support the idea that the CTGF-induced fibrosis pathway may be partially independent of TGF- β .⁵⁰

Recent studies have suggested the possibility that in PVR, some of the pathologic effects of the disease may be mediated through upregulation of vascular endothelial growth factor (VEGF). Although a minority of patients with PVR have been reported to have vitreous levels of VEGF in the low nanogram per milliliter range, the overall levels of CTGF and VEGF in PVR patients do not correlate with each other.⁵¹ To determine whether VEGF was involved in the pathogenesis of our experimental PVR model (injection of RPE+PDGF+rhCTGF), VEGF levels in the vitreous were measured by ELISA in six rabbits in which vitreous was available on d 0 as well as d 7 or d 14 after induction of PVR (results not shown). We found no statistically significant increase in VEGF expression in the vitreous at d 7 or d 14 compared with that in d 0 controls (paired *t*-test; *P* = 0.51 at d 7, *P* = 0.42 at d 14), thus it is unlikely that CTGF acts through VEGF in our model system.

RPE migration plays an important role in the pathogenesis of PVR.^{8,9} To our knowledge, this is the first study to demonstrate that full-length CTGF promotes RPE cell migration by chemotaxis. The result is consistent with studies reporting stimulation of cell adhesion and/or migration by CTGF or related peptides in different cell types, such as endothelial cells, vascular smooth muscle cells, fibroblasts, and chondrocytes.⁵²⁻⁵⁵ Recently, published studies have shown that CTGF stimulates metalloprotease expression in smooth muscle cells, activates phosphorylation of p^{44/42} mitogen-activated protein kinase, and stimulates RPE proliferation.^{51,56} Taken together, the results of these studies suggest that CTGF modulates wound healing by remodeling the microenvironment. Thus, it seems likely that CTGF has a central role in the development of fibrosis and as such may represent a useful therapeutic target.⁵⁷

PVR in animals has been established by vitreous injection of RPE cells, plasma, blood, growth factors (including PDGF), and other types of cells.^{5-9,58-60} However, previously published studies focused on the induction of PVR rather than on the formation of PVR fibrosis. In the present animal model, injection of CTGF 1 week after injection of RPE cells and PDGF induced strong fibrosis. No significant PVR or PVR fibrosis was induced with injection of CTGF alone or CTGF combined with RPE or adenovirus transfection of resting RPE with Ad CMV, GFP. This suggests that RPE cells must first be activated to respond to the stimulation of CTGF.⁶¹

In our study, RPE activation was stimulated by the addition of PDGF-BB. Although RPE cells express both α and β PDGF receptors, recent studies have suggested that PDGF receptor α plays a more critical role in the development of experimental and human PVR.^{62,63} Our use of PDGF-BB ensures activation of RPE, since PDGF-BB is an agonist for both α and β PDGF receptors, induces phosphorylation of p^{44/42} MAP kinase, and stimulates RPE chemotaxis.⁶⁴

In the present study, we showed that the RPE cells responded chemotactically to rhCTGF and increased collagen-1 mRNA and protein expression in vitro. We then demonstrated that the human PVR membranes contained CTGF and that there was a prominent accumulation of the N-terminal half fragment of CTGF in the vitreous of both humans and rabbits with PVR. Of importance, we found that when rhCTGF was injected into the vitreous of rabbits with mild PVR, the membrane became densely fibrotic. This is the first direct evidence, using an in vivo experimental system, that CTGF mediates pathologic intraocular fibrosis.

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