TGF-β and CTGF: pro-fibrotic factors in diabetic retinopathy

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Vitreous TIMP-1 Levels associate with Neovascularization and TGF-β2 Levels in the Clinical Course of Proliferative Diabetic Retinopathy

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ABSTRACT

Introduction
In proliferative diabetic retinopathy (PDR), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) cause blindness by neovascularization and subsequent fibrosis. This angio-fibrotic switch is associated with a shift in the balance between vitreous levels of CTGF and VEGF in the eye. Here, we investigated the possible involvement of other important mediators of fibrosis, tissue inhibitor of matrix metalloproteinases (TIMP)-1 and transforming growth factor (TGF)-β2 in the natural course of PDR.

Methods
TIMP-1, activated TGF-β2, CTGF and VEGF levels were measured by ELISA in 78 vitreous samples of patients with PDR (n=28), diabetic patients without PDR (n=24), and patients without diabetes with a macular hole (n=10) or macular pucker (n=16), and were related to clinical data, including degree of intra-ocular neovascularization and fibrosis.

Results
TIMP-1, CTGF and VEGF levels, but not activated TGF-β2 levels, were significantly increased in the vitreous of diabetic patients, with the highest levels in PDR patients. CTGF and the CTGF/VEGF ratio were the strongest predictors of degree of fibrosis. However, activated TGF-β2 correlated with TIMP-1 levels in diabetic patients with or without PDR, whereas TIMP-1 levels were associated with degree of neovascularization, like VEGF levels.

Conclusions
We confirm here our previous findings that retinal fibrosis in PDR patients is significantly associated with vitreous CTGF levels and the CTGF/VEGF ratio. In contrast, vitreous levels of TIMP-1, associated with those of activated TGF-β2, were linked with active neovascularization in diabetes, and not with fibrosis of the retina. Thus, TGF-β2 and TIMP-1 appear to have a role in the angiogenic phase rather than in the fibrotic phase of PDR.
**INTRODUCTION**

In proliferative diabetic retinopathy (PDR), a major cause of blindness,\(^1\) uncontrolled retinal neovascularization is followed by fibrosis, scarring, and tractional retinal detachment. PDR patients with established neovascularization and imminent fibrosis have a poor prognosis, despite aggressive laser treatment or surgical procedures. The major mediator of vascular leakage and angiogenesis in PDR is vascular endothelial growth factor (VEGF)-A, which is overexpressed in ischemic retina.\(^2,5\) Connective tissue growth factor (CTGF) is involved in the fibrotic phase of PDR where it acts as a mitogen for fibroblasts and induces increased ECM production.\(^6-10\) CTGF has been shown to be pro-fibrotic in various other organs, and is associated with pathological fibrosis, including vitreoretinal disorders, such as diabetic retinopathy.\(^11,12\) In a previous study, we reported that VEGF and CTGF levels in the vitreous of patients with PDR correlate with neovascularization and fibrosis, respectively, but that the ratio of CTGF and VEGF levels is the strongest predictor of fibrosis in these patients.\(^31\) We concluded that a shift in the balance between these growth factors causes the switch from angiogenesis to fibrosis in PDR, the so-called “angio-fibrotic switch”. Our concept predicts that anti-VEGF agents, when applied in advanced cases of PDR, may lead to increased fibrosis due to relative high CTGF levels, a phenomenon that indeed is observed in the clinic.\(^14\)

In the course of PDR development, VEGF and CTGF induce expression of many downstream mediators such as proteases and their inhibitors in the cells of fibrovascular membranes. Levels of one of these, tissue inhibitor of metalloproteinases (TIMP)-1, were previously found to be elevated in PDR together with that of matrix metalloproteinase (MMP)-9, an enzyme associated with extracellular matrix degradation.\(^15-20\) This led the authors to suggest a regulating role of TIMP-1 in the onset of neovascularization in PDR.

However, TIMP-1 has also been associated with fibrosis, and its expression is induced by TGF-β, a major causal factor in fibrosis and scarring processes in the skin and other organs, including the eye.\(^20-23\) TIMP-1 is one of the 4 members of the TIMP family, all natural inhibitors of the MMPs. TIMP-1 expression can be induced by many growth factors, including VEGF, TGF-β and CTGF.\(^24-26\) This strongly indicates that TIMP-1 may be involved in the fibrotic stage of PDR as well, and may even mediate the effects of CTGF in fibrosis in the eye. The inactive form of the TGF-β2 isoform is constitutively present in the human vitreous and may be activated by displaced retinal pigment epithelial cells, as occurs in proliferative vitreoretinopathy.\(^27\) Activated TGF-β2 is therefore a possible inducer of CTGF expression in proliferative vitreoretinopathies.\(^21,23,27\) CTGF expression is induced by TGF-β in retinal pericytes.\(^28\) Activated TGF-β2 has also been found to be increased in vitreous of PDR patients.\(^29\)

To investigate the possible roles of TIMP-1, TGF-β2, CTGF and VEGF in the progression of PDR and the angio-fibrotic switch in more detail, we investigated their vitreous levels in a series of diabetic patients with and without PDR and in non-diabetic controls, and associated protein levels to the degree of fibrosis and neovascularization.
MATERIALS AND METHODS

Patients
Seventy-eight vitreous samples of patients with macular hole or idiopathic macular pucker (control group, total N = 26), diabetic patients with macular hole or idiopathic macular pucker but without PDR (DM group, total N = 24), and diabetic patients with PDR (PDR group, N = 28) were collected. In the DM group, 22 patients had no clinical signs of DR, and 2 patients had mild non-proliferative DR. The study was conducted according to the tenets of the Declaration of Helsinki and informed consent was obtained from each patient. The study was approved by the institutional review board of the Academic Medical Center at the University of Amsterdam.

Clinical data, which allowed grading of fibrosis, activity of neovascularization, presence of haemorrhage, and presence and type of diabetes, were obtained both per-operatively using a standardized form and from the patient files. Fibrosis was graded as 0 when there was no fibrosis, as 1 when there were a few pre-retinal membranes (as limited as in macular pucker), as 2 when white preretinal fibrotic membranes with limited extension into the vitreous were present, and as 3 when abundant white membranes reaching into the vitreous body were observed. Neovascularization was graded as 0 when absent, as 1 (quiescent) when only non-perfused vessels were present, and as 2 (active) when there were perfused preretinal capillaries. Vitreous haemorrhage was graded as 0 when all media were clear and all fundus details were visible, and as 1 when media were clouded by haemorrhage.

Sample collection
Undiluted vitreous samples (0.5–1 ml) were obtained by using a vitrectome at the start of a three-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 in duplicate. The samples were kept at -80°C until assayed.

Enzyme-linked immunosorbent assays (ELISAs)
After thawing, vitreous samples were centrifuged at 20,000xg for 15 min at 4°C, and supernatant was collected. Concentrations of VEGF, TIMP-1 and activated TGF-β2 were determined by Quantikine ELISA assays according to the manufacturer’s protocol (R&D Systems, Minneapolis MS, USA). Concentrations of CTGF were determined by sandwich ELISA, using two distinct monoclonal antibodies specifically recognizing the N-terminal part of the CTGF protein (FibroGen, San Francisco CA, USA) as described previously. This assay detects both CTGF N-fragments as well as full-length CTGF. Purified recombinant human CTGF (FibroGen) was used as standard.

Statistical analysis
The growth factor levels in vitreous were tested for normal distribution using histograms and the Shapiro-Wilk test. VEGF showed a left skewed distribution. Therefore, VEGF data were log10 transformed where appropriate, and for vitreous levels of all proteins investigated geometric means were calculated. Differences between the groups in age were assessed by ANOVA. Differences in gender, diabetes type, degree of neovascularization, degree of fibrosis and haemorrhage were assessed by Chi-square or Fischer’s Exact test. Differences in growth factor levels were assessed...
by nonparametric tests (Kruskal-Wallis variance analysis, followed by pair wise comparison using
the Mann Whitney U test). Correlations between protein levels were expressed as Spearman’s
 correlation coefficient, with a ρ of 0.5 or greater considered relevant. Univariate and multiple
 ordinal logistic regression analyses were performed with degree of fibrosis and neovascularization
 as dependent variable, and associations were expressed as odds ratios with a 95% confidence
 interval. A two-tailed p-value <0.05 was considered to indicate statistical differences. All analyses
 were carried out using PASW Statistics (Version 18) software (SPSS, Chicago IL, USA).

RESULTS

Analysis of all patients

Patient characteristics and mean vitreous protein levels are listed in Table 1. Patients with PDR
 were significantly younger than the non-diabetic and diabetic patients with a macular hole or
 macular pucker.

Mean vitreous levels of CTGF, VEGF and TIMP-1, but not activated TGF-β2, were significantly
 higher in diabetic patients with a macular hole or macular pucker and in PDR patients than in
 non-diabetic patients (Figure 1). PDR patients had significantly higher levels of VEGF and TIMP-1,
 but not of CTGF and activated TGF-β2, than diabetic patients with macular hole or macular
 pucker. Univariate analysis of all 78 patients showed that CTGF, VEGF and TIMP-1 levels, and also
 activated TGF-β2, were significantly associated with the presence of diabetes (p-values <0.001,
 except for activated TGF-β2: p<0.05; data not shown).

Analysis of all diabetic patients

Since diabetes was associated with elevated levels of all proteins, we performed
 further statistical analysis on the data of the diabetic patients to exclude this
 confounder. Also, diabetic patients with and without PDR were analyzed separately.
 In diabetic patients, a significant correlation was found between the levels of TIMP-1 and
 activated TGF-β2 (ρ 0.5, p<0.001; data not shown). No relevant correlations were found between
 other proteins.

The multiple ordinal regression analysis which is adjusted, for the effects of other included
covariates, showed that CTGF, but not VEGF, TIMP-1 and activated TGF-β2, was significantly
 associated with degree of fibrosis (Table 2). An association with degree of neovascularization
 was found for VEGF and TIMP-1, but not for CTGF and activated TGF-β2. The effect of VEGF,
 however, was larger than the effect of TIMP-1.

Correlations of CTGF and TIMP-1 with degree of fibrosis and neovascularization are shown
 in Figure 2.

Analysis of patients with PDR

Additional regression analysis of the 28 patients with PDR revealed that CTGF levels were
 significantly associated with degree of fibrosis (OR 2.00, 95% CI 1.28-3.15, p<0.01), but the CTGF/
VEGF ratio was the strongest predictor of degree of fibrosis in this group (OR 4.08, 95% CI
1.68-9.90, p<0.01). TIMP-1 was significantly associated with degree of neovascularization in this
analysis (OR 1.04, 95% CI 1.00-1.07, p<0.05).
Table 1. Patient characteristics and data. VEGF data were not normally distributed. Therefore, geometric means with 95% confidence intervals were calculated for all proteins investigated.

<table>
<thead>
<tr>
<th>Patient characteristics (n=78)</th>
<th>Subcategory</th>
<th>Non-diabetic (n=26)</th>
<th>DM (no PDR) (n=24)</th>
<th>PDR (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Macular hole (n=10)</td>
<td>Macular hole (n=4)</td>
<td>PDR (n=28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pucker (n=16)</td>
<td>Pucker (n=20)</td>
<td>Pucker (n=20)</td>
</tr>
<tr>
<td>Age in years (mean ± SD)</td>
<td>n=</td>
<td>67.4 ± 5.3</td>
<td>71.9 ± 7.5</td>
<td>58.6 ± 12.3</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>16</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Patients with diabetes</td>
<td>Total</td>
<td>0</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>0</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>No haemorrhage (0)</td>
<td>26</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Haemorrhage (1)</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Degree of neovascularization</td>
<td>No neovascularization (0)</td>
<td>26</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Quiescent neovascularization (1)</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Active neovascularization (2)</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Degree of fibrosis</td>
<td>No fibrosis (0)</td>
<td>10</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Only few pre-retinal membranes (1)</td>
<td>16</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Some proliferative membranes (2)</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Abundant proliferative membranes (3)</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TIMP-1 (geom. mean, 95% CI)</td>
<td>14.1 ng/ml [10.3-19.3]</td>
<td>32.5 ng/ml [22.4-47.2]</td>
<td>76.7 ng/ml [58.1-101.3]</td>
<td></td>
</tr>
<tr>
<td>CTGF (geom. mean, 95% CI)</td>
<td>4.4 ng/ml [3.6-5.5]</td>
<td>7.3 ng/ml [5.6-9.6]</td>
<td>10.6 ng/ml [8.7-13.0]</td>
<td></td>
</tr>
<tr>
<td>VEGF (geom. mean, 95% CI)</td>
<td>9.8 pg/ml [8.7-111]</td>
<td>30.2 pg/ml [14.2-64.37]</td>
<td>625.5 pg/ml [413.7-945.7]</td>
<td></td>
</tr>
<tr>
<td>TGF-β2, activated (geom. mean, 95% CI)</td>
<td>85.4 pg/ml [533-137.6]</td>
<td>129.6 pg/ml [77.8-215.9]</td>
<td>151.2 pg/ml [96.5-236.9]</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Vitreous protein levels of CTGF (A), TIMP-1 (B), VEGF (C) and activated TGF-β2 (D) per group of patients that are non-diabetic (Con), diabetic without PDR (DM), and diabetic with PDR (PDR). Geometric means with 95% confidence intervals are shown. * p<0.01, ** p<0.001.

Again, TIMP-1 levels were significantly correlated with activated TGF-β2 in the PDR group ($\rho=0.5$, $p<0.05$; data not shown).

Analysis of diabetic patients without PDR

Again, in patients with diabetes but without PDR (DM group), a significant correlation was found between TIMP-1 and activated TGF-β2 levels (Figure 3A), and also between TIMP-1 and CTGF levels. No relevant correlations were found between the other proteins. Despite small numbers, TIMP-1 levels and also activated TGF-β2 levels were significantly higher in the diabetic patients with macular pucker (fibrosis grade 1) than in diabetic patients with macular hole (fibrosis grade 0; Figure 3B), a difference not observed in the non-diabetic control patients (data not shown). CTGF and VEGF levels did not differ significantly between macular hole and macular pucker patients whether the patients were diabetic or not.

DISCUSSION

In the present study, we confirmed our previous findings that the degree of fibrosis in retinas of PDR patients is significantly related to vitreous levels of CTGF and even stronger with the ratio of CTGF and VEGF levels.13,14 We also show here that the levels of a major protein involved in tissue remodeling, wound healing and fibrosis, TIMP-1, are increased in the vitreous of diabetic and
Table 2. Results of the multivariable ordinal logistic regression analysis (proportional odds model) with CTGF, TIMP-1, VEGF and activated TGF-β2 included in the model and degree of fibrosis and degree of neovascularization as responses in 52 diabetic patients with and without PDR.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Contrast</th>
<th>Degree of fibrosis</th>
<th></th>
<th>Degree of neovascularization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR [95% CI]</td>
<td>p Value</td>
<td>OR [95% CI]</td>
<td>p Value</td>
</tr>
<tr>
<td>CTGF</td>
<td>per unit increase</td>
<td>1.36 [1.18-1.58]</td>
<td>&lt;0.001</td>
<td>0.99 [0.87-1.12]</td>
<td>0.886</td>
</tr>
<tr>
<td>VEGF</td>
<td>per 10 fold increase</td>
<td>1.09 [0.56-2.09]</td>
<td>0.803</td>
<td>7.52 [2.47-22.92]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>per unit increase</td>
<td>1.01 [0.56-2.09]</td>
<td>0.803</td>
<td>1.00 [0.200-1.00]</td>
<td>0.030</td>
</tr>
<tr>
<td>act. TGF-β2</td>
<td>per unit increase</td>
<td>1.00 [0.56-2.09]</td>
<td>0.803</td>
<td>1.00 [1.00-1.01]</td>
<td>0.395</td>
</tr>
</tbody>
</table>

* CTGF, VEGF, TIMP-1, act. TGF-β2 in model

Figure 2. Association of CTGF (A,C) and TIMP-1 (B,D) with degree of neovascularization (A,B) and degree of fibrosis (C,D) in vitreous of 52 diabetic patients with and without PDR. The lines indicate the geometric means of protein levels per degree of neovascularization or fibrosis. CTGF correlated significantly with fibrosis (Spearman’s ρ 0.6, p<0.001), and TIMP-1 correlated significantly with neovascularization (Spearman’s ρ 0.6, p<0.001).

PDR patients, but it is associated mainly with active neovascularization rather than with fibrosis, in association with levels of activated TGF-β2.

We investigated whether TGF-β2, in addition to VEGF, acts as an upstream inducer of CTGF in PDR, and TIMP-1 as a downstream effector of CTGF in PDR. However, the relationship between the four proteins is complex. For example, TIMP-1 expression is known to be induced by TGF-β, but also by CTGF and VEGF.24,26 Feed-back mechanisms between VEGF and CTGF are also known: VEGF can induce CTGF, and CTGF can inhibit the actions of VEGF by direct
protein-protein binding. Despite these complex interactions, we were able to show novel associations between vitreous levels of these proteins and the degree of fibrosis and neovascularization of the retina that may help to understand their role in the development of PDR and the contribution to the angio-fibrotic switch.

First, we found that vitreous levels of CTGF, VEGF and TIMP-1 were significantly higher in patients with diabetes and in patients with PDR. Activated TGF-β2 levels on the other hand, did not significantly differ between the groups. Second, VEGF was associated with neovascularisation in diabetic patients. Although a role in active PDR has also been suggested for CCN2, the increased CCN2 levels in our PDR patients did not differ between quiescent and active neovascularization. CCN2 was associated only with fibrosis, as shown by us and others in previous reports. Third, TIMP-1 was associated with neovascularisation, and not with fibrosis. This suggests that in the natural course of PDR, developing from an angiogenic phase to a fibrotic phase via the angio-fibrotic switch, TIMP-1 has an early role in angiogenesis and may not act as a downstream mediator of CTGF in the fibrotic phase. Seemingly in contrast to the conclusion that TIMP-1 has a role in angiogenesis is our finding that in PDR patients, TIMP-1 correlated with active TGF-β2, a major growth factor known to cause fibrosis and scarring. However, we did not find a correlation of active TGF-β2 with fibrosis in PDR patients, indicating that this growth factor, like TIMP-1, has no role in the angio-fibrotic switch and the subsequent fibrotic phase of PDR.

Although not the main focus of this study, it is interesting to note that in the group of diabetic patients without PDR of which almost all had no retinopathy at all, TIMP-1 was also increased and correlated with activated TGF-β2. This suggests that the expression of TIMP-1 is under the control of TGF-β2 in diabetic patients with or without PDR. In the patients without PDR, TIMP-1 and activated TGF-β2 were both significantly higher in eyes with macular pucker than in macular holes, a pattern not seen in non-diabetic controls. This suggests that TIMP-1 and active TGF-β2 have a role in glial cell activation and pre-retinal fibrosis only in a diabetic environment, and that the pathogenesis of pre-retinal fibrosis may differ between diabetic and

Figure 3. Spearman correlation between levels of TIMP-1 and activated TGF-β2 in vitreous of 24 diabetic patients without PDR (A), and vitreous TIMP-1 levels in macular hole and macular pucker patients with diabetes (B). (A) TIMP-1 and activated TGF-β2 correlated significantly (Spearman’s ρ 0.5, p<0.01). (B) TIMP-1 levels were significantly higher in diabetic patients with pukers compared to macular holes. * p<0.01. The lines indicate the geometric means of TIMP-1 levels in each group.
non-diabetic subjects. Moreover, the role of activated TGF-β2 in early phases of DR rather than in the late phase of PDR fit in the role of TGF-β2 signaling in preclinical DR.28

We can only speculate how TIMP-1 functions exactly in the context and course of development of PDR. TIMPs are endogenous inhibitors of MMPs, which have an important role in connective tissue remodelling and in the degradation of the basal lamina and surrounding ECM during angiogenesis.19 Matrix remodeling, necessary for sprouting angiogenesis, is dependent on well-coordinated interactions between MMPs and TIMPs. TIMP-1 is found to be increased in PDR,17-20 and can inhibit MMP-9 activation in vitreous.15 Increased expression of TIMP-1 may therefore have a counter balancing effect on pro-angiogenic factors such as MMPs and VEGF, and thereby a regulatory anti-angiogenic effect. These observations in the literature are in line with our finding that TIMP-1 appears to have a role in the angiogenic phase rather than in the later scarring phases of PDR.

In summary, our study indicates that in the complex context of the development of PDR from an angiogenic phase to fibrosis and scarring, TIMP-1, a major regulatory protein involved in tissue remodeling and wound healing, is mainly involved in the process of angiogenesis after induction by VEGF and TGF-β2.

ACKNOWLEDGEMENTS

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