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

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General Discussion and Summary
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DR is a complex disease with a long asymptomatic pre-clinical phase (PCDR), in which hyperglycaemia induces various pathological changes in the retina, setting the stage for the vision-threatening clinical manifestations, such as vascular leakage, bleeding from newly formed vessels and retinal detachment as a consequence of fibrosis in proliferative DR (PDR). Several biochemical mechanisms have been proposed to modulate the pathogenesis of DR, including expression of growth factors such as vascular endothelial growth factor (VEGF), a major angiogenic factor in clinical DR. Growth factors with pro-fibrotic properties may also be critically involved, such as transforming growth factor (TGF)-β, with connective tissue growth factor (CTGF) as a downstream mediator. The studies in this thesis are aimed at further describing the role and interactions of these growth factors in PCDR and PDR.

In chapter 2, we review the role of CTGF in DR, in which tissue fibrosis plays an important role in two distinct phases. First, in PCDR, structural and functional extracellular matrix (ECM) alterations occur, including thickening of the basal lamina (BL) of retinal capillaries. BL thickening is a hallmark of early DR and may be critical in progression of the disease, as prevention of BL thickening may also prevent other early changes, such as loss of pericytes and development of acellular capillaries. Later, in the clinical phase, the switch from neovascularization to a fibrotic phase in PDR occurs, which is a wound healing-like fibrotic response.

CTGF, also known as CCN2, is a potent pro-fibrotic factor involved in ECM synthesis, and its levels are increased under diabetic conditions. CTGF functions not only as a downstream mediator of pro-fibrotic TGF-β signaling, but can also induce ECM synthesis independently.

In both stages of DR, CTGF has been shown to play a role. First of all, CTGF is increased in the diabetic retina and contributes to BL thickening in PCDR. In PDR, CTGF is associated with vitreoretinal fibrosis, and drives the angio-fibrotic switch in a critical balance with VEGF.

The main conclusion of this chapter is that CTGF-targeted therapy is a possible novel option to inhibit development of early disease as well as to prevent sight-threatening complications in advanced stages, warranting further studies.

Pre-clinical diabetic retinopathy

In chapter 3 and 4, we describe the effects of VEGF and TGF-β on the expression of CTGF and ECM-related factors in cultured retinal vascular cells. Our in vitro studies show differential induction of expression. In retinal endothelial cells (ECs), expression of CTGF was induced by VEGF, most likely independent of TGF-β, as CTGF expression was not directly induced by TGF-β in these cells. In retinal pericytes (PCs), however, expression of CTGF was induced by TGF-β, specifically through the ALKS-Smad2/3 receptor-complex and already at low concentrations. VEGF induced neither the expression of TGF-β nor CTGF in PCs, indeed suggesting that in these cells CTGF production has a function downstream of TGF-β.

Expression of the ECM component fibronectin (FN) was induced directly by VEGF and also by TGF-β, specifically via ALKS-Smad2/3, in both cultured ECs and PCs. However, gene expression and protein levels were higher in PCs. In general, PCs were much more responsive to TGF-β. Tissue inhibitor of metalloproteinase (TIMP)-1, a factor involved in ECM turnover, was induced in both types of retinal vascular cell by VEGF, but not by TGF-β.
In vivo, intravitreal injection of VEGF resulted in increased expression of pro-fibrotic factors (CTGF and TGF-β) and ECM-related factors (FN, TIMP-1) in the retina, similar to those in the retinal vascular cells. The major conclusions of these chapters are that VEGF and TGF-β have differential effects on retinal ECs and PCs.

In our next study in long term experimentally-induced diabetes in wild type (WT) mice and mice with CTGF-haploinsufficiency (CTGF +/-), we found that diabetes in WT mice induced the known early pathological changes of the retinal capillaries, i.e. BL thickening, loss of pericytes and formation of acellular capillaries (chapter 5). In contrast, the reduced levels of retinal CTGF protein in the CTGF +/- mice not only prevented diabetes-induced thickening of the BL after 6 to 8 months of diabetes, as we had shown before after 17 weeks of diabetes, but also completely prevented loss of pericytes and significantly reduced the formation of acellular capillaries. The conclusion of the studies described in this chapter is that CTGF has a major role in all the prominent early changes in the retina during PCDR.

Several experimental rodent studies have demonstrated the relevance of abnormal ECM synthesis and BL thickening in the development of DR, showing that inhibition of BL thickening by interfering with BL component production led to prevention of pericyte loss and acellular capillary formation. In line with these results, pericyte loss in our study may have been indirectly prevented by inhibition of BL thickening as a result of decreased retinal CTGF levels. However, CTGF could also have a direct role in pericyte loss in early DR by causing pericyte apoptosis or by allowing pericyte migration. This is supported by our earlier studies where we observed that in human early DR, a shift in the localization of CTGF protein occurs from a predominantly microglial location in the normal human retina to a predominantly pericyte location in diabetic patients.

The incomplete inhibition of acellular capillary formation in our model may imply that besides reduced CTGF levels, and despite a complete prevention of BL thickening and pericyte loss, other mechanisms are involved in vasoregression in diabetes. These may include direct effects of the formation of advanced glycation end products (AGEs), actions of adhered leukocytes, or disruption of the retinal neurovascular unit by apoptosis of neurons.

Our non-diabetic CTGF +/- mice showed that reduced retinal CTGF protein levels did not hamper the formation of a functional vascular network with proper recruitment of pericytes. This indicates that lowering of CTGF levels in the eye in itself is probably not harmful. Therefore, we conclude that targeting of CTGF can be a safe and effective way to prevent the development of DR, even as a long term preventive treatment in asymptomatic PCDR.

Clinical diabetic retinopathy

In patients with PDR, we have previously shown that vitreous levels of CTGF are associated with the clinical degree of vitreoretinal fibrosis. On the other hand, levels of VEGF, a major angiogenic factor in PDR, are associated with degree of neovascularization, and not with fibrosis. We have established previously that the ratio of CTGF and VEGF levels in the vitreous was the strongest predictor of fibrosis in PDR. These results led to the concept of the angio-fibrotic switch. During the course of PDR with high angiogenic VEGF levels, pro-fibrotic CTGF levels also increase, and when the balance between these two factors reaches a certain threshold ratio, the angio-fibrotic switch occurs and fibrosis is driven by the altered balance of these growth factors.
This concept predicts that intravitreal anti-VEGF treatment, which nowadays is routinely used in ocular diseases including DR, causes a shift in the CTGF/VEGF balance in favour of CTGF, leading to accelerated fibrosis. In an independent cohort of PDR patients, including patients treated with anti-VEGF antibodies (bevacizumab), we confirmed that CTGF and the CTGF/VEGF ratio are strong predictors of fibrosis (chapter 6). Anti-VEGF treated patients had higher CTGF levels and higher degrees of fibrosis in the retina. Furthermore, a significant increase in the degree of fibrosis was observed after treatment with bevacizumab. Given preoperatively as adjunct to vitreoretinal surgery, anti-VEGF treatment may increase the risk of post-operative fibrotic complications due to a balance shift towards CTGF. A major conclusion of this chapter is that adjuvant therapy targeted against fibrotic factors such as CTGF may be required in combination with anti-VEGF therapy.

In chapter 7, we investigated other possible mediators of fibrosis, tissue inhibitor of metalloproteinases (TIMP)-1 and activated TGF-β2, in the course of PDR. These proteins, together with CTGF and VEGF, were studied in vitreous samples of patients with PDR, but also in diabetic patients without clinical signs of (P)DR and non-diabetic controls. Compared to non-diabetic control patients, levels of TIMP-1, CTGF and VEGF, but not TGF-β2, were significantly increased in diabetic patients without PDR, representing in fact patients with PCDR as almost none had any clinical sign of DR at all. The highest levels of TIMP-1, CTGF and VEGF were found in PDR patients. In all diabetic patients, the degree of vitreoretinal fibrosis was associated with CTGF levels and the CTGF/VEGF ratio, and neovascularization with levels of VEGF. Of all 4 proteins examined in this study, correlations were found uniquely between TIMP-1 and TGF-β2 levels, in the group of all diabetic patients, and also in the subgroups DM without PDR and PDR. However, neither TIMP-1 nor TGF-β2 was associated with degree of fibrosis in the course of PDR. Interestingly, TIMP-1 appeared to be a modest but significant predictor of degree of neovascularization instead. This indicates that TIMP-1 may be more involved in the angiogenic phase of PDR, as a modulator of ECM turnover in the retinal vessels during neovascularization, rather than in the fibrotic phase after the angio-fibrotic switch has occurred.

Only in the group of diabetic patients without (P)DR did TIMP-1, and to a lesser extent also TGF-β2, show a correlation with fibrosis, as its levels were significantly higher in patients with a macular pucker than in patients with a macular hole. The conclusion of this chapter is that TIMP-1 and TGF-β2 may be involved in early stages of DR rather than late stages of DR.

In conclusion, we found differential pro-fibrotic effects of VEGF and TGF-β in models of PCDR, with CTGF as a downstream effector of both growth factors, depending on the vascular cell type in the retina. Furthermore, we showed that CTGF is involved not only in BL thickening, but also in other early changes, i.e. pericyte loss and vasoregression, in the development of DR. Together, this suggests that CTGF, which levels are indeed increased in vitreous of diabetic subjects without DR, may be a promising target for safe and effective prevention of the development of DR. Targeting of TGF-β, which induces CTGF in the retina, would be a less attractive option as this growth factor has other, beneficial, functions besides its pro-fibrotic properties.

In patients with PDR that received anti-angiogenic treatment by means of intravitreal injections of anti-VEGF antibodies, we showed that CTGF levels remain high and associate with vitreoretinal fibrosis, thereby increasing the risk of fibrotic complications such as tractional...
retinal detachment. This suggests that in this stage of the disease adjuvant therapy targeted against fibrotic factors is required in combination with anti-VEGF therapy. TGF-β and TIMP-1, two known mediators of fibrosis in other organs, were not involved in vitreoretinal fibrosis in PDR in our study, and therefore seem unsuitable as targets, whereas CTGF may well be such as target in PDR. Therefore, our studies emphasize the potential beneficial effects of anti-CTGF treatment in DR, either alone or in combination with anti-VEGF treatment.

REFERENCES
