Association of Connective Tissue Growth Factor With Fibrosis in Vitreoretinal Disorders in the Human Eye

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Objective: To investigate the expression of the profibrotic connective tissue growth factor (CTGF) in relation to severity of intraocular fibrosis and neovascularization in human vitreoretinal disorders for the identification of potential therapeutic targets to prevent fibrosis.

Methods: Concentrations of CTGF were measured by enzyme-linked immunosorbent assay in 119 vitreous samples from patients with proliferative diabetic retinopathy, proliferative vitreoretinopathy, epiretinal membrane, and macular hole. Clinical data, including degree of intraocular fibrosis and neovascularization, were collected using standardized forms.

Results: Multifactorial analysis revealed that only CTGF levels correlated highly significantly with degree of fibrosis in the various vitreoretinal disorders studied (P<.001; R²=47.7%). Likewise, variation in degree of fibrosis was best predicted by CTGF levels (P<.001).

Conclusion: The strong correlation between CTGF levels and degree of fibrosis in vitreoretinal disorders suggests that CTGF is an important factor in ocular fibrosis, similar to its role in pathologic fibrosis in other organs.

Clinical Relevance: Connective tissue growth factor may be a therapeutic target for prevention of sight-threatening vitreoretinal scarring in the eye.

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In both proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR), scarring is a final clinical outcome. In these pathologic conditions, fibrovascular or hypovascular fibrotic membranes eventually cause tractional retinal detachment, leading to blindness.1,2 Fibrosis and scarring also complicate the course of choroidal neovascularization in age-related macular degeneration (AMD), contributing to retinal degeneration and loss of vision.3 Age-related macular degeneration, PDR, and PVR represent the main causes of acquired blindness in the Western world.4-7 Although laser photocoagulation and conventional surgery, when applied early, may be beneficial for patients, prevention of fibrosis is an alternative approach to avoiding blindness. For the development of such a treatment, it is necessary to identify the mechanisms that cause fibrosis in pathologic eye conditions.

Connective tissue growth factor (CTGF) is a member of the CCN (CTGF, CYR61, and NOV) family of growth factors, which also includes WISP-1, WISP-2, and WISP-3.8-10 In the skin, lungs, and kidneys, CTGF has been identified as a major factor in physiological wound healing and in pathologic conditions associated with fibrosis.11-14 It mainly functions in these processes as an inducer of extracellular matrix formation.15-16 Furthermore, CTGF is a potent inducer of angiogenesis, at least in certain model systems.10,17-21 In preclinical models, CTGF has been identified as a potential target for antifibrotic therapy.8,22,23 In a rat model of unilateral urethral obstruction, CTGF antisense oligodeoxynucleotide treatment attenuated renal fibrosis.24 Currently, safety and tolerance of anti-CTGF antibodies are being tested in patients with idiopathic pulmonary fibrosis in a phase 1 clinical trial.25

Because of its promise as an antifibrotic target, studies investigating the role of CTGF in ocular fibrosis are mandatory. The CTGF protein and messenger RNA are up-regulated in surgically removed epiretinal and subtretinal fibrovascular membranes,26-28 in biopsy specimens of conjunctivae of patients with...
ocular cicatricial pemphigoid, and in the vitreous of patients with PDR with active neovascularization, all suggesting a role for CTGF in ocular neovascularization, ocular fibrosis, or both. To establish the role of CTGF in these processes more clearly, we studied the relationship between ocular CTGF levels and the severity of fibrosis and neovascularization in a large series of patients with vitreoretinal disorders.

**METHODS**

**PATIENTS**

The study was conducted according to the Declaration of Helsinki and approved by the institutional ethical review board for clinical studies of the Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands. Informed consent was obtained from each patient.

In total, 119 vitreous samples were obtained from 60 men and 59 women. The patients’ primary diagnosis included PDR (n=34), retinal detachment with or without PVR (n=46), macular hole (n=14), and macular pucker (n=25).

Clinical data, which allowed grading of the degree of fibrosis, severity of PDR, activity of neovascularization, degree of hemorrhage, and presence and type of diabetes mellitus, were obtained from preoperative ophthalmic and ultrasound examinations, patient files, and preoperative observations using standardized forms. Fibrosis in all retinal diseases, except for patients with retinal detachment with or without PVR, was graded as follows: 0, no fibrosis; 1, a few preretal membranes (as in macular pucker); 2, preretal membranes with some nonperfused membranes reaching into the vitreous; and 3, abundant nonperfused membranes reaching into the vitreous body. Fibrosis in patients with retinal detachment with or without PVR was graded according to the criteria of the Retinal Society Terminology committee. No proliferative activity was graded as 0, PVR grade a/b as 2, and PVR grade c/d as 3. Neovascularization was graded as 0 when absent, as 1 (quiescent) when only nonperfused gliotic vessels were present, and as 2 (active) when perfused preretal capillaries were present. Degree of hemorrhage was graded as 0 when all media were clear and all fundus details were visible, as 1 when the media were a little clouded but the fundus could still be examined, and as 2 when the optic disc was obscured by hemorrhage, and as 3 when fundus details could not be analyzed. All categorizing was performed by the same surgeon (H.S.T.), who was masked to the results of the CTGF enzyme-linked immunosorbent assay (ELISA).

**SAMPLE COLLECTION**

Samples of 0.5 to 1.0 mL of vitreous fluid were obtained at the beginning of a standard 3-port pars plana vitrectomy with the infusion line in position but not opened. The vitreous was transferred to sterile Eppendorf tubes and immediately frozen in dry ice. The samples were kept at –80°C until assayed.

**MEASUREMENT OF CTGF CONCENTRATION BY ELISA**

After thawing, vitreous samples were centrifuged at 20 800g for 15 minutes at 4°C, and supernatant was collected. The CTGF was measured in samples by means of sandwich ELISA using 2 distinct monoclonal antibodies specifically recognizing the N-terminal part of the CTGF protein (FibroGen Inc, San Francisco, Calif). Microwell plates were blocked with 100 µL of 1% bovine serum albumin in phosphate-buffered saline for 2 hours at room temperature and washed with wash buffer (phosphate-buffered saline with 0.05% Tween 20). Vitreous samples were diluted 5 times in assay buffer (50mM Tris buffer, pH 7.7; 0.1% bovine serum albumin; 4 mM magnesium chloride; 0.4M zinc chloride; 0.05% sodium azide; 50 mg/L of sodium heparin; 0.1% Triton X-100), and 50 µL of diluted sample was added to each well with 50 µL of biotinylated monoclonal antihuman CTGF detection antibody in assay buffer. Plates were incubated for 2 hours at 37°C, washed with wash buffer, and incubated with 100 µL per well of streptavidin-conjugated alkaline phosphatase (Jackson Immunoresearch, Cambridgeshire, England), 1 µg/mL in assay buffer, for 1 hour at room temperature. Plates were washed again with wash buffer, and 100 µL of substrate solution that contained 1 mg/mL of p-nitrophenyl phosphate (Sigma, St Louis, Mo) in diethanolamine buffer (1M diethanolamine, pH 9.8; 0.3M magnesium chloride; 0.02% sodium azide) was added to each well. After 20-minute incubation in the dark, absorbances were read at 405 nm on a microplate reader (BioRad Laboratories, Hercules, Calif). Purified recombinant human CTGF (FibroGen) was used as the standard. All measurements were performed in duplicate.

**STATISTICAL ANALYSIS**

The CTGF levels had a right-skewed distribution and were log transformed to obtain a normal distribution. For this reason, concentrations of CTGF are presented as geometric means (with 95% confidence intervals [CIs]). The effects of sex, age, diabetes, degree of neovascularization, degree of hemorrhage, and fibrosis score on CTGF levels were assessed by univariate and multifactorial analysis of variance (ANOVA). Because of interassay variability, ANOVA was performed with a correction for unequal variances. In the multifactorial analysis, a backward selection procedure was used. In addition, 2 multifactorial ordinal logistic regression analyses with backward selection were performed with fibrosis score or neovascularization activity as the dependent (outcome) variable. A 2-tailed P<.05 was considered statistically significant. All analyses were performed using SAS statistical software, version 9 (SAS Institute Inc, Cary, NC).

The characteristics of the 119 patients are shown in the Table. In univariate analyses, CTGF levels correlated significantly with degree of fibrosis (P<.001) and neovascularization activity (P=.04; Table). Moreover, the geometric mean CTGF levels exhibited a clear correlation with the degree of fibrosis: fibrosis grade 1, 11.63 ng/mL; grade 2, 16.79 ng/mL; and grade 3, 23.51 ng/mL (Figure 1). For each disease state, the mean CTGF level and degree of fibrosis increased simultaneously. Patients with a macular hole (mean, 9.15 ng/mL; 95% CI, 5.78-14.47) or macular pucker (mean, 11.63; 95% CI, 9.21-14.69) had significantly lower mean CTGF levels than patients with a retinal detachment with or without PVR (mean, 15.76; 95% CI, 11.25-22.09) or PDR (mean, 21.02; 95% CI, 17.31-25.53; Figure 2). Patients without neovascularization had significantly lower mean CTGF levels than patients with active neovascularization (mean, 13.59 vs 19.40, respectively; Figure 3). Sex, age, diabetes, and degree of hemorrhage were not significantly associated with CTGF levels.
Multifactorial ANOVA, including the 2 univariate variables significantly correlating with CTGF levels (ie, fibrosis and neovascularization), demonstrated that the most important variable correlated with variation in CTGF levels was degree of fibrosis; only degree of fibrosis remained in the model ($R^2 = 47.7\%$; Table). Likewise, variation in degree of fibrosis was best predicted by CTGF levels and to a lesser extent by neovascularization activity (multifactorial ordinal logistic regression model with fibrosis score as the dependent [outcome] variable resulted in $P < .001$ and $P < .009$ for CTGF and neovascularization activity, respectively). Neovascularization activity itself was best predicted by the presence of diabetes (multifactorial ordinal logistic regression model with neovascularization activity as the dependent [outcome] variable resulted in $P < .001$ for diabetes), whereas CTGF levels were not significantly related to diabetes ($P = .05$).

### Comment

Our study demonstrates for the first time, to our knowledge, that vitreous levels of CTGF strongly correlate with the presence and degree of fibrosis in several important vitreoretinal conditions. In contrast to intraocular neovascularization, fibrosis as a cause of vision loss and its potential as a therapeutic target in PDR and other pathologic vitreoretinal conditions has received relatively little attention in the literature. Moreover, the mechanisms involved in the pathogenesis of intraocular fibrosis are poorly understood. In agreement with observations in the skin, kidneys, and lungs, which have identified CTGF as an important causal factor in pathologic fibrosis, $^{11-14,33}$ our findings suggest that CTGF is also involved in the pathogenesis of intraocular fibrosis.

We did not investigate the source of the elevated CTGF protein levels in the eye, which may be either local production and/or leakage from the circulation through the defective blood-retinal barrier as found in conditions such as PDR and PVR. The theory that local production is the main source of the protein is suggested by the previous demonstration of CTGF messenger RNA in both fibrovascular membranes in PDR $^{28}$ and human retinal pigment epithelial cells. $^{36}$ However, regardless of the origin

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### Table. Patient Characteristics and Mean CTGF Levels

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of Patients (N = 119)*</th>
<th>CTGF, Geometric Mean (95% CI)</th>
<th>Univariate ANOVA $P$ Value†</th>
<th>Multifactorial ANOVA $P$ Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>59.3 ± 15.2</td>
<td>.92</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>.74</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60 (50)</td>
<td>15.32 (12.73-18.43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>59 (50)</td>
<td>14.71 (12.22-17.70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td></td>
<td>.25</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>No diabetes</td>
<td>79 (67)</td>
<td>13.96 (11.87-16.43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>13 (11)</td>
<td>17.14 (13.13-22.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>27 (22)</td>
<td>17.68 (13.43-25.14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neovascularization activity</td>
<td></td>
<td>.04</td>
<td>.92</td>
<td></td>
</tr>
<tr>
<td>No neovascularization (0)</td>
<td>85 (71)</td>
<td>13.59 (11.64-15.87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quiescent neovascularization (1)</td>
<td>12 (10)</td>
<td>19.05 (13.21-27.49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active neovascularization (2)</td>
<td>22 (19)</td>
<td>19.40 (14.71-25.59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree of hemorrhage</td>
<td></td>
<td>.23</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>No hemorrhage (0)</td>
<td>92 (77)</td>
<td>14.18 (13.16-16.53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media slightly clouded (1)</td>
<td>5 (4)</td>
<td>16.73 (8.16-34.29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optic disc obscured (2)</td>
<td>14 (12)</td>
<td>20.89 (9.63-70.59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No fundus details visible (3)</td>
<td>8 (7)</td>
<td>15.16 (9.83-23.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree of fibrosis</td>
<td></td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>No fibrosis (0)</td>
<td>27 (23)</td>
<td>9.57 (7.59-12.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only a few preretinal membranes (1)</td>
<td>25 (21)</td>
<td>11.63 (9.21-14.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Some proliferative membranes/PVR grade a/b (2)</td>
<td>34 (28)</td>
<td>16.79 (13.62-20.70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abundant proliferative membranes/PVR grade c/d (3)</td>
<td>33 (28)</td>
<td>23.51 (19.06-29.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; CTGF, connective tissue growth factor; NA, not applicable; PVR, proliferative vitreoretinopathy.

*Data are presented as number (percentage) unless otherwise indicated.
†Variable removed when $P > .05$ in the backward selection procedure.

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**Figure 1.** Geometric mean connective tissue growth factor (CTGF) levels in relation to degree of fibrosis. Error bars represent the 95% confidence intervals.
of vitreous CTGF, the strong correlation between CTGF levels and degree of fibrosis found in this study is likely to be biologically meaningful.

Our findings extend the findings of a study by Hinton et al, which showed elevated levels of CTGF N-terminal fragments in PDR with active neovascularization, whereas levels of full-length CTGF were low and did not differ among groups. In vivo CTGF is unstable, and proteolytic fragments of CTGF are found in human biological fluids. Recent literature provides evidence that the profibrotic activity of CTGF is mainly regulated by the N-terminal fragment. In our study, we used an ELISA that measures both whole CTGF and CTGF N-terminal fragments; therefore, it may be assumed that mainly N-terminal fragments contributed to elevated levels of CTGF in our samples.

Hinton et al suggested a relationship between CTGF N-terminal fragment levels and both neovascularization and fibrosis in PDR, processes that may be induced by CTGF. However, in their few cases with quiescent PDR, which were characterized by fibrosis and regressed neovascularization, CTGF N-terminal levels were not higher than in controls.

The specific aim of our study was to further investigate whether CTGF was preferentially related to degree of fibrosis or degree of neovascularization in PDR and other vitreoretinal conditions. In contrast to the findings of Hinton et al, multiple regression analysis in our large patient series clearly showed that CTGF correlates with the severity of fibrosis in each of these conditions rather than with the presence of neovascularization. Besides, we investigated CTGF levels in patients with PVR, which is mostly a nonvascular response in eyes with rhegmatogenous retinal detachment, resulting in tractional fibrotic membranes on the surface of the retina, and is an important cause of untreatable blindness. On the basis of the immunohistochemical demonstration of CTGF in PVR membranes, a role of CTGF in this condition has been previously suggested. Our study supports this role because the presence and grade of PVR strongly correlated

Figure 2. Connective tissue growth factor (CTGF) levels in relation to primary diagnosis and degree of fibrosis. The horizontal bars represent the geometric mean CTGF levels for each category. PDR indicates proliferative diabetic retinopathy, PVR, proliferative vitreoretinopathy.

Figure 3. Geometric mean connective tissue growth factor (CTGF) levels in relation to neovascularization activity. Error bars represent the 95% confidence intervals.

Figure 2. Connective tissue growth factor (CTGF) levels in relation to primary diagnosis and degree of fibrosis. The horizontal bars represent the geometric mean CTGF levels for each category. PDR indicates proliferative diabetic retinopathy, PVR, proliferative vitreoretinopathy.

Figure 3. Geometric mean connective tissue growth factor (CTGF) levels in relation to neovascularization activity. Error bars represent the 95% confidence intervals.
with CTGF levels. Hinton et al have suggested a hypothetical scenario for PVR development that included roles for hepatocyte growth factor, transforming growth factor β (TGF-β), and CTGF. Transforming growth factor β is a major growth factor known to cause fibrosis and scarring processes in the skin and other organs, including the eye. It uses CTGF as its main downstream mediator to induce fibrosis. The inactive form of the TGF-β isoform is constitutively present in the human vitreous and may be activated on contact with displaced retinal pigment epithelial cells, as in PVR. Activated TGF-β is therefore one possible inducer of CTGF expression in PVR.

However, in contrast to CTGF, TGF-β is not a suitable target for antifibrotic therapy because of its important physiological functions, such as maintaining an immunosuppressive ocular environment.

In patients with PDR, vascular endothelial growth factor A (VEGF-A) was found to be related to neovascularization activity. Since VEGF-A up-regulates CTGF in retinal vascular cells in vitro, the transition of neovascularization to fibrosis in the later stages of PDR may be regulated by the balance of VEGF-A and CTGF. Further studies are needed to investigate this possibility.

In summary, our study has demonstrated a strong correlation between CTGF levels and the degree of ocular fibrosis. Although associative in nature, our observations suggest that CTGF is causally involved in human ocular fibrosis. In line with applications of CTGF as an antifibrotic target in the lungs and kidneys, our study identifies CTGF as a potential target for therapeutic modulation of fibrosis in vitreoretinal disorders in the human eye.

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Author Contributions: Dr Schlingemann has full access to all the data in the study and takes final responsibility for the decision to submit for publication.

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outer-segment turnover. Spontaneous clinical resolution suggests active RPE phagocytosis. It is notable that previous articles\(^1,4\) have described more severely abnormal electro-oculogram results, possibly at a different or earlier stage of recovery; we have no other explanation for the apparent discrepancy. Previous studies have failed to show mutations in the VMD2 or RDS genes in affected patients that can be causative of inherited Best maculopathy and adult vitelliform macular dystrophy.\(^2,3\) This is in keeping with the absence of a family history in our patients and those previously described. In addition, the normal ERG results in case 3 precluded a diagnosis of Vogt-Koyanagi-Harada or paraneoplastic syndrome. Autofluorescence imaging is likely to be of help in the diagnosis and monitoring of acute exudative polymorphous vitelliform maculopathy and may further the understanding of its pathophysiology.

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**Correction**

Error in Figure. In the Laboratory Sciences article by Kuiper et al titled “Association of Connective Tissue Growth Factor With Fibrosis in Vitreoretinal Disorders in the Human Eye,” published in the October issue of the ARCHIVES (2006;124:1457-1462), some errors occurred in **Figure 2**. The figure is reprinted correctly as follows. We regret the error.

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**Figure 2.** Connective tissue growth factor (CTGF) levels in relation to primary diagnosis and degree of fibrosis. The horizontal bars represent the geometric mean CTGF levels for each category. PDR indicates proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy.

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