Regulation of Fibronectin-EDA through CTGF Domain-Specific Interactions with TGF β 2 and Its Receptor TGF β RII

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PURPOSE. To investigate the role of fibronectin containing extra domain A (FN-EDA) in the pathogenesis of proliferative vitreoretinopathy (PVR) and the regulation of FN-EDA by transforming growth factor (TGF)- β and connective tissue growth factor (CTGF) in retinal pigment epithelial (RPE) cells.

METHODS. Expression of FN-EDA in normal human retinas and PVR membranes was evaluated by immunohistochemistry. The effects of TGF β and CTGF on FN-EDA mRNA and protein expression in primary cultures of human RPE cells were analyzed at different time points by real-time PCR and Western blot, respectively. The interaction of CTGF with TGF β 2 or with its type II receptor TGF β RII was examined by ELISA, immunoprecipitation, and solid-phase binding assays.

RESULTS. FN-EDA was abundantly expressed in PVR membranes but absent from the RPE monolayer in normal human retinas. Treatment of RPE cells with TGF β 2 induced FN-EDA expression in a time- and dose-dependent manner, but CTGF alone had no effect. However, CTGF, through its N-terminal half fragment, augmented TGF β 2-induced expression of FN-EDA at the protein level. This effect was blocked by antibodies against TGF β 2 or TGF β RII. Interaction of TGF β 2 or TGF β RII with CTGF was dose dependent and specific. CTGF directly bound TGF β 2 and TGF β RII at its N- and C-terminal domains, respectively.

CONCLUSIONS. These findings suggest that CTGF promotes the profibrotic activities of TGF β acting as a cofactor through direct protein interactions and complex regulatory mechanisms. (*Invest Ophthalmol Vis Sci.* 2011;52:5068–5078) DOI:10.1167/iovs.11-7191

The retinal pigment epithelium (RPE) plays a pivotal role in both normal retinal homeostasis and in certain pathologic conditions. The normally quiescent nondividing RPE cells become activated in response to injury that disrupts this highly specialized monolayer of cells. Activation of RPE involves

growth factors, cytokines, and extracellular matrix (ECM) components, as the cells detach, migrate, and proliferate acquiring a macrophage- and fibroblast-like phenotype in an apparent attempt to repair the injury.¹ This RPE transdifferentiation is associated with altered gene expression, upregulation of growth factors, and profound changes in the ECM.² Excessive production of profibrotic growth factors and deposition of ECM components on both sides of the retina³ are critical features of several retinal disorders including proliferative vitreoretinopathy (PVR). Through a sequence of events that resembles a wound-healing response, PVR triggers the formation of epiretinal membranes that may contract and exert traction on the retina, leading to retinal detachment.

An important aspect of the wound-healing response is the establishment of a provisional ECM with the appropriate proteins and cytokines. Fibronectin (FN) is one of the earliest ECM components expressed; it mediates cellular adhesion and migration of RPE cells.⁴ From a family of widely distributed glycoproteins, FN is composed of two subunits (~220–240 kDa) that are cross-linked by disulfide bonds. Alternative splicing of the FN gene transcript results in several variants. One isoform (FN-EDA or EIIIA) includes an extra domain that is only present in cellular FN.⁵ The expression of FN-EDA is significantly increased at specific stages of embryonic development⁶ and during wound healing in the adult.^{6,7} FN-EDA is also present in abundance in fibrotic disorders such as alveolar,⁸ renal,^{9,10} skin,⁶ and liver fibrosis.^{11,12} However, the role of FN-EDA in retinal fibrosis and in PVR pathogenesis has not been studied.

Extracellular regulatory factors and intracellular *cis*- and *trans*-acting elements regulate the mechanisms of FN alternative splicing.^{13,14} Transforming growth factor (TGF)- β induces the expression of cellular FN^{14–16} and its receptor in fibroblasts.¹⁷ TGF β is a prototypic multifunctional growth factor that regulates cellular proliferation and differentiation, embryonic development, wound healing, and angiogenesis.¹⁸ Connective tissue growth factor (CTGF) shares many of these functions with TGF β . Depending on cell type, CTGF is involved in a wide variety of biological processes including mitogenesis, chemotaxis, angiogenesis, ECM production, tissue repair, and apoptosis.¹⁹ This functional similarity is consistent with the current hypothesis that CTGF may be a downstream mediator or a cofactor for TGF β .¹⁹ Both TGF β and CTGF have been implicated as key mediators of tissue fibrosis in various diseases.^{19,20} Overproduction of these profibrotic growth factors can result in excessive deposition of ECM, scar tissue, and fibrosis. Our laboratory has identified CTGF as a major mediator of retinal fibrosis in an experimental model of PVR.²¹ In this article, we investigate the regulation of FN-EDA by TGF β and CTGF in RPE cells and the combined action of these growth factors on mRNA and protein synthesis.

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METHODS

This study has been approved by the institutional review board (IRB) of the University of Southern California for using human fetal eye and human PVR membranes. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from all participants.

Cell Culture

RPE cells were isolated from human fetal eyes of 18 to 22 weeks' gestation (Advanced Bioscience Resources Inc., Alameda, CA) by soaking the eyes in PBS containing 5% penicillin/streptomycin; cleaning off excess tissue; and removing the cornea, lens, vitreous, and retina. The RPE choroidal/scleral tissue was placed in a 2% Dispase solution in PBS for 20 minutes at 37°C, mixed by pipetting up and down for 30 seconds, and filtered by passing through a 70 μ m, then a 40- μ m filter. RPE cells were recovered by centrifugation and resuspended in Dulbecco's minimum essential medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (VWR International, West Chester, PA) containing at least 25% heat-inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA). The cells were first plated on laminin-coated plates until ready to be divided; no further coating was necessary and cells were grown in 10% DMEM. The cells were confirmed to be RPE cells by immunocytochemical positivity for cytokeratin (>95%) and the lack of immunoreactivity for endothelial-cell-specific von Willebrand factor (Dako, Carpinteria, CA) and glial fibrillary acidic protein (Chemicon, Temecula, CA).

Cells from passages 2 to 4 (unless otherwise specified) were cultured in six-well plates. For growth factor regulation, the cells were cultured (~24 hours) in DMEM containing 10% FBS to 70% to 80% confluence, starved (24 hours) in serum-free medium, and stimulated (48 hours) with TGF β 1, TGF β 2 (R&D Systems, Minneapolis, MN), full-length human recombinant CTGF (rhCTGF), and N-terminal (rhN-CTGF) or C-terminal (rhC-CTGF) half fragments (FibroGen, Inc., San Francisco, CA). CTGF N- and C-terminal half fragments were generated by proteolysis of rhCTGF (FibroGen, Inc.).

For immunohistochemistry, cells grown in 24-well plates were washed with cold PBS and fixed in cold methanol. For analysis of protein expression, the supernatants and cellular homogenates were collected at various times. For antibody-blocking assays, anti-NCTGF (FG-3019, a fully human monoclonal antibody against N-CTGF; FibroGen, Inc.) and polyclonal TGF β 2 and TGF β R2 antibodies (R&D Systems) were used.

Immunohistochemistry

Immunohistochemistry was performed on surgically excised epiretinal membranes from six nondiabetic patients during vitrectomy surgery for PVR and on normal human retinas obtained postmortem from six adult donors. Cryostat sections of snap frozen membranes were fixed with fresh acetone, incubated in 0.3% hydrogen peroxide, and blocked with 1% BSA. Primary monoclonal antibody against human cellular FN-EDA (Accurate Chemicals and Scientific Corporation, Westbury, NY) was applied at 1:100 dilution. Biotinylated anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) secondary antibody was used at a 1:100 dilution. Antibody binding was detected using the ABC (Vectastain Elite) and AEC (3-amino-9-ethylcarbazole) substrate kits (Vector Laboratories). Sections were counterstained with Mayer's hematoxylin (American MasterTech, Scientific, Inc., Lodi, CA). Immunohistochemical staining of cellular FN-EDA was also performed in cultures of RPE cells for 48 hours after stimulation with TGF β 1 or -2, by using the same antibody against the EDA domain of cellular FN as indicated above.

Western Blot Analysis

Proteins were separated by 10% SDS-PAGE, transferred overnight onto PVDF membranes, and probed with anticellular FN-EDA monoclonal antibody at 1:500 dilution. Signals were visualized with a horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma, St. Louis,

MO) and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). Protein bands were quantified with image-analysis software (Scion Corporation; Frederick, MD) and normalized to no-treatment control samples after adjustment for background intensities.

RNA Isolation

The cells were lysed directly in the culture dish by adding 0.5 mL RNA isolation solution (0.3–0.4 mL/cm²; TRIzol LS Reagent; Invitrogen, Carlsbad, CA). The lysates were passed several times through a pipette and incubated at 22°C for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Cell homogenates were then transferred to tubes (Eppendorf, Fremont, CA) and frozen at –80°C overnight. The homogenized samples were thawed and mixed vigorously with 133 μ L chloroform (0.2 mL per 0.75 mL TRIzol; Invitrogen) for 15 seconds. After 15 minutes at 22°C, the samples were centrifuged (12,000g, 15 minutes, 4°C). The colorless upper aqueous phase was transferred into new tubes and mixed with 333 μ L isopropyl alcohol (0.5 mL per 0.75 mL TRIzol; Invitrogen) to precipitate the RNA. After 10 minutes at 22°C, the mixture was centrifuged (12,000g, 10 minutes, 4°C), and the pellet-free supernatant was then discarded. The pellet was washed with 667 μ L 75% ethanol (1 mL per 0.75 mL TRIzol), vortexed, and centrifuged (7500g, 5 minutes, 4°C). The RNA pellet was air dried for 10 minutes and dissolved in 20 μ L of DEPC-treated water. DNase treatment was performed with a DNA-free kit (Ambion, Austin, TX). The samples were stored at –80°C after the RNA concentration was determined at 260 nm.

DNase-treated RNA samples were transcribed to DNA (Reverse Transcription Quick Protocol; Promega, Madison, WI). The reaction containing 1 μ g of RNA and oligo(dT)₁₅ primer was incubated for 1 hour at 42°C, followed by heating for 3 minutes at 95°C.

Real-Time PCR

Human FN-EDA primers were designed using computer software (LC Probe Design; Qiagen Operon, Alameda, CA). The sequences of the primers are forward 5'-TCCAAGCGGAGAGAGT-3' and reverse 5'-GTGGGTGTGACCTGAG-3'. DNA was amplified and quantified relative to the *GAPDH* housekeeping gene on a thermal cycler (LightCycler FastStart DNA Master SYBR Green I reaction kit, instrument, and software; Roche Diagnostics GmbH, Mannheim, Germany). Melting curve analysis was used to assure that correct amplification data were obtained. PCR sensitivity was sufficient to detect FN-EDA mRNA in untreated cells.

Solid-Phase Binding Assay

Microtiter plates (Immulon; Thermo Electron Corp., Milford, MA) were coated with 100 μ L of 1 μ g/mL rhCTGF or its fragments (N, N-terminal half fragment, rhN-CTGF; C, C-terminal half fragment), rhTGF β 2, or rhTGF β R2 in 50 mM NaHCO₃ buffer (pH 9.6) at 4°C overnight, and blocked with 200 μ L binding buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2% BSA, 0.05% Tween 20) for 1 hour at 37°C. Biotinylated rhTGF β 2, rhTGF β R2 (R&D Systems), or rhCTGF were added to the wells in a total volume of 100 μ L binding buffer and incubated for 3 hours at 37°C. To confirm specificity, an equal amount of TGF β R2 (1 μ g/mL) was denatured at 95°C for 5 minutes before biotinylation. In addition, basic fibroblast growth factor (bFGF; R&D Systems) was biotinylated and used as a control. The wells were washed with binding buffer and then incubated with 100 μ L of alkaline phosphatase (AP)-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Bound AP was monitored using p-nitrophenyl phosphate AP substrate (Chemicon). Biotinylation of rhTGF β 2, rhTGF β R2, and rhCTGF was performed with sulfo-NHS-LC-biotin (Pierce, Rockford, IL), according to the manufacturer's protocol.

Immunoprecipitation

Immunoprecipitation was performed as described by the manufacturer (Pierce). Recombinant proteins TGF β 2, TGF β R2 (R&D Systems), and

CTGF or its fragments, N (domains 1 and 2) and C (domains 3 and 4; FibroGen, Inc.), were premixed in 50 μ L of immunoprecipitation buffer (0.025 M Tris, 0.15 M NaCl; pH 7.2) for 1 hour followed by the addition of 1 μ g/mL polyclonal anti-CTGF, anti-NCTGF (FG-3019; FibroGen, Inc.), anti-TGF β 2, or anti-TGF β RII (R&D Systems) and incubated overnight at 4°C. Antigen-antibody complexes were precipitated by protein A gel (Pierce) after a 2-hour incubation at 25°C. After several washes, immunocomplexes were eluted with electrophoresis loading buffer (Bio-Rad, Hercules, CA) and incubated for 5 minutes at 95°C. The supernatants were evaluated by SDS-PAGE as described above.

CTGF-TGF β Enzyme-Linked Immunosorbent Assay

The interaction of CTGF with TGF β 2 was determined by a direct sandwich ELISA using a monoclonal antibody targeted against TGF β 2 (R&D Systems). Plates were coated with 50 μ L of 1 μ g/mL rhCTGF in 0.05 M carbonate/bicarbonate buffer (pH 8.5) overnight at 4°C. After blocking for 2 hours with 150 μ L of 1% BSA and 0.1% azide in PBS, the plates were washed once with 150 μ L washing buffer (PBS+0.1% Tween). TGF β 2 detection antibody was added (4 μ g/mL) to each well. Samples (rhTGF β 2; R&D Systems) were added (50 μ L) in triplicate to the appropriate wells. To confirm specificity, an equal amount of TGF β 2 (156 ng/mL) was denatured at 95°C for 5 minutes before addition. The plates were covered and incubated on an orbital shaker for 1.5 hours at 4°C followed by a triple wash with 150 μ L washing buffer. AP-conjugated anti-mouse antibody (1:500; Vector Laboratories) was added for 1 hour at room temperature. AP substrate was added at 100 μ L per well (Sigma) for 30 minutes, and the absorbance was measured at 405 nm. The reaction was stopped with 50 μ L of 4 M NaOH, and the absorbance was reread. Values plotted represent optical density (OD) readings normalized to control.

RESULTS

The expression of FN-EDA in Human PVR Membranes and Normal Human Retinas

To determine the role of FN-EDA in PVR pathogenesis, we evaluated FN-EDA expression in surgically excised membranes from PVR patients and compared them to frozen sections of normal human retinas by immunohistochemistry. FN-EDA was strongly expressed in 85% (5/6) of PVR membranes (Fig. 1A). Immunoreactivity was prominently observed both within the extracellular matrix and within cells in cellular regions. Negative controls stained with nonimmune IgG had no immunoreactivity (inset). In normal human retina, FN-EDA was present

FIGURE 1. Detection of FN-EDA in human PVR membranes. Immunohistochemical staining of FN-EDA (red) in (A) surgically removed human PVR membranes or (B) normal human retina-choroid with a specific monoclonal antibody against the EDA domain of FN. Mouse IgG1 was used as a negative control and showed no staining (inset). Magnification, $\times 82.5$.

FIGURE 2. TGF β induced FN-EDA expression in RPE cells. Immunohistochemical staining of cellular FN-EDA was performed in cultures of RPE cells 48 hours after stimulation with (A) PBS control, (B) TGF β 1, or (C) TGF β 2 with a monoclonal antibody against the EDA domain of cellular FN. (D) Western blot showing FN-EDA protein expression in RPE cell supernatants after cells were stimulated with TGF β 1 and TGF β 2 for 48 hours. Arrowhead: ≈ 220 -kDa FN fragment. The experiment shown is representative of three.

along the walls of choroidal and retinal vessels, but the RPE monolayer was negative (Fig. 1B). The staining pattern was similar in all sections analyzed and absent in all retinal tissue except for the positive blood vessels.

Induction of FN-EDA Protein Expression in RPE Cells in Response to TGF β

We studied the effect of various growth factors that alter the expression of ECM molecules in RPE cells. Based on increasing evidence that TGF β upregulates the expression of FN-EDA in different cell types, we treated RPE cultures under serum-free conditions with either TGF β 1 or -2. FN-EDA protein was induced by both TGF β isoforms, as indicated by immunohistochemical analysis (Figs. 2A-C) using a specific monoclonal anti-human FN-EDA. Staining for FN-EDA was detected in cell cytoplasm and also as fibrillar structures surrounding the cells and to a smaller extent as short fibrils connecting the cells. FN-EDA immunoreactivity appeared stronger after stimulation with TGF β 2 (Fig. 2C) compared with TGF β 1 (Fig. 2B).

These results were confirmed by Western blot analysis in which FN-EDA protein was identified in RPE cell supernatants when stimulated with 10 and even more so with 30 ng/mL of TGF β (Fig. 2D). Similar results were observed in cellular homogenates but to a lesser extent than cell supernatants (results not shown). Induction of FN-EDA protein was greater by TGF β 2 than by TGF β 1, confirming the immunohistochemical observations (Fig. 2). Both TGF β isoforms stimulated FN-EDA protein expression, and since TGF β 2 is the prominent isoform in the eye,²² we conducted further experiments using TGF β 2 only.

Upregulation of FN-EDA Expression in RPE Cells in Response to TGF β 2

TGF β 2 induced the expression of FN-EDA protein in RPE cells in a time- and dose-dependent manner. Treatment of RPE cells with 60 ng/mL of TGF β 2 enhanced FN-EDA-secreted protein up to fourfold (Fig. 3A), as determined by Western blot analysis on RPE cell supernatants. FN-EDA protein expression increased with increased TGF β 2 concen-

FIGURE 3. TGFβ2 upregulates expression of FN-EDA. (A) Western blot for FN-EDA protein expression in RPE cell supernatants in response to increasing concentrations of TGFβ2 for 24 and 48 hours. Blots were probed with a monoclonal antibody against the EDA domain of cellular FN. Arrowhead: ≈ 220-kDa FN fragment. The experiment shown is representative of six. (B) Densitometric analysis of FN-EDA protein expression in RPE cells from six separate experiments. Data are expressed as the mean ± SD and statistical evaluation was made with Student's *t*-test, comparing TGFβ2-treated cultures to the respective no-treatment control (**P* ≤ 0.01, ***P* ≤ 0.001). (C, D) FN-EDA mRNA expression was determined by real-time PCR analysis of RNA isolated from RPE cells stimulated with increasing concentrations of TGFβ2 at 48 hours or with 30 ng/mL of TGFβ2 at indicated time. Data are measured as the mean ± SD (*n* = 9). Expression of FN-EDA mRNA was normalized to GAPDH and compared to no-treatment control cultures for statistical evaluation (#*P* < 0.05, **P* < 0.01, ***P* < 0.001).

trations and more than doubled with longer exposure in cell culture supernatants (Fig. 3B). Immunohistochemical analysis confirmed the dose-response-enhanced protein expression of FN-EDA in RPE cell cultures. Strong staining was detected in cell cytoplasm and in fibrillar structures surrounding the cells (results not shown).

TGFβ2 Induces FN-EDA mRNA in RPE Cells

To determine whether TGFβ2 also affects FN-EDA mRNA expression in a manner similar to its effect on FN-EDA protein expression, we performed real-time PCR on TGFβ2-treated RPE cells at various time points. TGFβ2, at concentrations as low as 1 ng/mL, induced the expression of FN-EDA mRNA up to fourfold. FN-EDA mRNA increased with increased TGFβ2 concentration and reached a plateau at 3 ng/mL (Fig. 3C). This effect was time-dependent as FN-EDA mRNA increased up to 4.7-fold after a 2-day treatment. Thus, maximum FN-EDA mRNA induction by 30 ng/mL of TGFβ2 was achieved at 48 hours (Fig. 3D).

Effect of CTGF on FN-EDA Protein Expression in RPE Cells

RPE cells were treated with various concentrations of rh-CTGF (FibroGen, Inc.). RPE cell supernatants were analyzed for FN-EDA expression by Western blot analysis. Contrary to our hypothesis, rhCTGF had no effect on FN-EDA expression, even with concentrations as high as 80 ng/mL (Fig. 4A).

Effect of CTGF and TGFβ2 on FN-EDA Protein Expression in RPE Cells

The unexpected finding that CTGF had no effect on FN-EDA expression prompted us to look at the combined effect of both growth factors on the expression of FN-EDA. RPE cultures were treated under serum-free conditions with increasing concentrations of TGFβ2 and CTGF for various times. CTGF enhanced the expression of TGFβ2-induced FN-EDA in RPE cells treated for 48 hours (Fig. 4B). This effect was concentration-dependent and was more pronounced at high TGFβ2 concentrations. Quantification of FN-EDA protein from five different experiments showed that this enhanced effect was statistically significant (*P* < 0.05; Fig. 4C).

Effect of CTGF and TGFβ2 on FN-EDA mRNA Expression in RPE Cells

To determine whether the synergistic effect of CTGF on TGFβ2-induced FN-EDA protein expression also affects FN-EDA mRNA expression, we used real-time PCR with specific FN-EDA primers. CTGF had no effect on TGFβ2-induced FN-EDA mRNA expression (Fig. 4D). The time course for FN-EDA mRNA in response to both growth factors in Figure 4E showed no effect of CTGF on TGFβ2-induced FN-EDA mRNA expression.

Effect of CTGF Fragments on FN-EDA Expression in RPE Cells

In view of emerging evidence that CTGF N- and C-terminal fragments have different functions,²³ we asked whether

FIGURE 4. CTGF augments TGFβ2-induced expression of FN-EDA in RPE cells. Western blot for FN-EDA protein expression in RPE cell supernatants stimulated with (A) various concentrations of CTGF compared to 30 ng/mL of TGFβ2 for 48 hours or with (B) increasing concentrations of TGFβ2 and CTGF for 48 hours. Arrowhead: ≈220-kDa FN fragment. The experiment shown is representative of six. (C) Densitometric analysis of FN-EDA protein expression in RPE cells from five separate experiments. Data are expressed as the mean ± SD and statistical evaluation was made with Student's *t*-test (**P* < 0.05, ***P* < 0.05). (D, E) FN-EDA mRNA expression was determined by real-time PCR analysis of RNA isolated from RPE cells stimulated with increasing concentrations of TGFβ2 and CTGF at 48 hours (**P* < 0.01) or at various time points. Data are measured as the mean ± SD. Expression of FN-EDA mRNA was normalized to GAPDH and compared to no-treatment control cultures for statistical evaluation (**P* < 0.01, ***P* < 0.001).

these fragments have differential effects on FN-EDA expression. RPE cells were treated with various concentrations of rhN- and C-CTGF (FibroGen, Inc.) and the equivalent equimolar amount of full-length CTGF. Just as with full-length rhCTGF, the fragments alone had no effect on FN-EDA expression. However, in the presence of TGFβ2, rhN-CTGF, but not rhC-CTGF, enhanced FN-EDA expression

similar to full-length CTGF (Figs. 5A, 5B). There was no significant difference in FN-EDA expression between rhN-CTGF and full-length in the presence of TGFβ2.

FN-EDA mRNA analysis demonstrated that the effect of CTGF fragments on FN-EDA was not transcriptionally regulated. The time course for FN-EDA mRNA in response to stimulation with either fragment and TGFβ2 (Figs. 5C, 5D)

FIGURE 5. Differential effects of CTGF fragments on TGFβ2-induced FN-EDA expression in RPE cells. (A) Western blot showing FN-EDA expression in RPE cell supernatants stimulated with 30 ng/mL TGFβ2 and 30 ng/mL N-terminal (N-30), C-terminal (C-30), or full-length (F-30 and F-60, respectively) rhCTGF at 48 hours; arrowhead: 220-kDa FN fragment. The experiment shown is representative of six. (B) Densitometric analysis of FN-EDA protein expression in RPE cells from six separate experiments. Data are expressed as the mean ± SD and statistical evaluation was made with Student's *t*-test (**P* < 0.02, #*P* < 0.01, ##*P* < 0.003). (C, D) FN-EDA mRNA expression was determined by real-time PCR analysis of RNA isolated from RPE cells stimulated with 30 ng/mL TGFβ2 and 30 ng/mL N- or C-terminal at the indicated time points. Data are expressed as the mean ± SD. Expression of FN-EDA mRNA was normalized to GAPDH and compared to no-treatment control cultures for statistical evaluation (**P* < 0.05, ***P* < 0.005).

showed no effect of CTGF fragments on TGFβ2-induced FN-EDA mRNA expression.

Effect of Inhibiting TGFβ2 or its Receptor on FN-EDA Expression in RPE Cells

To confirm the role of TGFβ2 on the synergistic effect of CTGF+TGFβ2 on FN-EDA expression, we used polyclonal

FIGURE 6. Blocking of FN-EDA expression by antibodies against TGFβ2 or TGFβRII. Western blot showing FN-EDA protein expression in RPE cell supernatants stimulated with 30 ng/mL of TGFβ2 and full-length rhCTGF for 48 hours and effect of treatment with anti-TGFβ2 or anti-TGFβRII antibodies. The experiment shown is representative of three.

antibodies against TGFβ2 or its receptor TGFβRII in RPE cell cultures treated with TGFβ2 and full-length rhCTGF. We found that both antibodies not only inhibited TGFβ2-induced FN-EDA expression but also the synergistic effect of CTGF on TGFβ2-induced FN-EDA expression (Fig. 6).

Effect of Inhibiting CTGF or its Fragment on FN-EDA Expression in RPE Cells

To further explore the synergistic effect of CTGF and TGFβ2 on FN-EDA expression, we used the CTGF domain-specific monoclonal antibody anti-NCTGF (the antibody against the N-terminal second domain), in RPE cell cultures treated with TGFβ2 and rhN-CTGF, rhC-CTGF, or full-length rhCTGF. For those experiments, we used the least amount of TGFβ2 (3 ng/mL) that could induce FN-EDA, to facilitate the identification of inhibitory effects and to minimize reagent waste. We found that anti-NCTGF inhibited rhN-CTGF enhancement of TGFβ2-induced FN-EDA expression but not that of full-length rhCTGF (Fig. 7). There was also the unexpected finding that rhC-CTGF appeared to inhibit FN-EDA expression alone and in the presence of anti-NCTGF. Polyclonal CTGF antibody had no effect on TGFβ2 upregulation of FN-EDA (results not shown).

CTGF Interactions with TGFβ2 and TGFβRII

These data suggested critical protein interactions of both N- and C-terminal fragments of CTGF in the synergy with TGFβ2 and complex regulatory mechanisms. We therefore conducted solid-phase binding assay, direct sandwich ELISA, and immunoprecipitation studies to determine possible CTGF interactions with both TGFβ2 and its receptor TGFβRII. Binding of rhTGFβRII and rhTGFβ2 to rhCTGF and its fragments was demonstrated by these methods. Interaction of TGFβ2 with CTGF was shown by ELISA to be dose dependent (Fig. 8A) and specific (Fig. 8B). By solid-phase binding assay, adding biotinylated CTGF to 0.1 μg/mL rhTGFβ2-coated plates (Fig. 8C) resulted in more than 10-

FIGURE 7. Blocking of FN-EDA expression by CTGF domain-specific monoclonal antibody. Western blot showing FN-EDA protein expression in RPE cell supernatants stimulated with 3 ng/mL TGFβ2 and 30 ng/mL N-terminal (N), C-terminal (C), or full-length (F) rhCTGF for 48 hours and the effect of treatment with the second-domain N-terminal antibody (anti-NCTGF). The experiment shown is representative of three.

FIGURE 8. Interactions between CTGF, TGFβ2, and TGFβRII. The binding of TGFβ2 to CTGF-coated plates was detected by direct sandwich ELISA showing (A) dose dependence (* $P < 0.005$, ** $P < 0.01$) and (B) specificity, as 156 ng/mL of denatured TGFβ2 did not bind to the CTGF-coated plates (* $P < 0.005$, *** $P < 0.0005$). Solid-phase binding assay showed binding of (C) biotinylated CTGF onto TGFβ2-coated plates (* $P < 0.005$) and (D) biotinylated TGFβ2 on CTGF-coated plates (** $P < 0.01$) which was inhibited (E) by second-domain N-terminal antibody (anti-NCTGF; * $P < 0.05$). Binding of biotinylated TGFβRII to CTGF-coated plates was also shown by solid phase binding assay to be (F) dose dependent (# $P < 0.05$, ## $P < 0.001$) and (G) specific as denatured TGFβRII did not bind to CTGF-coated plates ($P < 0.049$). (H) The reverse experiment showed results similar to those of biotinylated CTGF bound to TGFβRII-coated plates ($P < 0.001$). (I) Biotinylated-TGFβRII only bound onto C-terminal ($P < 0.05$) but not N-terminal CTGF-coated plates. Values plotted represent the mean $\pm$ SD from at least three separate experiments.

fold binding relative to control levels ($P < 0.005$), whereas the reverse experiment resulted in weaker binding (two-fold) of biotinylated TGFβ2 to 1 μg/mL rhCTGF-coated

plates (Fig. 8D, $P < 0.01$). To determine the site of interaction, CTGF domain-specific monoclonal antibody anti-NCTGF was added along with biotinylated TGFβ2 to 1

μg/mL rhCTGF-coated plates. We found that the second domain antibody anti-NCTGF inhibited binding of biotinylated TGFβ2 to rhCTGF (Fig. 8E; $P < 0.05$).

With a solid-phase binding assay, binding of biotinylated TGFβRII to rhCTGF was dose dependent and specific ($P < 0.05$) compared to control levels (Figs. 8F, 8G). The reverse experiment, adding biotinylated CTGF to 1 μg/mL of rhTGFβRII, had similar binding results (Fig. 8H). To determine the site of interaction, biotinylated TGFβRII was added to 1 μg/mL of either rhN-CTGF- or rhC-CTGF-coated plates and was found to bind the C-terminal fragment of CTGF ($P < 0.05$) but not the N-terminal domain (Fig. 8I). Addition of the second domain antibody anti-NCTGF along with biotinylated TGFβRII to 1 μg/mL rhCTGF-coated plates slightly reduced but did not significantly inhibit binding of biotinylated TGFβRII to rhCTGF (results not shown).

In vitro immunoprecipitation studies (Fig. 9) supported the binding assays shown in Figure 8. Polyclonal anti-TGFβ2 antibody immunoprecipitated rhTGFβ2 (Fig. 9A, lane 2), and this effect was blocked when anti-TGFβ2 antibody was preincu-

FIGURE 9. TGFβ2 and TGFβRII interaction with CTGF demonstrated by immunoprecipitation. Recombinant proteins at 1 μg/mL (A, TGFβ2 and CTGF; B, TGFβRII and CTGF) were used as antigens and immunoprecipitated by anti-TGFβ2 and anti-CTGF antibodies (A), or anti-TGFβRII and anti-CTGF antibodies (B); the proteins from immunoprecipitation were subjected to Western blot analysis using anti-TGFβ2 (A) or anti-CTGF antibodies (B). Bar graph represents densitometric analysis of Western blots (mean ± SD) from three independent experiments.

FIGURE 10. TGFβ2 and TGFβRII interaction with CTGF fragments demonstrated by immunoprecipitation. Recombinant proteins at 1 μg/mL, (A) TGFβ2 and CTGF fragments (N, N-terminal half fragment; D2, second domain of CTGF) or (B) TGFβRII and CTGF fragments (N, N-terminal; C, C-terminal half fragments of CTGF; D2, second domain; and D4, fourth domain of CTGF) were premixed in 50 μL of immunoprecipitation buffer for 1 hour followed by the addition of 1 μg/mL of the appropriate antibody (anti-NCTGF or anti-TGFβRII antibodies against CTGF N-terminal or TGFβRII, respectively) as indicated and incubated overnight at 4°C. Immunocomplexes were subject to Western blot analysis using anti-TGFβ2 (A) or anti-CTGF (B) antibodies.

bated with TGFβ2 peptide (Fig. 9A, lane 3). Polyclonal anti-CTGF antibody also immunoprecipitated rhTGFβ2 in the presence of rhCTGF (Fig. 9A, lane 6); and this effect was blocked when anti-CTGF antibody was preincubated with CTGF peptide (Fig. 9A, lane 7). Polyclonal anti-CTGF immunoprecipitated rhCTGF (Fig. 9B, lane 2). Polyclonal anti-TGFβRII antibody immunoprecipitated CTGF in the presence of TGFβRII (Fig. 9B, lane 4), and this effect was completely lost when anti-TGFβRII antibody was preincubated with its blocking peptide (Fig. 9B, lane 5).

To further confirm the site of CTGF interaction with TGFβ2 and TGFβRII, we performed a similar immunoprecipitation assay using CTGF N- and C-terminal domains and compared that to full-length CTGF. Just as shown by the binding assays, we found that TGFβ2 interacted with CTGF N-terminal half fragment (Fig. 10A, lane 1), specifically the second domain (Fig. 10A, lane 3), whereas TGFβRII interacted only with the C-terminal half fragment (Fig. 10B, lane 7). Other domains of CTGF could not be tested due to lack of appropriate antibodies.

In summary, our results demonstrated that FN-EDA is regulated by TGFβ2 in a dose- and time-dependent manner in RPE cell cultures and that this effect is transcriptionally regulated. CTGF alone had no effect on FN-EDA expression at either the mRNA or protein level. Co-stimulation of RPE cells with both growth factors showed that CTGF, specifically the N-terminal domain, synergistically enhanced TGFβ2-induced FN-EDA expression at the protein but not the mRNA level. Interaction of TGFβ2 with CTGF was shown by ELISA to be dose dependent and specific, and the site of interaction was determined by solid-phase binding assay to be the second domain of CTGF. By solid-phase binding assay, interaction of TGFβRII with CTGF

was also dose dependent and specific and the site of interaction was the C-terminal fragment of CTGF. Immunoprecipitation confirmed that CTGF interacts with TGF β 2 and its receptor TGF β RII at the N- and C-terminal domains, respectively. Therefore, CTGF may act as a cofactor through TGF β 2-TGF β RII interactions to augment FN-EDA expression in a concentration-dependent manner, indicating critical interactions between the two growth factors and complex regulation.

DISCUSSION

The intricate balance of ECM components after tissue injury is regulated by the coordinate interactions of various cells, cytokines, and matrix molecules. FN-EDA is an important factor in the establishment of a provisional ECM that mediates cellular adhesion and migration. The expression of FN-EDA is significantly increased during wound healing in the adult^{6,7} and in several fibrotic disorders.^{6,8-10,12} Although some studies have reported the presence of FN as a major component in preretinal membranes,²⁴ epiretinal membranes, and vitreous of PVR patients,²⁵ none of these studies specified the form of FN. Grisanti et al.²⁶ and Bochaton-Piallat et al.²⁷ reported that cellular FN was expressed in epiretinal membranes of PVR patients and suggested that FN was produced locally, possibly by cells involved in proliferative intraocular disorders. Consistent with these studies, our results demonstrate that FN-EDA is abundantly expressed in epiretinal membranes from PVR patients. We also found that FN-EDA is present in retinal and choroidal vessels but absent from neural retinal cells or the RPE layer of normal human retinal tissue. This is in agreement with a previous study that reported that FN and FN-EDA were synthesized locally in the human retina.²⁸ Since PVR membranes are avascular, the presence of FN-EDA in normal vasculature is unlikely to play a role in disease pathogenesis.

FN-EDA facilitates the initiation of cellular responses to injury and has been implicated in myofibroblast-like cell transdifferentiation in fibroblasts induced by TGF β 1¹⁵ and in resting liver epithelial cells.¹² The mechanisms by which FN-EDA induction of epithelial-mesenchymal transdifferentiation might lead to PVR are not well understood. The EDA fragment of FN activates gene expression and induces production of matrix metalloproteinases (MMP), resulting in matrix degradation and possible cellular phenotype alteration,²⁹ facilitating cellular migration and proliferation.

A large body of evidence supports a role for increased levels of the profibrotic growth factors TGF β and CTGF in the pathogenesis of ocular fibrosis. In PVR, high TGF β 2 levels were detected in vitreous samples^{22,30} and were correlated with intraocular fibrosis.²² The expression of CTGF in cellular and fibrotic regions of PVR membranes was demonstrated previously by our laboratory,³¹ and CTGF has been shown to promote intraocular fibrosis in animal models of PVR.²¹ Prolonged production of these profibrotic growth factors can result in excessive deposition of ECM, scar tissue, and chronic fibrosis. Also FN levels were found to be relatively high in vitreous aspirates from PVR patients.⁴

TGF- β upregulates the expression of the EDA splice variant form of FN primary transcript in cultured human skin fibroblasts,¹⁴ tubular epithelial cells,³² and injured liver endothelial cells.³³ TGF β has been reported to strongly induce and directly regulate CTGF expression. CTGF may therefore act as a downstream effector of TGF β and enhance binding of TGF β to its receptors.³⁴ Localization of these growth factors and FN-EDA in PVR membranes suggests a direct and local cause and effect response of myofibroblastic cells (transdifferentiated RPE) in response to injury.

We have established that TGF β upregulates FN-EDA protein expression in RPE cells in a dose- and time-dependent manner.

The basis of this effect was determined to be upregulation of FN-EDA mRNA within 24 to 48 hours, consistent with other studies in cultured human skin fibroblasts,¹⁴ sinusoidal endothelial cells,^{12,16} and tubular epithelial cells.³² The rather delayed FN-EDA mRNA response in RPE cells correlates with the protein expression.¹⁶ Fibronectin protein is shed from the cell surface and accumulates in the supernatant over time.³⁵ This delayed FN-EDA expression suggests that RPE cells must first be primed, to elicit a response to TGF β .

In contrast to published data in other cell types,³⁶⁻³⁹ CTGF, even at concentrations as high as 80 ng/mL, did not induce FN-EDA expression in RPE cultures. The delayed mRNA response and the failure of CTGF alone to induce FN-EDA may well be a protective mechanism of RPE cell response to injury, to hinder the overproduction and accumulation of FN-EDA in the eye. Based on our time- and dose-response results of FN-EDA upregulation by TGF β 2 (Fig. 3), we chose to use 30 ng/mL of TGF β 2 in subsequent experiments because that amount resulted in a more pronounced, quantitatively evaluable band of FN-EDA protein compared to the lower concentrations of TGF β 2 tested.

We hypothesized that both growth factors, TGF β and CTGF, would have a modified effect on FN-EDA expression since they are co-expressed in various fibrotic disorders, such as in scleroderma.⁴⁰ Indeed, CTGF enhanced TGF β -induced expression of FN-EDA protein in a concentration-dependent manner. Real-time PCR data indicated that this synergy was not transcriptionally regulated, suggesting that CTGF mediates its synergistic effects through protein-protein interactions.

As a member of the CCN family of immediate early genes, CTGF shares a modular structure of four distinct domains: an insulin-like growth factor binding domain, a cysteine-rich module designated as von Willebrand type c domain, a thrombospondin type 1 repeat, and a C-terminal cystine knot domain.⁴¹ The first two domains comprise the N-terminal which is separated from the C-terminal (third and fourth domains) by a proteolytically sensitive hinge region. Depending on cell type, CTGF is involved in a wide variety of biological processes, including mitogenesis, chemotaxis, angiogenesis, ECM production, cell attachment and survival, tissue repair, and apoptosis.¹⁹ No consistent signaling receptor has been identified for CTGF, but the literature suggests that CTGF mediates its effects through various integrins⁴² and through the low-density, lipoprotein-related protein (LRP).⁴³ Of note, RPE express functionally active LRP on the cell surface,^{44,45} suggesting that CTGF acts through this mechanism. Wu et al.⁴⁶ have demonstrated a CTGF-mediated signaling pathway through cell surface heparin sulfate proteoglycans and the tyrosine kinase receptor TrkA in human mesangial cells, but this has not been investigated in RPE.⁴⁶

Recently, it has been reported that CTGF domains have different functions with the N-terminal fragment involved in differentiation and the C-terminal fragment in proliferation.²³ Our data support the differential roles of CTGF fragments and suggest complex regulatory mechanisms resulting in variable effects. Enhancement of TGF β 2-induced FN-EDA by N-terminal CTGF in RPE cultures to levels similar to those of full-length CTGF implicates the N-terminal domain in fibrosis and suggests that almost all full-length CTGF activity is through the N-terminal. In fact, several studies reported the accumulation of the N-terminal fragment of CTGF in various fibrotic disorders including PVR,²¹ proliferative diabetic retinopathy,⁴⁷ scleroderma,⁴⁸ and diabetic nephropathy.⁴⁹ Therefore, there may be a direct correlation between levels of N-terminal CTGF and fibrosis.

Inhibition of the TGF β 2-induced FN-EDA expression, as well as the enhanced effect of CTGF on TGF β 2-induced FN-EDA expression by TGF β 2 or TGF β RII antibodies in RPE cell

cultures, suggest a primary role for TGF β 2 in FN-EDA regulation. However, CTGF or its fragments also contribute to FN-EDA regulation as CTGF domain-specific antibody did inhibit the synergistic effect partially (N-terminal). To understand the differential outcome of these inhibitory studies, we would have to consider possible differences in affinity, interaction dynamics, steric effects, and subsequent conformational changes due to binding of these antibodies. In addition, we know that TGF β 2 upregulates CTGF protein expression in RPE cell culture.⁵⁰ Once secreted, CTGF is rapidly degraded⁵¹ and thus endogenous N- and C-terminal fragments could act as agonists or antagonists, respectively. The C-terminal fragment of CTGF appears to have an inhibitory effect on TGF β 2 upregulation of FN-EDA at both 3 and 30 ng/mL of TGF β 2. The C-terminal fragment of CTGF could mediate this inhibitory activity by interacting with TGF β RII so that TGF β RII is no longer available to bind TGF β . This inhibitory effect would be promoted even further by blocking the N-terminal fragment of CTGF, which would result in blocking any endogenous N-CTGF. Since TGF β mediates its actions through CTGF-dependent and -independent pathways,^{51–53} we suggest that TGF β initiates the FN-EDA response and then later works in concert with CTGF to enhance this effect further.

Our findings for the first time provide evidence that the CTGF C-terminal fragment is a binding site for TGF β RII and suggest possible mechanisms by which CTGF may synergize with TGF β to augment its effects. We therefore hypothesize that CTGF acts as a cofactor whereby it binds TGF β RII at its C-terminal fragment and interacts with TGF β at its N-terminal fragment introducing conformational changes that result in a higher affinity of TGF β to its receptor. The exact epitope location and extent of binding are yet to be determined.

Other forms of FN may be differentially regulated by CTGF. Using ARPE-19 cells, Nagai et al.⁵⁴ showed that CTGF directly upregulates expression of FN and that this effect is mediated through activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway. It would be interesting to determine whether the TGF β /CTGF interactions identified in this manuscript also play a role in the regulation of other FN isoforms.

In summary, our results suggest differing and critical roles for CTGF fragments in modulating TGF β responses in RPE cells and provide a novel mechanism by which CTGF can act as a cofactor in mediating the profibrotic activities of TGF β through domain-specific interactions with TGF β and its receptor. Therefore, TGF β effects can be regulated through the modulation of CTGF and its fragments. In addition, our results, for the first time, implicate FN-EDA as an important factor in mediating TGF β and CTGF effects on RPE transdifferentiation, making FN-EDA a potential target for therapy in ocular fibrosis because of its relatively specific expression in tissue response to injury.

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