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

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Publication date
2012

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Connective Tissue Growth Factor is involved in Pericyte Loss and Vasoregression in Preclinical Diabetic Retinopathy

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Submitted for publication

ABSTRACT

Introduction

Early retinal vascular changes in the development of diabetic retinopathy (DR) include capillary basal lamina (BL) thickening, pericyte loss and development of acellular capillaries. Connective tissue growth factor (CTGF), a potent inducer of BL components, is upregulated early in diabetes, and diabetic mice lacking one functional CTGF allele ($CTGF^{+/-}$) do not show BL thickening. As the early events in DR may be interrelated, we hypothesized that CTGF levels could have a role not only in BL thickening but also in other pathological changes of the retinal capillaries.

Methods

We studied the effects of long-term streptozotocin-induced diabetes on retinal capillary BL thickness, numbers of pericytes and the development of acellular capillaries in $CTGF^{+/+}$ and $CTGF^{+/-}$ mice.

Results

Reduction of retinal CTGF prevented BL thickening of retinal capillaries and pericyte dropout after long-term diabetes (6-8 months). Furthermore, in contrast to diabetic wild type mice, formation of acellular capillaries was not significantly increased in diabetic $CTGF^{+/-}$ mice.

Conclusions

We conclude that CTGF is necessary for the three major phenomena of diabetes-induced retinopathy in rodents. Inhibition of CTGF in the eye may therefore be protective against the development of DR.

INTRODUCTION

The vision-threatening clinical manifestations of diabetic retinopathy (DR) are preceded by a long pre-clinical phase (PCDR). During the 5-15 years of PCDR, hyperglycaemia induces a number of pathological changes in the retinal vasculature,[1] among which diffusely increased permeability, thickening of the retinal capillary basal lamina (BL), loss of pericytes, degeneration of endothelial cells and neurons, and the development of acellular capillaries.[2] The acellular capillaries eventually develop into expanding areas of capillary non-perfusion, retinal ischaemia and other clinical signs of DR.

The exact sequence of the pre-clinical events and their relative importance in the development of DR are not clear yet. Loss of pericytes that maintain capillary stability and regulate homeostasis of the endothelium,[3] is an early event in the diabetic retina. It is associated with altered activity of factors controlling survival and differentiation of pericytes in interaction with the endothelium, such as platelet-derived growth factor (PDGF)-B and transforming growth factor (TGF)-β.[4,5] The angiopoietin (Ang)/Tie-2 system is also involved, in which Ang-2 is upregulated early in diabetes and is associated with pericyte dropout in retinal capillaries in DR.[6-8] Loss of pericytes leads to reduced numbers of endothelial cells and ultimately to the formation of non-perfused acellular capillaries.[5-9]

In addition to pericyte loss, another early structural change is thickening of the basal lamina (BL) around capillaries of the inner retina. This is the result of extracellular matrix (ECM) remodelling leading to increased deposition of BL components such as collagen type IV, laminin and fibronectin (FN),[10] and occurs in both diabetic animals and humans.[11-15] Inhibition of diabetes-induced BL thickening in rodent models by modulation of BL components has been shown to prevent diabetic vascular changes, such as retinal pericyte loss, formation of acellular capillaries, and vascular leakage.[16-18] These findings suggest that BL thickening may be critical in the further development of DR into the clinical phase.

Connective tissue growth factor (CTGF) is a member of the CCN family of matricellular proteins and is also known as CCN2.[19] It is a potent pro-fibrotic factor involved in ECM synthesis, and its levels are increased under diabetic conditions.[20,21] CTGF functions as a downstream mediator of TGF-β signaling and may act as a co-factor for the pro-fibrotic actions of TGF-β,[22] but can also induce ECM synthesis independently.[23]

Increasing evidence points at an important role of CTGF in the pathogenesis of both preclinical and proliferative DR.[24-31] In the retina of rodents, CTGF is upregulated in streptozotocin (STZ)-induced diabetes, as well as after intravitreal injection of VEGF and after systemic infusion with advanced glycation end products (AGEs).[24,26] CTGF protein is expressed in pericytes in the retina of diabetic humans with early DR but not in normal retina.[27] In cultured retinal vascular cells, CTGF expression is induced by VEGF in pericytes and endothelial cells and by TGF-β in pericytes but not in endothelial cells.[24,25] Moreover, in vitreous of diabetic patients without clinical signs of DR, CTGF levels are increased (Van Geest et al., submitted). In patients with proliferative DR (PDR), CTGF is associated with fibrosis and, in a critical balance with VEGF, with the induction of the angio-fibrotic switch.[28-30]

We have previously reported that in PCDR, mice lacking one functional CTGF allele ($CTGF^{+/-}$) did not show the BL thickening observed in diabetic wild type mice after 17 weeks

of experimentally induced diabetes.[31] As a recent study in $CTGF^{-/-}$ mice embryos suggests that CTGF is required for normal levels of FN expression during development, and also for pericyte recruitment, in part by potentiating PDGF signaling,[32] we hypothesized that CTGF levels may not only have a role in BL thickening in PCDR, but also be involved in pericyte loss and the other known pathological changes in PCDR. Therefore, we studied the effects of CTGF haploinsufficiency on retinal capillary BL thickness, numbers of pericytes and endothelial cells in retinal capillaries, and on the development of acellular capillaries in long-term experimentally induced diabetes in wild type ($CTGF^{+/+}$) mice and $CTGF^{+/-}$ mice.

METHODS

Animal experiments

Animal experiments were performed with the approval of the Animal Ethics Committee of the University of Amsterdam and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

$CTGF^{+/-}$ mice, in which exon 1 of one of the CTGF alleles was replaced by a neomycin-resistance gene, were crossed back on a C57BL/6 background (Harlan, Horst, The Netherlands) [33]. Male and female mice of the 8th generation or later were used in the present study, and were compared with male and female $CTGF^{+/+}$ littermates. Each group contained approximately as many males as females. Genotyping was performed on 100 ng DNA isolated from earmarks following a standard procedure using the following primers: 5'-TGTGTAGGACTTCATTCAGTTCT-3', 5'-GTCTGTGATCGCAGCTCACTC-3' and 5'-ATGGCCGCTTTTCTGGATTC-3', resulting in a 400 base pair product for the wild type, and a 560 base pair product for the CTGF-neomycin construct.

Diabetes was induced by means of a single intraperitoneal injection of 200 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA), dissolved in 100 mM sodium citrate buffer, pH 4.5. Control animals were injected with buffer alone. Hyperglycaemia was determined at 3 days after injection by measurement of blood glucose levels (Medisense Precision Xtra; Abbott, Bedford, IL, USA). Non-responders were injected with a second dose of STZ. Slow-release insulin pellets (Linshin, Scarborough, ON, Canada) were implanted to stabilize the condition of the diabetic animals at 5 days after STZ injection. Mice were monitored daily for weight loss and their clinical condition. Blood was withdrawn by cheek puncture for glucose measurements at 1, 3, 6 and 8 months after induction of diabetes, and on indication. Blood glucose levels >20 mmol/l warranted reimplantation of a slow-release insulin pellet. In general, mice received an insulin pellet each 4-6 weeks. Glycosylated haemoglobin (HbA_{1C}) was measured on automated HA8140 HPLC analysers (Menarini Diagnostics, Florence, Italy). Animals were kept on standard laboratory chow with daily addition of mash food. All mice were housed in a temperature- and humidity-controlled room with a 12-hour light/dark cycle.

Mice were killed at 6 to 8 months after induction of diabetes by a ketamine-xylazine-atropine injection. One eye of each animal was enucleated and snap frozen in liquid nitrogen and stored at -80°C until analysis, and the other eye of each animal was immediately fixed for electron microscopy (EM). Numbers of animals are shown in Table 1.

Table 1. Control and diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice have similar characteristics and the same degree of diabetes.

	CTGF+/+		CTGF+/-	
	Control	**Diabetic**	**Control**	**Diabetic**
Number of mice (% male) in 6 months groups	8 (50)	9 (56)	7 (43)	7 (57)
Number of mice (% male) in 8 months groups	8 (50)	11 (64)	8 (50)	10 (50)
Body weight (g)	28.3 ± 1.2	24.7 ± 0.7*	29.7 ± 1.6	24.9 ± 0.7*
Plasma glucose (mmol/l)	10.3 ± 2.5	22.8 ± 5.3*	10.8 ± 2.9	22.6 ± 6.1*
HbA_{1c} (%)	3.6 ± 0.3	6.6 ± 2.1*	3.7 ± 0.2	6.1 ± 1.4*

Data of animals are means ± SD. * p<0.05 diabetic versus control mice. No significant differences between $CTGF^{+/+}$ and $CTGF^{+/-}$ mice.

Western blot analysis

Retinas of 4 $CTGF^{+/+}$ and 4 $CTGF^{+/-}$ adult surplus mice were isolated and pooled per genotype in 100 µl lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1x phosphatase inhibitors and 1x Complete Protease Inhibitors; Roche Biochemicals, Almere, The Netherlands). Samples were homogenized with a pestle and vigorous vortexing and frozen overnight. Samples were thawed and vortexed and then centrifuged at 4°C for 20 minutes at 14,000x*g*. Supernatants were collected in new vials and stored at -80°C. The protein concentration of each sample was determined with a Bradford assay kit (Bio-Rad).

Western blots were performed as described previously.[25] Sixty µg of protein was separated on a 12.5% SDS-PAGE gel and rabbit-polyclonal anti-CTGF (ab6992; Abcam, Cambridge, UK) was used in a dilution of 1:1,000 overnight at 4°C to detect CTGF. Rabbit anti-β-actin antibody (1:10,000; Sigma) was used on the same blots for loading control. Horseradish peroxidase-conjugated secondary anti-rabbit antibody (Sigma) was used to detect protein bands by digital scanning of the enhanced chemoluminescence (ECL) signal (Roche) with an image-capturing device (LAS-3000; Fujifilm Corporation, Tokyo, Japan). All Western blot experiments were performed in duplicate.

Electron microscopy

Eyes for EM were prepared as described.[31] Briefly, eyes were fixed in freshly-prepared McDowell's fixative in 0.1 M sodium cacodylate buffer, pH 7.4, directly after enucleation. Retinas were extracted and also fixed in McDowell's fixative in 0.1 M sodium cacodylate buffer, pH 7.4. Then, the retinas were washed in 0.1 M sodium cacodylate buffer, pH 7.4, and were postfixed in 1% osmium tetroxide (Merck, Haarlem, The Netherlands) in bidistilled water for one hour, subsequently dehydrated, and embedded in Epoxy resin (Epon LX-812; Merck). Ultrathin sections were cut from the resin block on a Reichert-Young ultramicrotome equipped with a diamond knife. Sections were mounted on copper slot grids.

Images were made of transversally sectioned capillaries in and around the inner nuclear layer, in the middle sector of the retina between the optic disc and the peripheral retina. Of each retina, approximately 20 images were taken at a magnification of 11,500x on a FEI Technai 12 (FEI; Eindhoven, The Netherlands).

Measurement of BL thickness

Measurements of the thickness of BLs of retinal capillaries were performed as described.[31] Twelve EM images of capillaries were randomly selected per individual retina and a grid with 20 equally spaced radial lines was overlaid as described by Cuthbertson and Mandel (Figure 1A).[34] Where the radial lines intersected the BL, BL thickness was measured, using a validated software package (CapiScope; KK Technology, Devon, England).

The BL of the capillaries was divided in domains (Figure 1A). The "outer" and "inner" domains were defined according to Fischer and Gärtner.[35] The "outer" BL is positioned outside the endothelial cell (eBL) and/or pericyte (pBL) adjacent to surrounding glial tissue. The "inner" BM is positioned in between endothelial cell and pericyte as joint BL (jBL). Measurements were taken by a blinded observer, only when the cell membranes adjacent to the BL were visible as distinct dark lines.

Retinal digest preparations

The number of endothelial cells and pericytes in capillaries of the inner retinal vasculature were determined as described.[8] Retinas of diabetic and non-diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice were fixed in 4% paraformaldehyde for 24–48 h. Then, retinas were incubated in bidistilled water for 30 min. Subsequently, the retinas were transferred to a solution of 3% trypsin dissolved in 0.2 M Tris buffer, pH 7.4, for 2–3 h. The retinas were washed with bidistilled water, and isolated retinal vasculature was flat mounted on objective slides. The samples were stained with periodic acid Schiff base and hematoxylin.[36]

Quantitative retinal morphometry

Analysis of pericyte coverage of the retinal vasculature and numbers of endothelial cells present in the vasculature was carried out in the retinal digest preparations (Figure 1B).[4] In

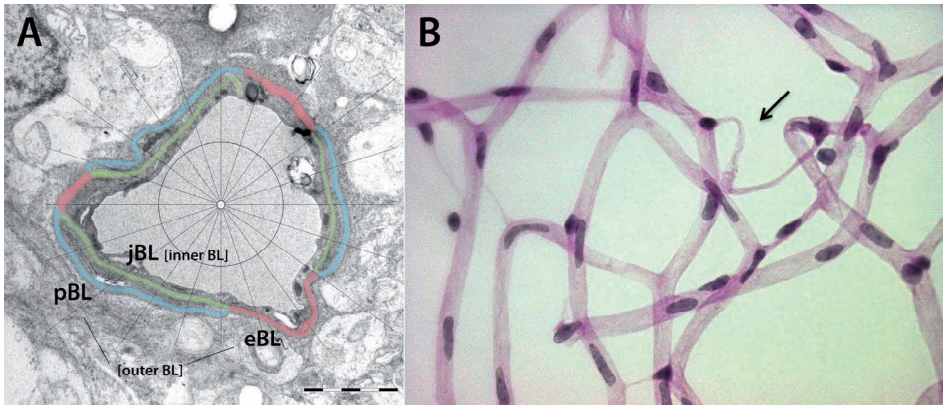

Figure 1. (A) EM image of a retinal capillary analyzed for BL thickness. The different basal laminas (BLs) are defined as outer BL, consisting of an endothelial BL domain (eBL; red) and a pericyte BL domain (pBL; blue), and as inner BL or joint BL (jBL; green) in between endothelial cells and pericytes. Bar = 2 µm. (B) LM image of the retinal vasculature in a digest preparation. *Arrow* indicates acellular capillary. Magnification: 400x.

brief, total numbers of pericytes and endothelial cells were counted in ten randomly selected fields of the retina using an image analyzing system (CUE-2; Olympus Opticals, Hamburg, Germany), and their numbers were normalized to the relative capillary density (numbers of cells per mm^2 of capillary area).

For analysis of vasoregression profiles, numbers of acellular capillary segments were counted according to an established method in ten randomly selected fields as described previously.[4] Samples were evaluated in a blinded fashion.

Statistical analysis

Data are presented as mean ± SD. Differences between groups were analyzed by one way-ANOVA with the Bonferroni post-hoc method for multiple comparisons. For BL measurements, mean BL thickness was first calculated per capillary and then averaged per mouse. Statistical analysis was performed using GraphPad Prism software version 5.02 (GraphPad Software, San Diego, CA, USA). For all comparisons, a value of $p<0.05$ was considered to be significant (two-tailed).

RESULTS

Clinical characteristics of mice with long-term STZ-induced diabetes

Experimentally induced diabetes in both $CTGF^{+/+}$ (wild type) and $CTGF^{+/-}$ mice with STZ resulted in persistent hyperglycaemia, increased HbA_{1C} levels and decreased body weight (Table 1). Body weight, blood glucose levels and HbA_{1C} levels did not differ between wild type and $CTGF^{+/-}$ mice at 6 and 8 months. Therefore, mean values are given for all mice in each experimental group for both time points.

Retinal CTGF protein levels

Total retina protein levels of CTGF as determined by Western blot analysis were significantly lower in $CTGF^{+/-}$ mice as compared to $CTGF^{+/+}$ mice (Figure 2). Inactivation of one CTGF allele reduced CTGF protein levels in the retina by 35%.

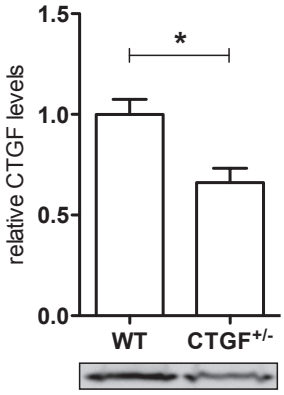

Figure 2. Retinal CTGF protein levels are decreased in $CTGF^{+/-}$ mice. Western blot analysis of retinal CTGF protein showing 35% reduction in $CTGF^{+/-}$ mice as compared to wild type mice. β-Actin was used as loading control. *N* = 4 animals per group. Data are presented as mean ± SD, * $p<0.05$.

CTGF haplo-insufficiency prevents diabetes-induced BL thickening of retinal capillaries

Retinal capillary BL thickness measurements did not differ significantly at 6 and 8 months of diabetes within any of the experimental groups. Therefore, measurements were combined for both time points of long-term diabetes. As expected, in diabetic CTGF$^{+/+}$ mice after 6 to 8 months of STZ-induced diabetes, the thickness of the retinal capillary BL was significantly increased as compared to the non-diabetic wild type mice (Figure 3). More specifically, the outer BL thickness was increased (Figure 3A), which could be attributed solely to increased thickness of the endothelial BL (Figure 3B). The pericyte BL and the inner BL were not significantly thickened (data not shown).

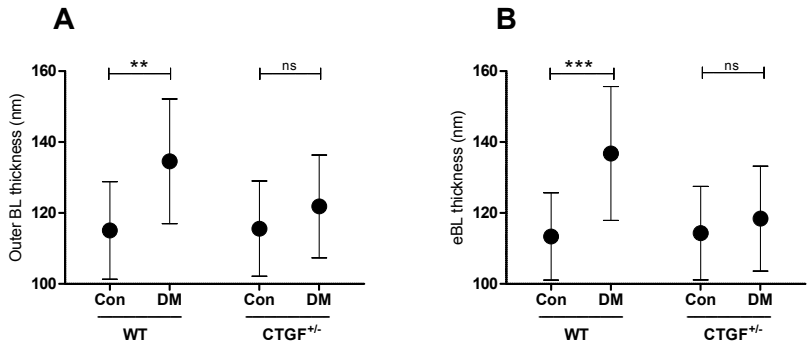

Figure 3. CTGF haplo-insufficiency prevents diabetes-induced BL thickening. Basal lamina (BL) thickness measurements in nanometers of retinal capillaries in diabetic (DM) and non-diabetic control (Con) CTGF$^{+/+}$ (WT) and CTGF$^{+/-}$ mice after 6 to 8 months of diabetes. (A) Thickness of the outer BL, as defined by the BL outside the endothelial cell and/or pericyte. (B) Endothelial cell BL thickness. *N* = 14-15 animals per group. Data are mean ± SD. ** p<0.01, *** p<0.001.

In contrast, BL thickness was the same in diabetic CTGF$^{+/-}$ mice and non-diabetic CTGF$^{+/-}$ mice (Figure 3). BL thickness did not significantly differ between non-diabetic wild type mice and CTGF$^{+/-}$ mice.

CTGF haplo-insufficiency prevents pericyte loss and decreases formation of acellular capillaries

The numbers of pericytes per mm^2 of capillary area were similar in non-diabetic CTGF$^{+/+}$ and CTGF$^{+/-}$ mice. At 8 months of diabetes, a significant 15% loss of pericytes was observed in the diabetic CTGF$^{+/+}$ mice as compared to the non-diabetic wild type mice (Figure 4A). In contrast, in the CTGF$^{+/-}$ mice, the number of pericytes was unaffected by diabetes. In areas without vasoregression, endothelial cell numbers did not differ between CTGF$^{+/+}$ and CTGF$^{+/-}$ mice (Figure 4B). No effect of diabetes was found on EC proliferation in areas unaffected by vasoregression, both in wild type and CTGF$^{+/-}$ mice.

The number of acellular capillaries was significantly increased in diabetic versus non-diabetic CTGF$^{+/+}$ mice (Figure 5). In contrast, in the diabetic CTGF$^{+/-}$ mice, the number of acellular capillaries was not significantly different from the non-diabetic controls.

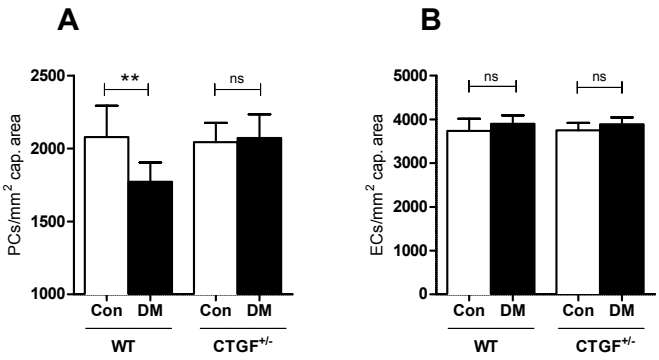

Figure 4. CTGF haplo-insufficiency prevents diabetes-induced pericyte loss. Pericyte (A) and endothelial cell (B) numbers in diabetic (DM) and non-diabetic control (Con) CTGF$^{+/+}$ (WT) and CTGF$^{+/-}$ retinas after 8 months of diabetes. Pericyte coverage was reduced by 15% only in DM WT. Endothelial cells numbers were not affected by diabetes or genotype. *N*= 7-9 animals per group. Data are mean ± SD. ** p<0.01.

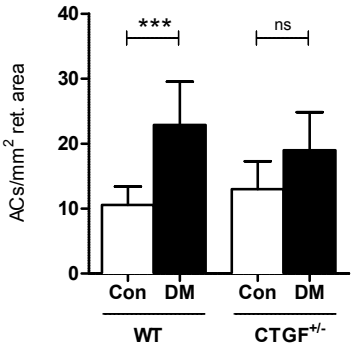

Figure 5. CTGF haplo-insufficiency decreases diabetes-induced formation of acellular capillaries. Presence of acellular capillaries (ACs) in diabetic (DM) and non-diabetic control (Con) CTGF$^{+/+}$ (WT) and CTGF$^{+/-}$ retinas after 8 months of diabetes, presented as numbers per unit area of retina (mm^2). *N*= 7-9 animals per group. Data are mean ± SD. *** p<0.001.

DISCUSSION

This study not only confirms our previous finding that CTGF haplo-insufficiency prevents diabetes-induced BL thickening in PCDR in mice, it also shows for the first time that pericyte loss is prevented and that formation of acellular capillaries is decreased when CTGF levels

in the diabetic retina are reduced. Our findings indicate a major role for CTGF in these early pathological changes in DR.

The diabetic state of $CTGF^{+/-}$ mice in this study was comparable to that of the diabetic $CTGF^{+/+}$ mice, as indicated by similar blood glucose and HbA_{1c} levels. However, there was a distinct genotype effect on retinal CTGF protein levels, as the $CTGF^{+/-}$ mice had lower CTGF protein levels in the retina. This subtotal reduction in CTGF levels was sufficient to prevent thickening of the BL in $CTGF^{+/-}$ mice with long-standing diabetes. This confirms and extends the observations of our previous study in mice at 17 weeks of diabetes, which also showed increased eBL thickening in wild type mice, but not in $CTGF^{+/-}$ mice.[31] The observed thickening of specifically the endothelial BL and not the pericyte BL in both studies may be explained by our previous observation that under diabetic conditions in vitro, CTGF induces expression of FN,[37] and that incorporation of FN in the ECM by endothelial cells is higher than by pericytes.[38]

In long-standing diabetes, CTGF is involved in structural and functional ECM alterations, including BL thickening, which lead to microvascular diabetic complications such as nephropathy.[21,39-42] Several experimental rodent studies have demonstrated the relevance of abnormal ECM synthesis and BL thickening in the development of DR, showing associations between inhibition of BL thickening with interference in BL component production and prevention of pericyte loss and acellular capillary formation.[17,18,43] In this respect it is not unlikely that a changed composition rather than the absolute thickening of the BL plays a major role in causing the progressive morphological changes in the mouse diabetic retina.

In fact, in our present study, reduced retinal CTGF levels not only inhibited BL thickening, but also prevented diabetes-induced pericyte loss. We observed previously that in human early DR, a shift in CTGF protein occurs from a predominantly microglial location in the normal human retina to a predominantly pericyte location in diabetic patients.[27] Taken together, these observations suggest that CTGF has a role in pericyte loss in PCDR. This role may be either direct or indirect, by causing pericyte apoptosis or by allowing pericyte migration.[44] That this role may be indirect via CTGF-induced changes in the ECM is supported by the literature, as in diabetic conditions in vitro, CTGF is involved in pericyte detachment and anoikis, a form of apoptosis resulting from loss of cell-ECM interactions.[45] Also, in developing vasculature in mouse embryos lacking the CTGF gene, pericyte recruitment was impaired in association with decreased FN levels.[32]

In addition to prevention of pericyte loss, CTGF haplo-insufficiency reduced the development of acellular capillaries. The incomplete inhibition of acellular capillary formation in our model implies that in the presence of reduced CTGF levels, and despite a complete prevention of BL thickening and pericyte loss, other mechanisms can cause vasoregression in diabetes. These may include direct effects on endothelial cells of AGE formation, actions of adhered leukocytes, or disruption of the retinal neurovascular unit by apoptosis of neurons.[26,46-48]

Our studies identify CTGF as a possible modifier of DR incipience in this rodent model. This may be also relevant for human DR, and is supported by our recent observation of increased vitreous levels of CTGF in human diabetic patients without any clinical signs of DR (Van Geest et al., submitted).

Our $CTGF^{+/-}$ mice had reduced retinal CTGF protein levels, but BL thickness, the number of pericytes, endothelial cells and acellular capillaries were not different between non-diabetic

wild type and CTGF$^{+/-}$ mice. This shows that a functional vascular network is established with proper recruitment of pericytes, even when only one functional CTGF allele is present. This indicates that lowering of CTGF in the eye in itself is probably not harmful. Thus, targeting of CTGF may be a safe and effective way to prevent the development of DR, even as a long term preventive treatment in the asymptomatic preclinical stage of this major eye disease.

Contribution Statement

RJVG, HPH, RG, IK, CJFVN and ROS planned the study. HPH, RG, IK, CJFVN and ROS supervised the conduct of the study, data sampling and data analysis. RJVG, JWL, AD, FP, KB, KAH, IMCV, DVDG and ND performed experiments or data sampling and data analysis. RJVG prepared the manuscript and all authors contributed and approved the final manuscript.

This study was financially supported by grant 2005.00.042 from the Diabetes Fonds Nederland.

JWL was supported by the Dutch Kidney Foundation (grant C05.2144).

FP was supported by the Deutsche Forschungsgemeinschaft (DFG GRK 880 'Vascular Medicine').

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