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Biochemical and Biophysical Research Communications 358 (2007) 209–214
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Adenoviral-mediated inhibition of connective tissue growth factor in Rat-2 cells

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Received 13 April 2007
Available online 25 April 2007

Abstract

Connective tissue growth factor (CTGF) is a profibrotic factor shown to induce extracellular matrix production and angiogenesis, two processes involved in the development of diabetic retinopathy (DR). In this study we tested the effect of a recombinant adenovirus encoding for a CTGF antisense oligonucleotide (rAdASO) on the levels of transforming growth factor-β (TGF-β) induced expression of CTGF in Rat-2 fibroblasts. Using semi-quantitative RT-PCR, there was a 2-fold increase in CTGF message induced by TGF-β. Western blot and immunocytochemical analyses revealed a significant increase in CTGF protein level. This upregulation of CTGF by TGF-β was inhibited by infection with rAdASO. These findings indicate that infection of the Rat-2 cells with rAdASO was effective in decreasing TGF-β-induced CTGF expression. These results indicate that this viral vector might have therapeutic potential to control elevated CTGF levels that occur in DR.

Keywords: CTGF; TGF-β; Adenovirus; Antisense oligonucleotide; Rat-2 fibroblasts

Connective tissue growth factor (CTGF) is a 38 kDa cysteine-rich matricellular protein belonging to the CCN family of proteins, whose members include Cyr61, CTGF, Nov, Wisp1, Wisp2, and Wisp3. The CCN family of proteins have many diverse functions, which are cell-type and cell-context dependent. They stimulate mitosis, adhesion, apoptosis, extracellular matrix (ECM) production, growth arrest, and migration [1]. These proteins have been implicated in biological processes such as development, angiogenesis, wound healing, chondrogenesis, fibrosis, osteogenesis, nerve conduction, muscular contraction, and tumorigenesis [2,3]. CCN proteins could be targets for diagnosis and therapy, with possible applications in vasculogenesis, chondrogenesis, osteogenesis, nerve conduction, and muscle contraction [3]. CTGF, in particular, has been shown to be a potent inducer of ECM, and the levels of CTGF have been linked to the severity of the disease [2,4–7]. Elevated levels of CTGF have been found in many pathological conditions, such as fibrotic skin disorders, scleroderma, diabetic nephropathy, pancreatic ductal adenocarcinoma, and diabetic retinopathy (DR) [4,13,22–25].

TGF-β1, a key regulator of inflammatory and immune processes, is known to induce CTGF [6] and is increased during hyperglycemia [22]. CTGF is a downstream target of TGF-β [6] and is activated by TGF-β in fibroblasts and endothelial cells causing collagen synthesis and proliferation of extracellular matrix components [8–11]. The goal of these studies was to test whether the TGF-β mediated induction of CTGF expression could be inhibited by a novel recombinant adenovirus encoding for a short CTGF-antisense oligonucleotide (rAdASO) in embryonic rat fibroblasts (Rat-2). While previous studies used a similar approach using human or animal cell lines [16–18], we chose to test a rat fibroblast cell line because the findings could then be applied to examine the effect

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.04.109

of the adenovirus in retina of diabetic rats, a commonly used model of hyperglycemia [22].

Materials and methods

Cell cultures. Rat-2 fibroblasts were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with sodium bicarbonate (1.5 g/L, Gibco) and 10% Fetal Bovine Serum (FBS, Invitrogen). Rat-2 cells were seeded at a density of 5.8×10^5 cells in T-25 tissue culture flask (Falcon, BD) and allowed to adhere overnight. Cells were then incubated in reduced serum medium (0.5% FBS) for 18 h prior to TGF-β treatment. Rat-2 cells infected with recombinant adenovirus were incubated in reduced-serum medium containing the recombinant adenovirus at multiplicity of infection (MOI) of 5000 for 24 h prior to TGF-β treatment. Then cells were incubated with reduced serum media containing TGF-β (0.1 ng/ml, R&D Systems) for 4, 6, 8, and 17 h prior to harvesting. After TGF-β treatment cells were trypsinized and pelleted. Cell pellets were washed in ice cold RNase-free Phosphate Buffer Solution (PBS, Ambion) and centrifuged at 3000 rpm for 10 min at 4 °C and then used for total RNA isolation or protein extraction. All experiments were repeated at least three times and each sample was in triplicate. Results were analyzed by the Student's *t* test.

Recombinant adenovirus. Recombinant antisense CTGF oligonucleotide adenovirus (rAdASO) was generously provided by Dr. B. Chaqour, Department of Anatomy and Cell Biology, SUNY Downstate Medical Center. The antisense oligonucleotide (ASO) is a 300 bp fragment of mouse CTGF cDNA (that spans 25 nucleotides of the 5′ untranslated region and 275 nucleotides downstream of the start codon). The ASO fragment cloned in the rAdASO showed about 93% homology to the rat CTGF sequence. The virus was amplified and purified by Vector Biolabs (Philadelphia, PA). A control adenovirus expressing Green Fluorescent Protein (GFP) was purchased from Vector Biolabs and used as a negative control in all viral experiments. The recombinant adenovirus was expanded in Human Embryonic Kidney Cells (HEK 293) and viral concentration was determined using the AdenoVator Vector System (Qbiogene). A virus titer of 1×10^9 was achieved. Using the GFP virus, the optimum virus dose for infection of cells was determined to be 5000 infectious units (ifu)/cell. This virus dose is comparable to that obtained in Rat-2 cells by Whitlock et al. [21]. HEK 293 were cultured in Minimum essential Eagle Medium (MEM, Sigma) supplemented with sodium bicarbonate (1.5 g/L, Gibco), sodium pyruvate (1.0 mM, Sigma), and 10% heat inactivated horse serum (Invitrogen).

Total RNA isolation and semi-quantitative reverse transcriptase PCR. Total RNA was isolated from Rat-2 cells using Trizol reagent (Invitrogen) and its concentration and purity were determined by spectrophotometry. The RNA integrity was verified by electrophoresis on 1.2% denaturing formaldehyde gels. RNA preparations were treated with recombinant DNAse-1 (DNAse-free kit, Ambion). One microgram of total RNA was transcribed using SuperScript III reverse transcriptase (Invitrogen). For each PCR an equal amount of cDNA (1/10 of the reaction) was used. PCR was performed in 25 μl reactions containing 0.2 μM each primer and 2.5 U Platinum *Pfx* DNA polymerase (Invitrogen). The number of cycles was optimized depending on the particular mRNA abundance and chosen to select PCR amplification on the linear portion of the amplification curve to avoid saturation effect. Aliquots of 15 μl were analyzed by electrophoresis on a 2% agarose gel (Fisher Scientific), the bands were quantified by densitometric scanning of band intensities and normalized to the levels of the housekeeping gene 18 S rRNA using Image J 1.32 gel analysis software (National Institutes of Health, NIH). CTGF amplicon size was 405 bp, forward primer GACAGCTTGTGGCAAGTGAA, reverse primer TTCCTCGTGGAAATCTGACC, GenBank Accession No.: BC072503; 18 S rRNA amplicon size was 488 bp, forward primer TC AAGAACGAAAGTCGGAGG, reverse primer GGACATCTAAGG GCATCACA [12].

Western blot analysis. Rat-2 fibroblasts were homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 1% Triton X-100), with protease inhibitor cocktail (PIC, Sigma), by passing them several times through a 20-guage needle (BD). After homogenization samples were cleared by centrifugation at 15,000 rpm for 30 min at 4 °C. Cleared lysates were transferred to fresh tubes. Protein concentration was measured using bicinchoninic acid colorimetric assay (BCA kit, Pierce). Samples were fractionated on 4–15% gradient Tris–HCl SDS–PAGE Ready Gels (Bio-Rad). Fifty micrograms of total protein was loaded in each lane. Proteins were electroblotted onto nitrocellulose membrane (Amersham Biosciences) in Borate Transfer-buffer (10 mM Sodium Borate) for 90 min at 60 mAmps at room temperature. Membranes were blocked in 5% dried non-fat milk (Carnation) in Tris buffered saline with 0.1% Tween (TBS–T) for 1 h at room temperature. The membranes were incubated with gentle rocking overnight at 4 °C with anti-mouse CTGF polyclonal antibody (Torrey Pines) diluted in 5% non-fat milk in TBS–T (1:1000). After several washes in TBS–T, membrane was incubated for 1 h with donkey anti-rabbit IgG linked to horseradish peroxidase (HRP, Amersham Biosciences) diluted in TBS–T (1:5000). The bands were visualized with enhanced chemiluminescence using ECL plus Western blotting detection reagents (Amersham Biosciences). Protein bands were scanned with Epson Perfection 2400 and analyzed using Image J Software (National Institutes of Health, NIH). Nitrocellulose membranes were stripped by incubating the membranes for 15 min at room temperature in Restore™ Western Blot Stripping Buffer (Pierce). Membranes were washed in TBS–T, tested for complete removal of bound primary and HRP-conjugated antibodies, then re-probed for GAPDH using a rabbit polyclonal antibody (Santa Cruz, 1:1000 in 5% non-fat milk in TBS–T) and processed for immunodetection as described above.

Immunocytochemistry. Rat-2 cells were seeded at a seeding density of 1.6×10^5 cells on dual chamber tissue culture slides (LabTek) and allowed to adhere overnight. Cells were incubated in reduced serum medium (0.5% FBS) for 18 h prior to TGF-β treatment. The medium was replaced with reduced serum medium containing TGF-β1 (0.1 ng/ml) and cells were incubated for 4, 6, and 8 h. Cells were fixed in ice-cold acetone for 10 min, followed by post-fixing in Zamboni's fixative (2% Paraformaldehyde and 15% Picric Acid) for 2 min. Slides were then washed in 0.1 M PBS three times for 5 min each, incubated for 20 min in PBS containing 0.3% Hydrogen peroxide (Fisher), and washed twice in PBS. Slides were incubated in blocking buffer [PBS containing 3% horse serum (Gibco) and 0.01% Saponin (Sigma)] for 30 min, washed twice in PBS for 5 min and incubated with antibody against CTGF (1:100 diluted in PBS) at 4 °C overnight in a humid chamber. Next day, slides were washed twice in PBS for 5 min, incubated in the dark at room temperature for 1 h with Alexa Fluor 488 conjugated anti-rabbit (Molecular Probes) IgG (diluted 1:200 in PBS), washed twice in PBS for 5 min, covered with two drops of Vectashield Solution (Vector Laboratories, Inc.) and coverslipped.

Confocal microscopy. Confocal images were obtained using a Radiance 2000 confocal microscope (Bio-Rad) attached to a Zeiss Axioskop microscope (Carl Zeiss Inc.). Images 540×540 pixels were obtained and processed using Adobe Photoshop 6.0 (Adobe Systems).

Results

To determine whether TGF-β-induced CTGF mRNA in Rat-2 fibroblasts, semi-quantitative reverse transcriptase-PCR (RT-PCR) was performed on RNA samples isolated from Rat-2 cells treated with different concentrations of TGF-β (0.1, 1, and 10 ng/ml) at 4 and 17 h, respectively. Treatment of Rat-2 cells with TGF-β for 4 h resulted nearly a 2-fold increase in CTGF message (Fig. 1). At 17 h the levels of CTGF mRNA were not significantly different from that at 4 h (data not shown), indicating that the effect of TGF-β on CTGF was not changing with prolonged incubation times. This observation confirms previous reports of TGF-β mediated CTGF expression in

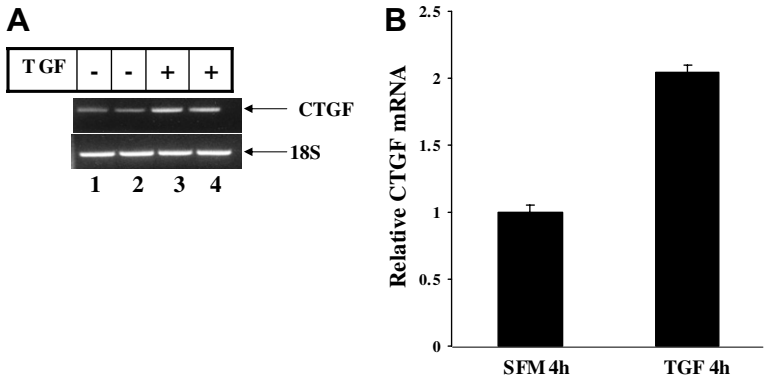

Fig. 1. TGF-β induced a 2-fold increase of CTGF message in Rat-2 fibroblasts grown in Serum Free Medium (SFM). (A) Semi-quantitative RT-PCR analysis of CTGF and 18S expression in cells incubated without (lanes 1 and 2) or with TGF-β (0.1 ng/ml) (lanes 3 and 4) for 4 h. (B) Densitometric analysis of CTGF expression in control and treated Rat-2 cells incubated with TGF-β. 18S used as housekeeping gene.

other types of fibroblasts [1] and demonstrates, for the first time, that this effect occurs in Rat-2 cells.

To ascertain whether the upregulation of CTGF mRNA was accompanied by an increase in protein, Rat-2 cells were treated with TGF-β (0.1 ng/ml) for 4, 6, and 8 h. CTGF protein levels, detected using Western blot, revealed increased CTGF protein levels at all time points with a maximal induction of CTGF protein seen after 6 h of stimulation with TGF-β. In unstimulated cells, no measurable levels of CTGF protein could be detected using Western blot analysis (Fig. 2A). Immunocytochemical analysis confirmed the increase in CTGF protein levels at all time points (4, 6, and 8 h.) in TGF-β treated cells (Fig. 3).

To determine that TGF-β-induced expression of CTGF is inhibited by the antisense oligonucleotide (ASO), Rat-2 fibroblasts were infected with rAdASO. The levels of CTGF mRNA expression in cells infected with rAdASO in the absence of TGF-β and in cells infected with recombinant GFP adenovirus (AdGFP) were similar to that of uninfected cells (Fig. 4A). In contrast, the level of CTGF mRNA decreased 4-fold in cells infected with rAdASO and exposed to TGF-β as compared to non-infected cells incubated with TGF-β (Fig. 4). Endogenous or basal levels of CTGF in Rat-2 cells did not significantly change upon infection with rAdASO or AdGFP. These data clearly show that rAdASO acts specifically upon the TGF-β-induced CTGF expression.

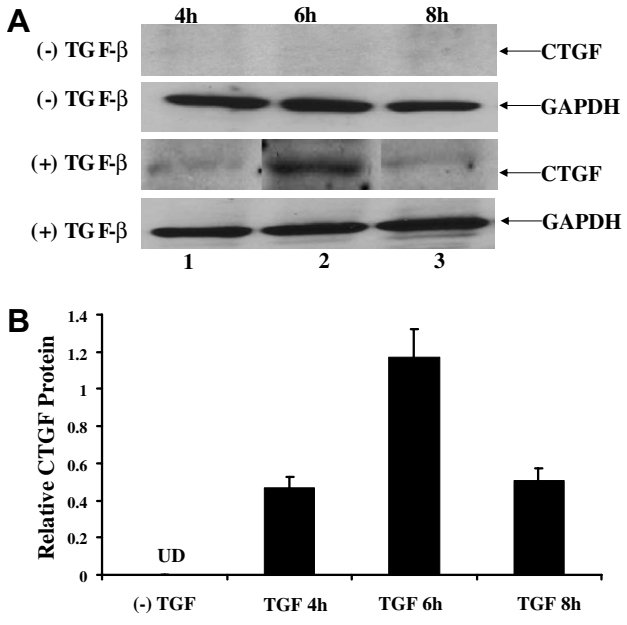

Fig. 2. TGF-β-induced CTGF protein in Rat-2 fibroblasts. (A) Western blot analysis for CTGF and loading control (GAPDH) in Rat-2 cells incubated without or with TGF-β (0.1 ng/ml) for 4, 6, and 8 h. CTGF protein level in cells incubated in the absence of TGF-β were undetectable. (B) Densitometric analysis of relative CTGF protein levels. UD = undetectable. Maximal protein induction was detected after 6 h of TGF-β treatment.

Discussion

CTGF, a proadhesive matricellular protein, promotes fibroblast proliferation, migration, adhesion, and extracellular matrix production [10] and plays a role in formation of blood vessels, bone and connective tissue [13]. In several human-derived cell lines CTGF mRNA expression is upregulated by TGF-β [5,14]. This induction, however, is cell-type specific, since it occurs in connective tissue cells but not in epithelial cells [15]. The regulation of CTGF expression by TGF-β is controlled at the level of gene transcription, and involves members of Smad family of proteins [16].

CTGF expression increased in rat retina during hyperglycemia ([22] and Winkler et al., unpublished observations), supporting the view that this molecule is involved in the appearance of the pathological signs of diabetic retinopathy. Since diabetic rats are a classical model of DR, it was important to establish whether rat cells responded to a novel recombinant adenovirus designed to inhibit CTGF expression. For that purpose, Rat-2 fibroblasts were used in the present report to examine the TGF-β-induced CTGF

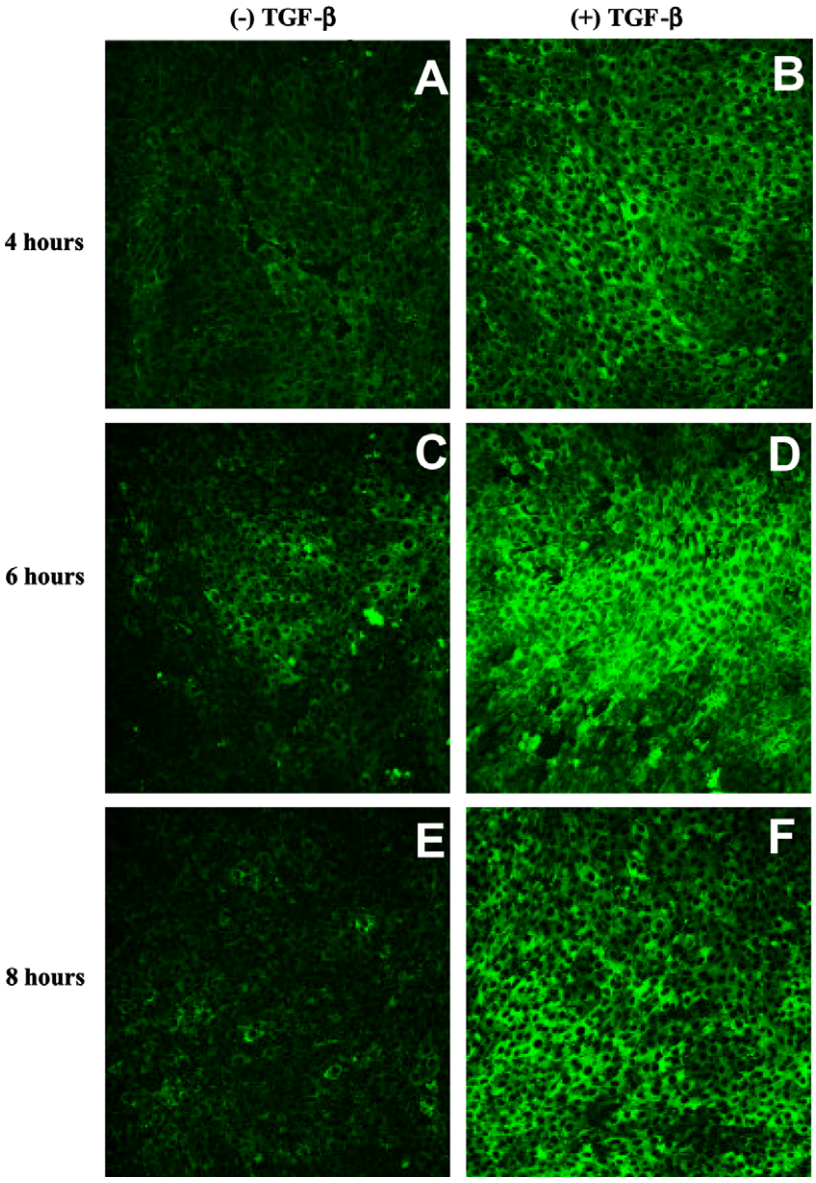

Fig. 3. TGF-β increased CTGF protein in Rat-2 fibroblasts. Immunostaining for CTGF in cells treated without (A, C, and E) or with TGF-β (0.1 ng/ml) (B, D, and F) for 4 h (A and B), 6 h (C and D), and 8 h (E and F). Most intense CTGF staining was seen after 6 h of TGF-β treatment.

expression profile and to test the effectiveness of the recombinant adenovirus.

We demonstrate that TGF-β induces CTGF expression at the transcription and protein levels in a time- and concentration-dependant manner. While TGF-β was added to the Rat-2 cells for different incubation time periods (4, 17, 24, and 48 h, data not shown); there was no significant increase in CTGF expression between these time points. Subsequent experiments were performed by stimulating cells for 4 h. These results are in agreement with Colwell et al. who showed an 11-fold increase of a TGF-β targeted gene (Wnt-4) after a mere 2 h incubation of primary fetal mouse fibroblasts with TGF-β [18].

Recombinant adenovirus (rAd) is one of the most extensively used vectors in gene therapy to date [19] because it has high replication and infection efficiencies. The recombinant adenovirus (rAdASO) tested in the present study, which encodes for a 300 bp antisense mouse CTGF cDNA fragment, was used to interfere with CTGF gene expression in cultured embryonic Rat-2 fibroblasts. Semi-quantitative RT-PCR analysis indicated that rAdASO is capable of causing at least a 4-fold decrease in TGF-β-induced CTGF gene expression. In the absence of TGF-β, rAdASO caused a statistically insignificant decrease in the basal level of CTGF and there was a minimal increase in CTGF expression in non-stimulated cells when infected with AdGFP, which could be attributed to cytokine mediated response caused by any adenoviral infection [20].

Adenoviral constructs do not integrate into the host genome and they might elicit adversary host immune responses [19], which may limits their long-term usefulness as gene therapy. On the other hand, a positive outcome

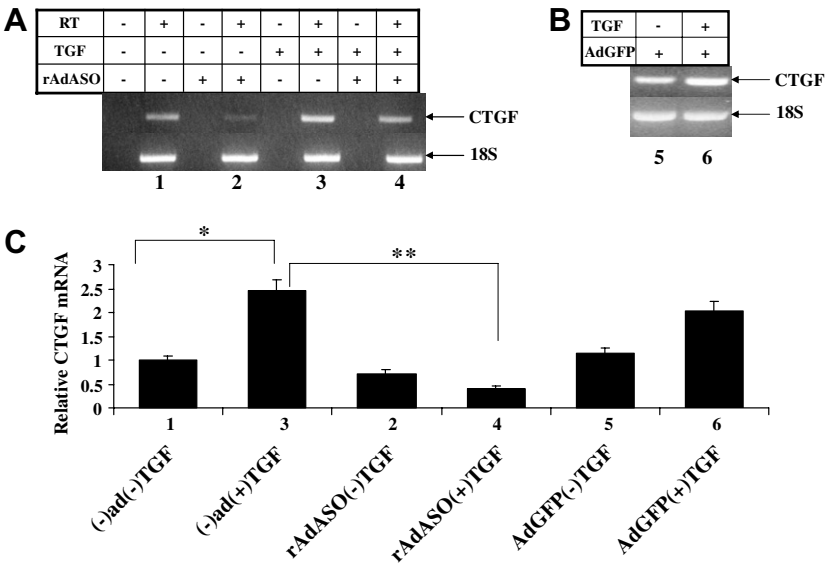

Fig. 4. Treatment of Rat-2 fibroblasts with rAdASO decreased CTGF message in cells maintained in reduced serum with TGF-β. (A) Semi-quantitative RT-PCR analysis of CTGF and 18S expression in Rat-2 cells without TGF-β and without adenovirus (lane 1), without TGF-β and with rAdASO (lane 2), with TGF-β (0.1 ng/ml) and without adenovirus (lane 3), and with TGF-β (0.1 ng/ml) and rAdASO antisense adenovirus (lane 4). (B) Representative gel showing CTGF mRNA levels in cells treated with control adenovirus (AdGFP) in the presence (lane 5) and the absence (lane 6) TGF-β. (C) Densitometric analysis of CTGF expression in control and virus treated Rat-2 cells (rAdASO and GFP). 18S used as housekeeping gene. Note a decrease in CTGF message in TGF-β treated cells when infected with the rAdASO. Reactions in which reverse transcriptase (RT) was omitted did not yield any product indicating negligible genomic DNA contamination. There was an insignificant difference in CTGF mRNA levels between mock, non-infected, and cells infected with a control virus expressing the green fluorescent protein (AdGFP). Numbers in histogram correspond to lane numbers in the RT-PCR gels. *Significant $p < 0.003$, **highly significant $p < 0.0001$.

may occur if the decrease in the severity of DR could be induced by a single adenoviral treatment in parallel to tight glycemic control. In conclusion, the data presented in this report documents the inhibition of CTGF expression by a novel antisense vector and suggest a promising approach to ameliorate any of the changes linked to upregulation of CTGF that occur in patients with diabetic retinopathy (DR) and different fibrotic diseases.

Acknowledgment

This work was supported with funds from the Juvenile Diabetes Research Foundation (pilot project).

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