Accumulation of NH$_2$-Terminal Fragment of Connective Tissue Growth Factor in the Vitreous of Patients With Proliferative Diabetic Retinopathy

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**OBJECTIVE** — To evaluate the expression of connective tissue growth factor (CTGF) and its fragments in the vitreous of patients with proliferative diabetic retinopathy (PDR) and to localize CTGF expression in associated preretinal membranes.

**RESEARCH DESIGN AND METHODS** — Vitreous was obtained from 24 patients with active PDR, 4 patients with quiescent PDR, and 23 patients with other retinal diseases and no diabetes, including 5 patients with vitreous hemorrhage. Enzyme-linked immunosorbent assay was used to determine levels of whole CTGF and its NH$_2$- and COOH-terminal fragments. Preretinal membranes from three patients with active PDR were stained immunohistochemically for the presence of CTGF and cell type–specific markers.

**RESULTS** — A significant increase in NH$_2$-terminal CTGF fragment content was found in vitreous samples from patients with active PDR when compared with samples from nondiabetic patients ($P < 0.0001$) or patients with quiescent PDR ($P = 0.02$). Levels of NH$_2$-terminal CTGF were also greater in vitreous samples from diabetic patients with vitreous hemorrhage compared with samples from nondiabetic patients with vitreous hemorrhage ($P = 0.02$). Vitreous levels of whole CTGF were similar in all groups. COOH-terminal fragments of CTGF were not detected. CTGF immunoreactivity was predominantly localized to smooth muscle actin–positive myofibroblasts within active PDR membranes.

**CONCLUSIONS** — NH$_2$-terminal CTGF fragment content is increased in the vitreous of patients with active PDR, suggesting that it plays a pathogenic role or represents a surrogate marker of CTGF activity in the disorder. The localization of CTGF in myofibroblasts suggests a local paracrine mechanism for induction of fibrosis and neovascularization.

Diabetes Care 27:758–764, 2004

Diabetic retinopathy is the most common cause of new-onset blindness in adults aged 20–74 years (1). The pathophysiology of the disorder is characterized by retinal microvascular dysfunction; however, all retinal cell types are ultimately affected (2). Proliferative diabetic retinopathy (PDR) is an important cause of severe vision loss in these patients. In PDR, neovascularization of the optic disc and retina occurs in association with the development of fibrovascular membranes at the vitreoretinal interface (3). Examination of vitreous fluids from patients with PDR has provided considerable data implicating a wide variety of locally synthesized growth factors in disease initiation and progression (4–13). Patients with PDR have increased levels of proangiogenic growth factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor, and platelet-derived growth factor (5–10), and decreased levels of antiangiogenic growth factors, including pigment epithelium-derived growth factor and somatostatin (11–13).

Much less is known about the factors that control the development of retinal fibrosis. A polymorphism in the gene encoding transforming growth factor (TGF)-β has recently been identified as a strong genetic risk factor for PDR (14). Whereas some studies have shown increased levels of total or latent TGF-β in the vitreous of patients with PDR, other studies have shown that the amount of active TGF-β is actually decreased, indicating deficient TGF-β activation (15–18). This finding suggests that other fibrogenic factors are operative in PDR.

Over the past decade, connective tissue growth factor (CTGF) has emerged as the major profibrotic growth factor associated with a wide variety of fibrotic disorders (19). CTGF (Mr 38,000) is a secreted, cysteine-rich polypeptide that was originally identified from conditioned medium of human umbilical vein endothelial cells (20). It is expressed by a variety of cells, including fibroblasts, epithelial cells, and smooth muscle cells (21,22). CTGF is rapidly upregulated after serum or TGF-β stimulation (23) and is believed to function as a downstream mediator of TGF-β action on fibroblasts by stimulating cell proliferation and ex-
tracellular matrix (collagen 1 and fibronectin) deposition (24). CTGF has also been reported to bind and modulate the action of TGF-β (25). Fragments of CTGF have been shown to accumulate in tissue culture or body fluids and these fragments may retain biologic activity (22,26–28). Of importance to this study, CTGF is also a pro-angiogenic factor with activity both in vitro and in vivo (29–31). In addition, CTGF has been reported to interact with VEGF and affect its activity (32).

Little is known about the role of CTGF in proliferative retinal disease. CTGF expression has been demonstrated in cytokeratin-positive stromal cells within epiretinal membranes from patients with proliferative vitreoretinopathy associated with rhegmatogenous retinal detachment (33). It was recently reported that CTGF is upregulated in the retinas of rats with streptozotocin-induced diabetes (34). These animals demonstrated preproliferative changes in their retinas with increased vascular permeability and increased VEGF expression (34). In vitro studies show that VEGF upregulates CTGF mRNA expression in retinal endothelial cells (35,36). Thus, these studies provide support for the idea that CTGF could be involved in the pathogenesis of diabetic retinopathy in the absence of increased active TGF-β.

In the present study, we determined the levels of CTGF and its fragments in the vitreous fluids of patients with quiescent and active PDR and nondiabetic patients with other retinal disorders. We then histologically localized the expression of CTGF in preretinal membranes from patients with PDR.

**RESEARCH DESIGN AND METHODS**

**Vitreous samples and preretinal membranes**

All procedures conformed to the Declaration of Helsinki for research involving human subjects. The Institutional Review Board of the University of Southern California approved the project, and informed consent was obtained from all participants. Vitreous was sampled by one of the authors (J.L.) at the time of pars plana vitrectomy. 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. Samples were obtained from four patients with quiescent PDR, 10 patients with uncomplicated active PDR, 11 patients with active PDR with vitreous hemorrhage, and 3 patients with active PDR with iris neovascularization or neovascular glaucoma. PDR stage was classified as active if there was active capillary neovascularization on the retinal surface or fresh vitreous hemorrhage with neovascularization. Quiescent PDR was defined by the presence of fibrotic, regressed, retinal neovascularization after photoagulation. Vitreous samples from nondiabetic patients with retinal disease were obtained from six patients with choroidal neovascularization, seven patients with macular hole, five patients with epiretinal membrane/macular pucker, and five patients with vitreous hemorrhage resulting from trauma (n = 3), retinal tear (n = 1), or central retinal vein occlusion (n = 1).

The average patient age was 60 years; 54% of all of the patients were women, and 54% of the diabetic patients were insulin dependent. There was no significant difference in the age or sex of patients in the PDR group versus those in the nondiabetic group.

Preretinal membranes were excised from three patients with active PDR at the time of vitrectinal surgery. They were snap frozen within 1 h of removal and stored at ~80°C. Samples of normal retina were obtained from three Oregon Eye Bank donor eyes without retinal disease. Samples were snap frozen and stored at ~80°C.

**Enzyme-linked immunosorbent assay**

Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed to determine the content of whole CTGF and NH2- and COOH-terminal CTGF fragments (FibroGen, South San Francisco, CA) in the vitreous supernatants. Pairs of CTGF-specific monoclonal antibodies were selected for capture and detection of full-length recombinant human CTGF (rhCTGF), NH2-terminal rhCTGF, and COOH-terminal rhCTGF. ELISAs were developed to measure full-length CTGF, full-length CTGF + NH2-terminal CTGF fragments, and full-length CTGF + COOH-terminal CTGF fragments. Spike-recovery experiments using rhCTGF demonstrated quantitative detection in vitreous fluid and assay sensitivities were determined to be in the range of 0.6 ng/ml. Standard curves were generated using rhCTGF. CCN gene family members cyr61 and nov were not detected in the assays. Vitreous sample content of NH2-terminal CTGF fragments was determined by subtracting the content of full-length CTGF from the content of full-length CTGF + NH2-terminal CTGF fragments. Vitreous sample content of COOH-terminal CTGF fragments was determined similarly. In contrast, previous CTGF studies using ELISA measures were not able to quantitatively distinguish CTGF fragments from whole CTGF (37).

**Statistical methods**

SAS (SAS Institute, Cary, NC) was used for all analyses. ANOVA and independent sample Student’s t tests were used to compare continuous variables, whereas Fisher’s exact and χ² tests were used to compare categorical variables. Multiple comparison Student’s t tests with Bonferroni correction were used for pairwise comparisons when an ANOVA P value was statistically significant. The accepted level of significance for all tests was α < 0.05.

**Immunohistochemistry**

Tissues were cryostat-sectioned at 6 µm, air dried, rehydrated with phosphate-buffered saline (pH 7.4), and blocked with 5% normal goat serum for 15 min. Each of the subsequent steps was followed by three washes with phosphate-buffered saline. Sections were sequentially incubated with anti-CTGF rabbit polyclonal antibody (60 min) (FibroGen, South San Francisco, CA), biotinylated secondary anti-rabbit antibody (1:400; Vector Laboratories, Burlingame, CA), and streptavidin peroxidase. The red reaction product was developed using an aminoethyl carbazole kit (Zymed, South San Francisco, CA). Slides were counterstained with hematoxylin and mounted with glycerin-gelatin medium.

**Double-label confocal immunofluorescence**

Thawed 6-µm frozen sections were air dried, fixed with reagent grade acetone for 5 min, and washed with Tris buffer (pH 7.4). After endogenous peroxide was blocked by treatment with 0.3% hydrogen peroxide, sections were blocked for 15 min with 1% BSA (Sigma, St. Louis, MO) in Tris buffer. For double labeling, sections were incubated first with poly-
coma (NVG). Values are means ± SD. Intravitreal hemorrhage, or active PDR with iris neovascularization (INV) or neovascular glaucoma; active PDR with vitreous hemorrhage and PDR with vitreous hemorrhage alone may directly contribute to the increased NH2-terminal CTGF levels, we compared samples with vitreous hemorrhage and PDR with vitreous hemorrhage samples from patients without diabetes; active PDR with vitreous hemorrhage samples showed significantly higher levels of NH2-terminal CTGF (15.68 ng/ml) than nondiabetic samples with vitreous hemorrhage (6.42 ng/ml) (P = 0.02). The relative abundance of NH2-terminal fragment over whole CTGF was examined by calculating NH2-terminal/whole CTGF ratios (Table 1).

Figure 1—Levels (ng/ml) of whole CTGF (white) and NH2-terminal CTGF fragment (black) determined by ELISA (ng/ml) in vitreous samples. Nondiabetic patients with retinal disease include choroidal neovascularization (CNV), macular hole, epiretinal membrane/macular pucker (ERM/pucker), and vitreous hemorrhage. PDR is divided into quiescent PDR, active PDR (without intravitreal hemorrhage, iris neovascularization, or neovascular glaucoma), active PDR with intravitreal hemorrhage, or active PDR with iris neovascularization (INV) or neovascular glaucoma (NVG). Values are means ± SD.

RESULTS

CTGF ELISA

Whole CTGF values (Fig. 1, Table 1) were similar in all groups (2.34 ng/ml in active PDR, 2.20 ng/ml in quiescent PDR, 2.61 ng/ml in other retinal disease; P = 0.8). COOH-terminal CTGF fragment was not detected above the lower threshold of the ELISA in any samples. Interestingly, there was a wide range in values for the NH2-terminal fragment of CTGF (Fig. 1, Table 1). NH2-terminal CTGF levels in vitreous samples from patients with PDR (12.59 ng/ml) were significantly greater than those from patients with retinal disease and no diabetes (6.01 ng/ml) (P < 0.0001). The highest levels of NH2-terminal CTGF were found in patients with active PDR (inclusive of those with hemorrhage, iris neovascularization, or neovascular glaucoma) (13.66 ng/ml), and these values were significantly greater than those found in patients with quiescent PDR (6.13 ng/ml) (P = 0.02). To explore the possibility that vitreous hemorrhage alone may directly contribute to the increased NH2-terminal CTGF levels, we compared samples with vitreous hemorrhage and PDR with vitreous hemorrhage samples from patients without diabetes; active PDR with vitreous hemorrhage samples showed significantly higher levels of NH2-terminal CTGF (15.68 ng/ml) than nondiabetic samples with vitreous hemorrhage (6.42 ng/ml) (P = 0.02). The relative abundance of NH2-terminal fragment over whole CTGF was examined by calculating NH2-terminal/whole CTGF ratios (Table 1).

N-fragment/whole CTGF ratios are greatest in active PDR samples (inclusive of those with hemorrhage, iris neovascularization, or neovascular glaucoma; ratio of 6.7), and these ratios are significantly greater than those seen in nondiabetic samples (ratio of 2.5; P < 0.0001) or quiescent PDR (ratio of 2.9; P = 0.05).

CTGF expression in preretinal membranes

Each of the PDR membranes showed discrete areas of neovascularization, spindle cell proliferation, and fibrosis (Fig. 2A). Although normal retinas from donor eyes did not show CTGF immunoreactivity (results not shown), each of the PDR membranes showed cellular immunoreactivity for CTGF in areas of spindle cell proliferation with weaker staining of the extracellular matrix (Fig. 2A, inset). Double staining with cell type-specific antibodies showed that most of the CTGF-immunoreactive cells were SMA-positive myofibroblasts (Fig. 2B–D). Rare astroglial cells positive for glial fibrillary acidic protein and endothelial cells positive for CD31 were also immunoreactive for CTGF (results not shown).

CONCLUSIONS—PDR pathogenesis is multifaceted; neovascularization and fibrosis play important roles in loss of vision due to retinal vascular complications, retinal distortion, and traction retinal detachment (3). Angiogenesis and fibrosis are highly correlated in PDR; therefore, CTGF, a growth factor that is both proangiogenic and profibrotic, was studied as a potential mediator of these effects. Analysis of whole CTGF content in the vitreous samples showed no difference among the disease groups of patients with or without diabetes.

CTGF is unstable in vivo, and proteolytic fragments of CTGF are found in human biologic fluids and in supernatants of tumor cell cultures (26–28). When the content of CTGF fragments was measured in vitreous samples, NH2-terminal CTGF fragment was present at much higher levels in patients with PDR than in nondiabetic patients with other retinal diseases (P < 0.0001). NH2-terminal fragment of CTGF was highest in vitreous samples from patients with active PDR (quiescent PDR versus active PDR, P = 0.02). A ratio >1 of [NH2-terminal CTGF/whole CTGF] in each of the disease groups and especially active PDR suggests
Table 1—The levels of CTGF (whole, NH2-terminal, or NH2-terminal/whole) determined by ELISA assay (ng/ml) in undiluted vitreous samples obtained at surgery from patients without diabetes or with PDR

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<td>6.13 (3.15–9.11)</td>
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Data are means (95% CI). Nondiabetic groups: choroidal neovascularization (CNV), macular hole (MH), epiretinal membrane (ERM)/macular pucker, vitreous hemorrhage (VH). PDR groups: quiescent PDR, active PDR with no neovascularization or vitreous hemorrhage (no NV/VH), active PDR with iris neovascularization or neovascular glaucoma (INV/NVG), active PDR with vitreous hemorrhage (VH). *ANOVA or Student’s t-test was used for comparison of means; †multiple pairwise comparisons with a Bonferroni adjustment: N-CTGF, active PDR (VH) greater than quiescent PDR and all nondiabetic groups (P = 0.02 quiescent PDR, 0.003 CNV, 0.01 MH, 0.003 ERM, 0.01 VH). N/Whole, active PDR (no NV/VH) and active PDR (VH) greater than nondiabetic CNV (P = 0.01 and 0.02, respectively).

that these diseases may be associated with increased CTGF proteolysis. Matrix metalloproteinase (MMP) has been implicated in the pathogenesis of PDR, and several MMPs (MMP-1, -3, -7, and -13) are able to process CTGF into fragments (38). Although vitreous samples from patients with PDR show increased MMP-9 activity (39), a complete analysis of MMPs known to cleave CTGF has not been performed in PDR. The ratio of NH2-terminal CTGF/whole CTGF in normal vitreous is unknown. We evaluated this ratio in cadaveric vitreous from 10 patients without retinal disease (Oregon Eye Bank), and a ratio of 0.9 (95% CI: 0.8–1.1) was found (results not shown), suggesting the possibility of less proteolysis in the nondiseased state; however, this result must be viewed with caution because we do not know whether post-mortem changes influence the level of CTGF or its fragments in vitreous fluid.

The role that NH2-terminal CTGF plays in the pathogenesis of PDR is unknown. CTGF contains four functional modules: an insulin-like growth factor–binding domain, von Willebrand type C (or CR) domain, thrombospondin type 1 repeat domain, and cystine knot (CT) domain (21–23). The first two domains comprise the NH2-terminal fragment; these are separated from the third and fourth domains, which comprise the COOH-terminal fragment by a proteolytically sensitive hinge region (38). It has recently been shown that the CR domain of CTGF binds TGF-β, resulting in enhanced TGF-β receptor binding and TGF-β activity (25). Therefore, even though the vitreous of patients with active PDR may have decreased levels of active TGF-β, the increased levels of NH2-terminal CTGF could increase TGF-β activity. Selective accumulation of NH2-terminal CTGF fragment may occur because the NH2-terminal region of CTGF is more stable than its intact precursor to MMP digestion (38); therefore, its accumulation represents a surrogate marker of CTGF production and turnover.

COOH-terminal fragments of CTGF were not detected in any of the vitreous samples, suggesting that the COOH-terminal fragments are unstable or localized in another compartment. Another possibility is that all possible COOH-terminal fragments are not detected by the assay system used. Smaller (10-kDa) fragments of COOH-terminal CTGF have
CTGF in diabetic retinopathy

Figure 2—Immunoperoxidase staining for CTGF of a preretinal surgically excised PDR membrane using polyclonal anti-CTGF antisera, aminoethyl carbizole as the chromogen (red), and hematoxylin as a nuclear counterstain (blue). The membrane shows distinct regions of neovascularization (arrowheads), fibrosis (asterisks), and stromal cell proliferation (arrows). The stromal cells show immunoreactivity for CTGF (A and inset), with weak staining of the associated extracellular matrix. Adjacent tissue sections viewed using confocal microscopy show strong stromal cell immunofluorescence for CTGF (B) and smooth muscle actin (C). Merged images (from B and C, shown in D; overlap appears yellow) show that most CTGF staining colocalizes in SMA-positive cells. Bar = 50 microns (inset A magnified 4× further).

been found in biologic fluids, suggesting that this fragment may be subject to further proteolytic digestion beyond that found for MMPs (26–28,38). It has been shown that CTGF can bind VEGF$_{165}$ in the COOH-terminal domain and that the resulting complex inhibits the angiogenic activity of VEGF$_{165}$ (32). The preferential degradation of CTGF by certain MMPs then reactivates VEGF released from the complex, suggesting that CTGF may be an important regulator of VEGF function (38).

The accumulation of NH$_2$-terminal CTGF fragment in the vitreous of patients with active PDR suggests local synthesis of CTGF in the proliferative preretinal tissues; however, a contribution from serum or blood cannot be entirely ruled out. It is unlikely that blood alone is a major contributor, because PDR patients with vitreous hemorrhage had significantly more NH$_2$-terminal CTGF in their vitreous than nondiabetic patients with vitreous hemorrhage due to a variety of etiologies ($P = 0.02$). A specific role for CTGF in nondiabetic causes of retinal neovascularization with hemorrhage such as that complicating central retinal vein occlusion cannot be evaluated from this study due to the small sample size.

The possibility that diabetes and its metabolic alterations promote greater retinal expression of CTGF than other disorders with retinal neovascularization should be further evaluated. Recent studies in rats with streptozotocin-induced diabetes showed increased retinal CTGF expression in retinas with preproliferative disease (34). Interestingly, patients with choroidal neovascularization complicating age-related macular degeneration did not show increased vitreous levels of NH$_2$-terminal CTGF in our study, although immunohistochemical studies show that CTGF is expressed locally within surgically excised choroidal neovascular membranes (40). The lack of accumulation of NH$_2$-terminal CTGF in the vitreous of these patients may result from poor diffusion or transport of CTGF across the intact retina from the subretinal space to the vitreous. In a similar way, VEGF is prominently expressed in subretinal neovascular membranes in macular degeneration patients, but analysis of vitreous samples from these patients does not show increased VEGF levels (41).

Prominent CTGF immunoreactivity was seen in SMA-positive stromal myofibroblasts in epiretinal PDR membranes, suggesting local production. Previous studies of CTGF expression in biopsies from patients with diabetic nephropathy and nondiabetic patients with proliferative vitreoretinopathy also showed prominent expression in myofibroblasts, suggesting that this cell type may be a common source of CTGF expression (33,42). In further support of local CTGF production in the retina, a recent in situ hybridization study of three fibrotic epiretinal membranes from patients without diabetes showed local expression of CTGF mRNA within epiretinal membrane tissue (43). It would be interesting to know whether the weak extracellular staining of CTGF in the membranes represents intact CTGF or CTGF fragments; however, the antisera used in the present study do not make this distinction.

There is considerable evidence that CTGF is a major downstream effector of TGF-β in many cell types (21,23,24,30), including retinal endothelial cells (44). CTGF was localized to myofibroblasts in the present study, and myofibroblastic cells in PDR membranes have been shown to express both TGF-β and TGF-β receptors (45). Because levels of active TGF-β may be decreased in the vitreous of patients with active PDR, other mechanisms of regulation must also be considered. Other agents that play a role in active PDR and also regulate CTGF include VEGF, advanced glycosylation end products, mechanical stress, hypoxia, high glucose, and hyperinsulinemia (35,36,46–49).
The preferential accumulation of NH2-terminal CTGF fragment in PDR suggests an important role for this growth factor in retinal fibrosis and neovascularization. Further studies will determine the functional significance of this CTGF cleavage product in PDR and its potential as a target for therapeutic intervention.

Acknowledgments—This study was supported by grants from the Arnold and Mabel Beckman Foundation and from Fibrogen, by an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, and by National Institutes of Health Grants EY-03040 and EY-01545.

We thank Howard Ying, MD, for helpful discussions about experimental design and specimen collection, Ernesto Barron for assistance in preparation of the figures, and Susan Clarke for editorial review.

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CTGF in diabetic retinopathy


