· Original article ·

CTGFsiRNA ameliorates retinal cells apoptosis in the streptozotocin-induced diabetic rat

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Abstract

• AIM: To detect the effect of CTGF on the apoptosis in the diabetic retina with small interfering RNAs (siRNA) targeting with CTGF.

• METHODS: A total of 60 rats were divided into six groups including control group, diabetic 4,8,12,16 weeks group, and interference group. Diabetic rats were induced by STZ intra-peritoneal. At 4, 8, 12, 16 weeks after diabetic setting up, retinas were obtained from control, diabetic rats and diabetic animals treated by intravitreal injection of CTGFsiRNA to suppress the expression of CTGF mRNA. Retinal cells apoptosis was detected by Tunnel staining and mRNA expression of CTGF was analyzed by RT-PCR.

• RESULTS: The levels of CTGF and the apoptosis in the retinas of diabetic rats were significantly higher than those in the controls. Apoptosis occurred at 4 weeks after a diabetic model setting up, became serious with the diabetes developing, while CTGF elevated at 8 weeks. The cell apoptosis counts increased to 25.8cells/mm^2 at 24 weeks of diabetes. SiRNA-mediated inhibition of CTGF mRNA resulted in a significant decrease in apoptosis. Significant correlations were found between CTGF and apoptosis in the retina.

• CONCLUSION: These results suggest that CTGF might be involved in retinal cells apoptosis which is a characteristic of early diabetic retina. siRNA targeting CTGF seems to have the advantage of ameliorating retinal cells lost.

• KEYWORDS: apoptosis; CTGF; retina; diabetets
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INTRODUCTION

Diabetic retinopathy is one of the most common complications of diabetes and a leading cause of vision loss, ultimately resulting in an advanced stage of proliferative retinopathy with neovascularization, fibrovascular proliferation and retinal detachment. At the cellular level, diabetes alters the function and structure of all retinal cell types[1]. The pathogenesis of diabetic retinopathy includes glucose-mediated microvascular damage[2-4] and alterations of the neural retina, impaired glial reactivity and apoptotic cell death of retinal cells have been observed in cases of short-term experimental diabetes and in humans with diabetes[5]. Animal studies show accelerated apoptosis of retinal neurons[6], glial activation[7], microvascular cells[8], photoreceptors[9] and microglial cell[10]. It is important to characterize the early pathological processes in the diabetic neural retina before the onset of vascular pathology. More recently, a novel, cysteine rich secreted protein CTGF has been associated with diabetic retinopathies, which is postulated to have prosclerotic, angiogenic and apoptosis induction[11] properties[12] in other tissues. However, there have been few previous reports that have linked the elevated expression of CTGF in diabetic retinas to pathological changes. To investigate the role of diabetes-enhanced CTGF expression in retinal cell apoptosis, diabetic rats were treated with CTGFsiRNA intravitreal injection.

MATERIALS AND METHODS

Materials Wistar rats were purchased from Animal Laboratories of China Medical University. Throughout the study animals were given access to food and water in metabolic cages. All animal procedures were in accordance with guidelines set by the Animal Experiment Committee of the China Institutes for Biological Sciences. Wistar rats, male, weighing 180-200g, were randomly divided into six groups: control group, diabetic groups at 4 weeks (DM4W), 8 weeks (DM8W), 16 weeks (DM16W), 24 weeks (DM24W) and DM16W interfered with CTGFsiRNA group ($n=10$).

Methods

Streptozotocin-induced diabetic rat model Experimental diabetes was induced by intraperitoneal injection of β-cell toxin streptozotocin (60mg/kg). Immediately prior to use, streptozotocin was dissolved in cold 0.1mol/L citrate buffer, pH 4.5. Control rats received an injection of 0.1mol/L citrate buffer alone. Blood glucose (BG) levels were measured before and 72 hours after the STZ injection, urinary glucose (UG) measured consequently the first three days. Only the animals with UG above +++, blood glucose levels >16.7mmol/L were considered diabetes. Body weight, UG, BG and glycated hemoglobin were measured weekly. At 4, 8, 16, 24 weeks of diabetes, ten rats were randomly selected from the normal control and diabetic groups and killed with a lethal dose of

Table 1 The sequences of the siRNAs targeting rat CTGF gene

Target gene	Sequence
CTGF	sense: 5'-CAACUAUGAUGCGAGCCAATT-3'
	antisense: 5'-UUGGCUCGCAUCAUAGUUGGG-3'
Scrambled siRNA	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense: 5'-ACGUGACACGUUCGGAGAATT-3'

GenBank accession number NM_022266.

Table 2 General characteristics of non-diabetic and diabetic rats at 4, 8, 16 and 24 weeks after STZ injection

		Weight (g)	Blood glucose (mmol/L)	Urinary glucose
4 weeks	Non-diabetic	276 ± 11.56	3.84 ± 0.31	(-)
	Diabetic	245 ± 8.39	23.23 ± 0.24[b]	(+++)
8 weeks	Non-diabetic	389 ± 12.41	4.42 ± 0.24	(-)
	Diabetic	264 ± 8.12	24.32 ± 0.35	(+++)
16 weeks	Non-diabetic	503 ± 13.54	5.26 ± 0.43	(-)
	Diabetic	305 ± 9.21[b]	19.83 ± 0.55[b]	(+++)
24 weeks	Non-diabetic	627 ± 25.48	6.21 ± 0.32	(-)
	Diabetic	256 ± 14.53[b]	23.42 ± 0.69[b]	(+++)

[b] $P < 0.01$, the difference is significant.

pentobarbital sodium. Eyes from each rat were rapidly enucleated, one being snap-frozen in liquid nitrogen and stored in -80℃ for the consequent RT-PCR, while the contralateral eye was fixed at 40g/L paraformaldehyde for apoptosis and immunohistochemistry.

Preparation of CTGFsiRNA A double-stranded rat CTGF-siRNA were synthesized by personnel at GenePharma (Shanghai, China), as described previously[13,14]. The sequence of siRNAs targeting rat CTGF gene are shown in Table 1. The resultant siRNA was purified, quantified and suspended in water at a concentration of 50ng/μL, and 0.5μL (10 picomoles) siRNA for CTGF was combined with 0.5μL siRNA transfection reagent (GenePharma Co. Ltd. Shanghai, China) for 20 minutes before injection according to the manufacturer's instructions. Control injection was 1.0μL PBS. The doses of CTGFsiRNA used in the present study were chosen according to studies.

Intravitreous injections Ten diabetic rats at 16 weeks after the diabetic model setting up were performed intravitreal injection eyes as previously described[15]. Rats were anesthetized with an intraperitoneal injection of 65mg/kg pentobarbital sodium (Sigma, USA). A topical anesthetic (5g/L tetracaine hydrochloride, Santen, Japan) was administered and the pupils were dilated with 10g/L tropicamide before inserting a 30-gauge needle just 2.0mm posterior to the limbus to avoid lens damage. 1.0μL CTGFsiRNA was injected in right eyes using a 1mL Hamilton syringe. All fellow eyes were injected with Control injections. Then 5g/L topical Tobra-Dex ointment (Alcon, USA) applied to the injected eye for preventing the infection. Rats were killed 1 day later, and the eyes were removed. CTGF mRNA levels and apoptosis were examined in retinas.

RNA Extraction and Reverse Transcription-polymerase Chain Reaction Retina tissues were collected from different groups. RNA was extracted from the retina using Trizol (Invitrogen), RT-PCR was performed according to the manufacturers' instructions. PCR protocol: 2 minutes at 94℃, followed by 32 cycles of 30 seconds at 94℃, 30 seconds at 56℃ and 1 minute at 72℃, 2 minutes at 72℃. Relative concentrations of DNA in each specimen was semiquantitated on an Automated Imaging System (Alphainnotech ChemiImager 5500, USA) by the integral density of the product bands, which was then normalized to the density of β-actin. The data are expressed as the mean transcript/β-actin ratio ± SD. The primer of CTGF: 5'-TGTGAAGACATACAGGGCTAA-3' 5'-GTTCTCACTTTGGTGGGATAG-3'.

***In situ* Cell Death Detection** Apoptotic cells were detected by TUNEL assay with an *In Situ* Cell Apoptosis Detection Kit (Boster, Wuhan, China) according to the kit manufacturer's instructions. After deparaffinization, the sections were treated with proteinase K, incubated with TUNEL reaction mixture and peroxidase-conjugated antibody, stained with the diaminobenzidine solution. To quantify the number of TUNEL-labeled nuclei, we obtained counts and averaged them from five different randomly selected areas of a given coverslip, using an eyepiece graticule grid that represented an area of 400μm × 400μm. Thus, to convert values to cells/mm^2, each averaged value multiplied by 6.25 (ie. 2.5 × 2.5). Ten coverslips were analyzed for each treatment and values statistically compared for differences.

Statistical Analysis All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed by a Student's *t*-test using special software (SPSS for windows, ver. 10.0; SPSS, Inc.). $P < 0.05$ was considered significant.

RESULTS

Animal Characteristics Fifty rats were intraperitoneal injection of the β-cell toxin streptozotocin (60mg/kg). All the animals with UG above +++, blood glucose levels > 16.7mmol/L were induced to diabetes. The mean weight, blood glucose, was significantly different between non-diabetic and diabetic animals(Table 2). Diabetic rats gained hyperglycemia

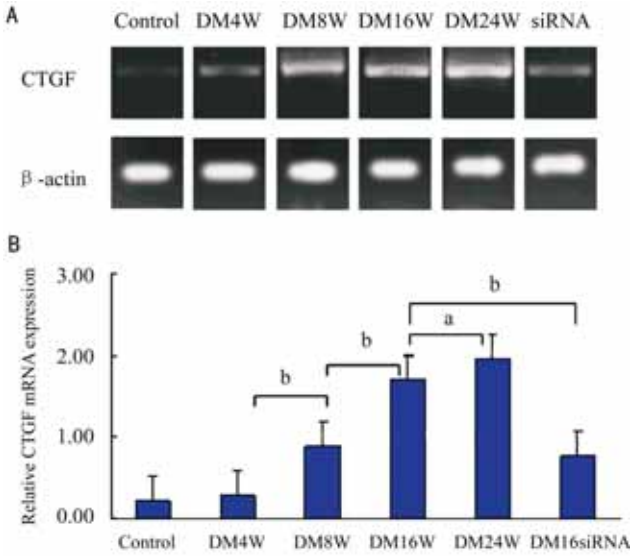

Figure 1 Expression CTGF mRNA in retina of normal rat, diabetic rats and diabetic retina ($n = 10$), the difference is significant ($^aP < 0.05$, $^bP < 0.01$).

and increased urinary glucose compared with the normal rats of the control group. At 4 weeks there is no significant effect on body weight and from 8 weeks on, the difference is significant. Throughout the experiment, it was not noted of inflammation, retinal detachment, or vitreous hemorrhage in any of the rats.

Effect of Diabetes on CTGF We compared with CTGF mRNA expression levels of different groups. There was seldom in the normal retina and DM4W group, and became stronger in diabetic rat in DM8W ($P < 0.05$). CTGF expression levels were increased twofold. DM16W and DM24W ($P < 0.01$) groups steadily. Figure 1 shows CTGF gene expression in the retina. The statistical analysis demonstrates that CTGF mRNA expression in the diabetic retina is up regulated. The difference is significant shown in the Figure 1 ($^aP < 0.05$, $^bP < 0.01$). The error bars show the standard deviation for each group. CTGFsiRNA was injected intravitreously in diabetic rats to make the CTGF gene silence. So we chose 16-week groups to inhibit the CTGF expression by intravitreal CTGFsiRNA injection. The results of these experiments showed that the inhibitory efficiency of CTGFsiRNA were 55%. Such inhibition of gene expression were significant ($P < 0.01$), as determined by Student's t-test.

Retinal Cells Apoptosis and the Correlation with CTGF The expression of apoptosis was examined by Tunel staining and retina samples were processed from control, DM4W, DM8W, DM16W, DM24W and DM16W interfered with CTGFsiRNA groups. DM16W was also the non-interfered group compared with interfered with CTGFsiRNA group. The TUNEL-positive nuclei were identified by a brown reaction product and were found in all regions of the retina. After only 4 weeks, diabetic retinas had seldom TUNEL-positive nuclei in ganglion cell layer compared to the control. At 8 weeks, the diabetic retinas had more TUNEL-positive nuclei than the control (Figure 2). The cell of apoptosis count is 12.6 cells/mm^2 and the positive stained included ganglion cells and the glial cell.

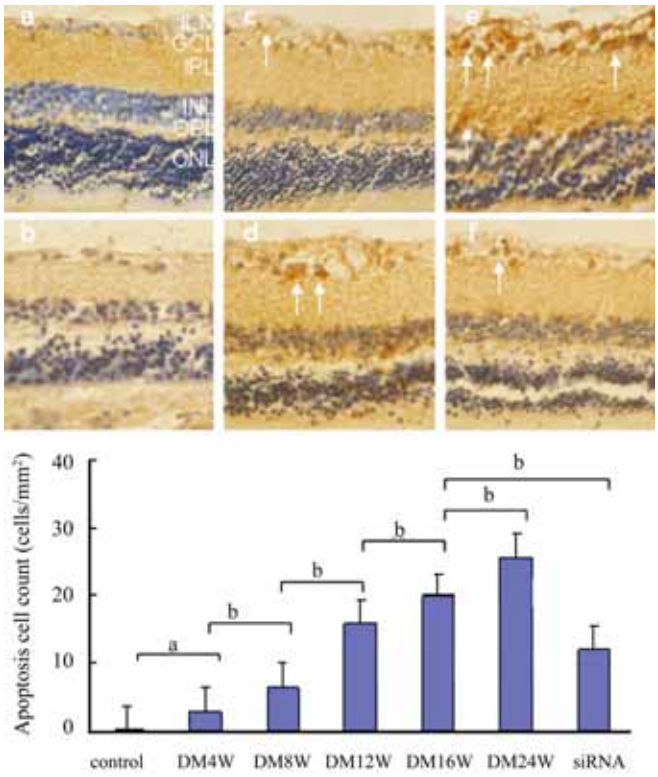

Figure 2 Tunel positive cells (arrows) appeared in diabetic retina a: normal retina; b: diabetic 4 weeks; c: diabetic 8 weeks; d: diabetic 16 weeks; e: diabetic 24 weeks; f: diabetic 16 weeks interfereced by CTGFsiRNA. The internal limiting membrane (ILM), the ganglion cell layer (GCL), the inner plexiform layer (IPL), the outer plexiform layer (OPL), and the outer nuclear layer (ONL). The difference is significant ($^aP < 0.05$, $^bP < 0.01$).

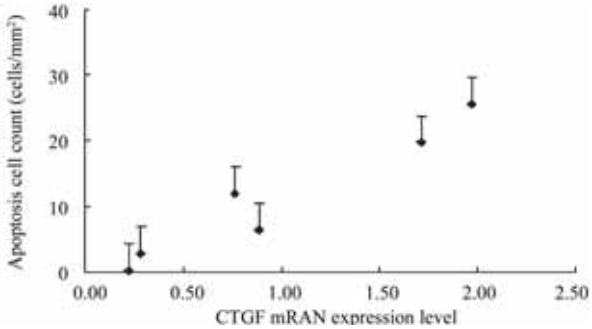

Figure 3 The correlations of CTGF mRNA by RT-PCR and apoptosis by Tunel assay.

At 24 weeks of diabetes TUNEL-positive nuclei localized in all regions of the retina, including vascular endothelial cells and the cells in inner nuclear layer, and the cell apoptosis counts increased to 25.8cells/mm^2. There is a downregulation of apoptosis in interfered retina compared with un-interfered retina. The CTGFsiRNA protects the retina from apoptosis, compared with the un-interfered retina, in which the difference is significant ($P < 0.01$). Next we tested for a relationship between the apoptosis and CTGF mRNA in retina. As evidence above, the correlations between the apoptosis expression and CTGF expression ($r = 0.871$, $P = 0.011$, Figure 3). There was a significant correlation between the apoptosis and the expressions of CTGF in diabetic retina.

DISCUSSION

The present study indicates there is an increased expression of the CTGF gene level and the apoptosis of early diabetes and the degree of increasing became stronger with the diabetic development. The diabetic retinas showed apoptosis in ganglion cells layer chiefly. We can come to a conclusion that this change is a very early marker of diabetes-induced retinal changes and it occurred before the onset of visible vascular lesions. Then we used siRNA targeting with CTGF to silence the CTGF gene, which is a valuable tool for investigating the function of gene products in tissues[16-18]. CTGFsiRNA could effectively down-regulate the expression of CTGF in diabetic rat retinas, and a significant inhibition of apoptosis in the retina occurred by the interfered with CTGFsiRNA. There was a strong correlation between apoptosis and CTGF in diabetic rat retinas. So we demonstrated CTGF might be affected on the apoptosis in diabetic retina of rat.

Increased apoptosis is implicated in several other diabetic complications such as neuronial apoptosis in neuropathy, cardiomyocyte apoptosis in cardiomyopathy, and mesangial cell apoptosis in nephropathy. Diabetes can affect capillaries[19], neurons, and glia[19] within the retina and alters the function and structure of all retinal cell types. Much evidence had shown the apoptosis in the ganglion cells layer, the inner nuclear layers[20], and photoreceptors and microvascular cells[19] in diabetic retina. Direct diabetes damage to glial cell or neuronal metabolism would directly impact neurotransmission[21] and may lead to apoptosis of retinal neurons and visual field defects. Indeed, retinal axons are lost before the onset of visible vascular lesions[22]. Recent reports also demonstrate that impaired local responses on the multifocal electroretinograms predict subsequent development of vascular lesions[23]. Vision depends on neuronal function, so most forms of vision impairment with clear ocular media must include neuronal dysfunction definitely. Further work is needed to determine how alterations in ganglion, glial, microglial, and neuronal cell interactions reduce the quality of vision.

CTGF is a cysteine-rich matricellular protein belonging to the CCN family of proteins, which have many diverse functions such as angiogenesis, fibrosis and apoptosis and so on. Recently, various studies have shown that CTGF expression at the mRNA or protein level in retina has previously been demonstrated in vivo in diabetic rat[24] and human[25], as well as in cultured retinal microvascular cells[26]. CTGF has been shown to be upregulated in the retina together with endothelial cell death. These are believed to be the result of metabolic changes caused by hyperglycemia and advanced glycation end products (AGEs)[27]. Overexpression of CTGF in cultured human aortic smooth muscle cells, a cell type closely related to pericytes and mesangial cells induces apoptosis by activating caspase 3[28]. Moreover, the involvement of Cyr61 and CTGF in pericyte detachment and anoikis was implicated in the pathogenesis of DR[29]. Cyr61- and CTGF-induced apoptosis was mediated through the intrinsic pathway and involved the expression of genes that have been functionally grouped as p53 target genes. Expression of the matrix metalloproteinase-2 gene, a known target of p53, was increased in pericytes overexpressing either Cyr61 or CTGF. Inhibition of matrix metalloproteinase-2 had, at least in part, a protective effect against Cyr61- and CTGF-induced apoptosis. Thus, it is possible that up-regulation of CTGF may contribute to inducing apoptosis, especially in the vascular endothelium cells, ganglion cells, perhaps included the glial cells. This up-regulation leads to the loss of retinal cells as a critical early event. And then the vascular cells affected suggest that cell loss may be a direct result of a consequence of widespread vascular disease in the etiology of diabetic retinopathy[30]. But Alistair J *et al* indicated that most of the apoptotic cells in the retina are not endothelial cells or pericytes. Judging from such different findings, there might be an unidentified mechanism modulating the apoptosis in the diabetic retina.

Together, these studies leave little doubt that apoptosis is the earliest detectable changes in diabetes. Regardless of whether the initial events begin in blood vessels or neural cells, the clinical stage of diabetic retinopathy manifest cellular, histological, and functional features of a retinal neuropathy[31]. To the best of our knowledge, there is no evidence that a primary, selective defect in vascular cells is sufficient to cause diabetic retinopathy. Clearly, it is essential to treat both the vascular and neural elements of the retina to preserve vision. This concept permits a new paradigm for understanding the mechanism of vision impairment in diabetes and provides therapeutic targets that are directly linked to vision[32,33]. In summary, this study suggests that CTGF may be involved in apoptosis which is a characteristic of early diabetic retina. siRNA targeting CTGF seems to have the advantage of ameliorating retina apoptosis directly or indirectly. This study provides evidence treatment strategies that intravitreous injection of siRNA containing potentially therapeutic transgenes treatment[34,35]. However, it remains unclear how up-regulated expression of CTGF in the diabetic retina and the exact mechanism leading to apoptosis in STZ rats should be further investigated. Meanwhile, we must be cautious in interpreting these findings, because animal models of diabetic retinopathy do not exhibit advanced retinal lesions such as those seen in the man.

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CTGFsiRNA 改善 STZ 诱导糖尿病大鼠视网膜细胞凋亡

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摘要

目的:探讨 CTGF 对糖尿病大鼠视网膜内细胞凋亡的影响。

方法:将 60 大鼠分为对照组,糖尿病 4,8,12,16wk 组和干预组。糖尿病大鼠应用 STZ 腹腔注射诱导成模。干预组大鼠于糖尿病成模后 16wk 玻璃体腔注射 CTGFsiRNA 来造成 CTGF 基因的沉默。取各组视网膜组织,应用 RT-PCR 检测视网膜内的 CTGF 基因表达,Tunnel 法检测视网膜凋亡细胞的情况。

结果:糖尿病组视网膜内 CTGF 表达和凋亡细胞数较正常组明显增加。凋亡发生在建模后 4wk,并随着时间的延长而加重,在 24wk 时,凋亡细胞数为 25.8 个/mm^2。CTGF 表达于 8wk 时出现升高,到 16wk 时升高更明显。CTGFsiRNA 处理后凋亡细胞数明显降低。视网膜内 CTGF 和细胞凋亡之间具有明显的相关性。

结论:CTGF 参与了糖尿病视网膜早期病变的细胞凋亡机制,CTGFsiRNA 有利于改善糖尿病早期视网膜由于凋亡所致的细胞丢失。

关键词:凋亡; CTGF; 视网膜; 糖尿病