Proteolytic Processing of Connective Tissue Growth Factor in Normal Ocular Tissues and during Corneal Wound Healing

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PURPOSE. Connective tissue growth factor (CTGF) is a fibrogenic cytokine that is up-regulated by TGF- β and mediates most key fibrotic actions of TGF- β , including stimulation of synthesis of extracellular matrix and differentiation of fibroblasts into myofibroblasts. This study addresses the role of proteolytic processing of CTGF in human corneal fibroblasts (HCF) stimulated with TGF- β , normal ocular tissues and wounded corneas.

METHODS. Proteolytic processing of CTGF in HCF cultures, normal animal eyes, and excimer laser wounded rat corneas were examined by Western blot. The identity of a 21-kDa band was determined by tandem mass spectrometry, and possible alternative splice variants of CTGF were assessed by 5' Rapid Amplification of cDNA Ends (RACE).

RESULTS. HCF stimulated by TGF- β contained full length 38-kDa CTGF and fragments of 25, 21, 18, and 13 kDa, while conditioned medium contained full length 38- and a 21-kDa fragment of CTGF that contained the middle "hinge" region of CTGF. Fragmentation of recombinant CTGF incubated in HCF extracts was blocked by the aspartate protease inhibitor, pepstatin. Normal mouse, rat, and rabbit whole eyes and rabbit ocular tissues contained abundant amounts of C-terminal 25- and 21-kDa fragments and trace amounts of 38-kDa CTGF, although no alternative transcripts were detected. All forms of CTGF (38, 25, and 21 kDa) were detected during healing of excimer ablated rat corneas, peaking on day 11.

CONCLUSIONS. Proteolytic processing of 38-kDa CTGF occurs during corneal wound healing, which may have important

implications in regulation of corneal scar formation. (*Invest Ophthalmol Vis Sci.* 2012;53:8093–8103) DOI:10.1167/iov.12-10419

Corneal scarring is a major cause of haze and impaired vision. After corneal trauma, stromal scarring is the result a complex cascade of multiple growth factors, cytokines, chemokines, and proteases. Immediately after epithelial damage, the process of healing is initiated by multiple cytokines and growth factors, including IL-1, TNF- α , bone morphogenic proteins 2 and 4 (BMP2, BMP4), epidermal growth factor (EGF), platelet derived growth factor (PDGF), TGF- β , and connective tissue growth factor (CTGF).^{1,2} The TGF- β system has been established as a key scar-promoting growth factor system.^{2,3} CTGF, a 38-kDa cysteine rich cytokine, is a downstream mediator of the fibrotic action of TGF- β .

The structure of CTGF is similar to other CCN proteins in that it contains a C-terminal domain, hinge region and N-terminal domain. The N-terminal domain contains two modules; the insulin-like growth factor (IGFBP) binding module that is predicted to bind IGF, and the von Willebrand factor type C repeat, which has been implicated as a binding site for TGF- β family members modulating their activity.⁴ Within the C-terminal domain, there are two modules; a thrombospondin type 1 module that is likely involved in binding to sulfated glycoconjugates⁵ and the CT module, which is similar to that found in TGF- β , PDGF, and nerve growth factor and allows dimerization of these proteins.^{6,7} We previously reported the expression level of CTGF was elevated in rat corneas after ablation.⁸

Fragments of CTGF were first reported in biological tissues by Brigstock et al.⁹ in pig uterine flushings. Since this discovery, 10-, 12-, 16-, 18-, 19-, 20-, 24-, and 31-kDa fragments of CTGF have been identified in different cell types, tissues, and body fluids.^{10–16} Recently, Tall et al.¹⁶ reported a novel 31-kDa fragment of CTGF that lacked the N-terminal sequence in the insoluble extracts of cultures of human corneal fibroblasts (HCF) when grown on different extracellular matrix components (collagen, fibronectin, and vitronectin), but not when grown on plastic.

The fragmentation of CTGF became more biologically important when Grotendorst and Duncan¹⁷ found the N-terminal and C-terminal fragments had distinct and mutually opposing effects on cells, either stimulating proliferation (C-terminal) or stimulating differentiation (N-terminal). To further define the biochemical processing of CTGF in ocular tissues, we characterized the fragmentation patterns of CTGF in cultures of HCF and established an aspartate protease is responsible for cleavage of 38-kDa CTGF. Unexpectedly, we found a 21-kDa fragment that may be the abundant form in

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normal mouse, rat, and rabbit ocular tissues and in rat corneas during healing of excimer ablation wounds.

MATERIALS AND METHODS

Sources of Growth Factors and Antibodies

Recombinant human (rh)CTGF was prepared by the Grotendorst laboratory using a baculovirus expression system.¹⁸ Antibodies were either purchased from US Biological (Swampscott, MA) and Santa Cruz Biotechnology (Santa Cruz, CA) or produced from the University of Florida Interdisciplinary Center for Biotechnology Research Monoclonal Core. Donkey-anti rabbit, donkey-anti goat, and donkey-anti mouse secondary antibodies labeled with an infrared dye were purchased from Li-cor Biosciences (Lincoln, NE). Streptavidin labeled with an infrared dye was also purchased from Li-cor Biosciences.

SDS-PAGE and Western Blots

Sensitivity of the different antibody detection during Western blot was measured by making serial dilutions of the rhCTGF from 150 ng/well to 0 ng/well. Samples were analyzed on 12% NuPAGE bis-Tris gels (Invitrogen, Carlsbad, CA). To detect CTGF, gels were blotted onto polyvinylidene difluoride (PVDF) membranes using iBlot transfer system (Invitrogen) following the manufacturer's guidelines. Blots were probed with primary antibody to CTGF and followed by infrared labeled secondary antibody. Band detection was performed using the Odyssey Infrared Imaging System (Li-cor Biosciences). C-terminal monoclonal and hinge region monoclonals antibodies were tested for specificity by using CTGF knockout and heterozygous CTGF mice embryo homogenates obtained from Karen Lyons, MD (University of California, Los Angeles).

Human Corneal Fibroblast Cell Culture

Cultures of HCFs were established by outgrowth from corneal explants, obtained in compliance with the tenants of the 2012 Declaration of Helsinki, as described previously.¹⁹ Briefly, epithelial and endothelial cells were removed from corneas, the stroma was cut into cubes of approximately 1 mm³, placed in culture medium consisting of equal parts Dulbecco's Modified Eagle Medium (DMEM), with 4.5 g/L of glucose and 1 g/L of L-glutamine (Invitrogen, Grand Island, NY). Medium was supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Grand Island, NY). Cells from cultures between passages 2 and 5 were used for all experiments.

TGF-β1 Stimulation of CTGF

To stimulate the expression CTGF, HCFs were place in serum-free medium for 48 hours. After 48 hours, the medium was replaced by 5 ng/mL of TGF-β1 (Sigma, St. Louis, MO) in DMEM. At various time points (0, 1, 6, 12, 18, 24, 48, and 72 hours) protein samples were collected from the medium, and a protease inhibitor cocktail (cocktail III; Calbiochem, Darmstadt, Germany) and 0.5 mM EDTA were added to the medium. Cell lysates were collected with the addition of PBS supplemented with 0.1% TritonX-100⁸ and the previously mentioned protease inhibitors cocktail. Samples were concentrated down using Centriprep Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Billerica, MA). Concentrated media and extracts were stored at -20°C until further analysis. Three biological replicates were performed per time point.

SDS-PAGE and Western Blots of CTGF from HCF Time Course

The concentration of total protein for all samples was measured using the NanoDrop Spectrophotometer (Thermo Scientific, Rockford, IL).

The same concentration of total protein per well (20 ug) was loaded per sample. SDS-PAGE was performed as described above. Detection for CTGF was performed as described above with the US Biological polyclonal antibody. The intensity of each band was determined using the area under the curve function in the ImageJ software (National Institutes of Health, Bethesda, MD). A recombinant CTGF standard (concentration of 300 ng) was run on each gel. Variation between Western blots were normalized by comparing individual bands (38, 25, 21, 18, and 13 kDa) to the rhCTGF at 200 ng (an internal blot control) in order to remove variation of antibody binding or exposure times of the blots. The relative band intensities were compared for statistical significance using ANOVA followed by Tukey's Post-hoc using GraphPad Prism (GraphPad, La Jolla, CA). A *P* value of less than 0.05 was considered significant.

Immunoprecipitation and Identification of 21 kDa CTGF Band

Immunoprecipitation of CTGF was conducted using Direct Immunoprecipitation Kit (Thermo Scientific). Briefly, the US Biological polyclonal antibody was coupled with AminoLink Plus Coupling Resin (Thermo Scientific) following manufacturer's instructions. Five aliquots of same sample were applied over the column to purify the CTGF from the cell culture extract. The five elutions were combined and then were concentrated using DNA 110 Speed Vac (Thermo Scientific). The concentrated immunoprecipitated CTGF samples were rehydrated in distilled water. The sample was run on SDS-PAGE as previously described. Silver staining was performed using SilverQuest Silver Stain (Invitrogen) following the manufacturer's instructions. The 38- and 21-kDa bands were cut out of the gel and then they were destained following manufacturer's instructions. Samples were sent to ProtTech Inc. (Norristown, PA) to perform tandem mass spectroscopy (NanoLC-MS/MS).

Identification of the Protease Class that Cleaves CTGF from HCF Extracts

To stimulate the expression CTGF and proteolysis, HCFs were place in serum-free medium for 48 hours. After 48 hours, the medium was replaced by 10 ng/mL of TGF-β in DMEM for 24 hours. Medium was removed and discarded. The cells were rinsed three times with PBS. The corneal fibroblasts were then scraped off the flask and collected in 1.5-mL PBS. The collected cells were pelleted by centrifugation at 14,000 rpm for 10 minutes. Supernatant was collected and pellet was solubilized 0.1% TritonX-100. Stock concentrations of the following protease inhibitors were made as follows: 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride (AEBSF; 100 mM in water), Aprotinin (80uM in water), Bestatin (5 mM in DMSO), E-64 (1.5 mM in dimethyl sulfoxide [DMSO]), Leupeptin (2 mM in water), EDTA (0.5 M in water), and Pepstatin (1 mM in dimethyl sulfoxide [DMSO]). The proteases inhibitors were diluted to the above concentration to model the concentrations used in the protease inhibitor cocktail (cocktail III) from Calbiochem. When the assay was performed these stock solutions were diluted 1:1000. Recombinant CTGF (US Biological) was combined with the supernatant and incubated for 0 or 1 hour at 37°C. Proteolysis was stopped by the addition of a protease inhibitor cocktail (Calbiochem) and 0.5 mM EDTA. SDS-PAGE and Western blot was performed as previously described. Band detection was performed using the Odyssey Infrared Imaging System (Li-cor Bioscience). Band intensity was determined using the area under the curve function from ImageJ software. Band intensity between blots were normalized as described above. Three biological replicates were performed. The relative band intensities were compared using Student's *t*-test using GraphPad Prism. A *P* value of less than 0.05 was considered significant. The differences between the 0 and 1 hour of the normalized band intensities were assessed. Then the differences from different treatment groups were compared with the untreated by using Student's *t*-test using GraphPad Prism.

FIGURE 1. Detection of rCTGF using several different antibodies. **(A)** US Biological rabbit polyclonal, mouse hinge region monoclonal, and mouse C-terminal monoclonal, were all tested for sensitivity using rCTGF. **(B)** Specificity of the hinge region monoclonal and C-terminal monoclonal were tested by analyzing the ability of the antibody to detect CTGF in heterozygous (+/-) and homozygous (-/-) mouse tissue homogenates.

Mouse, Rat, and Rabbit Eye Homogenates

Frozen whole mouse, rat, and rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR). Ten mouse eyes were frozen in liquid nitrogen and combined into a steel piston homogenizer. The eyes were shattered by the force placed on the piston. The eye homogenates were collected by placing 0.1% TritonX-100⁸ in PBS and a protease inhibitor cocktail III and 0.5 mM EDTA to the homogenizer and collecting all of the samples. To assess a harsher detergent, whole eyes were also homogenized in an ionic detergent consisting of 1% SDS in PBS with the protease inhibitor cocktail III and 0.5 mM EDTA. Samples were centrifuged at 5000x g for 10 minutes to extract any pieces of tissue that were not homogenized. Each species had 10 different eyes homogenized together to produce whole eye homogenates. The eye structures (cornea, retina, iris, sclera, lens, and vitreous) of the rabbit eye were individually dissected out of the eye. Ten of each of the eye structures were processed as described above.

SDS-PAGE and Western Blots of Whole Eye Homogenates

SDS-PAGE was performed as described above. To detect CTGF, gels were blotted onto PVDF membranes using iBlot transfer system (Invitrogen) following the manufacturer's guidelines. Blots were incubated with primary antibody, biotinylated US Biological rabbit anti-CTGF. The biotinylated US Biological rabbit anti-CTGF, biotinylated hinge region monoclonal, and biotinylated C-terminal monoclonal were diluted to a concentration of 10 µg/mL in Odyssey Blocking Buffer containing 0.1% Tween. Blots were incubated in infrared-labeled streptavidin diluted 1:5,000 in Odyssey Blocking Buffer with 0.2% Tween 20 and 0.01% SDS. Band detection was performed using the Odyssey Infrared Imaging System.

5' Rapid Amplification of cDNA Ends (RACE) of Mouse and Rat RNA

Total RNA was extracted from mouse and rat whole eyes using the RNA-Bee Reagent (Tel INC, Lake Forest, CA) following manufacturer's instructions. The mRNA was purified from the total RNA by using the MicroPoly(A)Purist Kit (Ambion, Grand Island, NY) following the manufacturer's instructions. The 5' RACE was performed using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA) following the manufacturer's instructions. The primers were as follow: Mouse CTGF 5' RACE primer: 5' GGCTTGGCAATTTTAGG CGTCCGGAT 3' and Rat CTGF 5' RACE primer: 5' GGCTTGGCGATT TAGGTGTCCGGAT 3'. Bands were identified on a 1% agarose gel.

Rat Corneal Ablation

Adult male Sprague Dawley rats were used for this study, and the procedure was performed in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Using the technique that we previously described,⁸ precise, reproducible, central corneal ablations were created in rat corneas using a Nidek EC-5000 Excimer laser (Nidek, Fremont, CA). Briefly, rats were anesthetized with isoflurane/oxygen inhalation, proparacaine eye drops were applied to achieve local anesthesia of corneas, and both eyes of each rat were ablated to a depth of 80 µm with creating a 4.4-mm diameter central epithelium to stroma injury. The ablation conditions were specifically designed to remove all of the corneal epithelial cell layers and some of the stroma to simulate photorefractive keratectomy (PRK). Both eyes of each rat were ablated. The animals were killed at 0, 1, 6, 12, 18, 24 hours and 4, 7, 11, and 21 days after ablation. Four rat corneas per time point were collected. At the various time points after excimer laser ablation, the animals were euthanized, and the corneas were then excised. Corneas were homogenized using 1-mL glass homogenizers in

FIGURE 2. Western blot analysis of cell extracts from HCF cells stimulated with TGF- β 1. (A) Western blot analysis detected expression of lower molecular weight forms of CTGF using the US Biological polyclonal antibody. (B) Quantification of individual CTGF bands (38, 25, 21, 18, and 13 kDa) were performed from three replicates and significance is indicated by * ($P < 0.05$). MW stands for molecular weight standard.

0.1% TritonX-100 in PBS and protease inhibitor cocktail III and 0.5-mM EDTA were added to each sample. SDS-PAGE and Western blot analyses were performed as described above. Each band intensity was determined by using the area under the curve function in ImageJ software. The relative band intensities were compared for statistical significance using ANOVA followed by Tukey's Post-hoc using Graph-Pad Prism. A P value of less than 0.05 was considered significant.

RESULTS

Antibody Sensitivity and Detection

The sensitivity of each antibody was determined by running a standard curve of the recombinant CTGF protein. The polyclonal antibody, US Biological, had the greatest level of detection at 23 ng of total CTGF protein. With respect to the monoclonal antibody detection, the hinge region antibody had the greatest level of detection at 50 ng of total CTGF protein, whereas, the C-terminal antibody had the lowest level of detection at 100 ng of total CTGF protein (Fig. 1). To determine monoclonal specificity, heterozygous CTGF mice homogenates and homozygous CTGF knockout mice homogenates were probed using the C-terminal and hinge region monoclonal antibodies. Both antibodies detected the 38-kDa band in the heterozygous CTGF mice homogenates. Neither monoclonal detected a band at 38 kDa for the homozygous CTGF knockout mice homogenates.

TGF- β 1 Stimulation of CTGF in HCF

After the antibodies were tested on recombinant CTGF protein for sensitivity and specificity, we measured the time course of CTGF expression after stimulation with TGF- β 1. HCF cells were serum starved for 48 hours and then stimulated with TGF- β 1. At 0, 1, 6, 12, 18, 24, 48, and 72 hours the media and cell extract were collected and analyzed by Western blotting. In the cell extracts (Fig. 2), the full length (38 kDa) CTGF protein was detected at all time points from 0 hours to 72 hours peaking at 18 hours. At 18 hours there was 1.6 times more full length (38 kDa) CTGF than at 0 hour, though the difference was not statistically significant. A 21-kDa CTGF fragment was detected 1 hour post stimulation and peaked at 12 hours. The increase in the level of the 21-kDa CTGF fragment at 12 hours fragment was statistically significantly compared with earlier and later time points, with the 12 hour being 37 times greater than the 0 hour time point. At 72 hours after stimulation, the detection level of the 21-kDa fragment returned to the 0 hour level. A 25-kDa fragment was also detected and had a peak at forty eight hours but it was not determined to be statistically significant. Finally, two other fragments, 18- and 13-kDa CTGF fragments were detected throughout the treatment times, 0 hours to 72 hours. There was no significant change in intensity of these two bands following TGF- β 1 treatment. Therefore, we conclude that these fragments are not produced in response to the addition of TGF- β 1.

FIGURE 3. Composite Western blot analysis of conditioned media from HCF cells stimulated with TGF-β1. (A) Western blot analysis of the time course detected lower molecular weight forms of CTGF using the US Biological polyclonal antibody. (B) Quantification of individual CTGF bands (38, 21, 18, and 13 kDa) was performed from three replicates. P of 0.01 < P < 0.05 indicated by * and 0.001 < P < 0.0099 indicated by **.

CTGF is a secreted protein, therefore, the conditioned media from the HCF stimulated with TGF-β1 were analyzed (Fig. 3). An unexpected 50-kDa band was detected in the conditioned media. The greatest detection occurred at time 0, and it was at least 2.6-fold higher than any of the other time points ($P < 0.05$). The full length (38 kDa) fragment protein was initially detected at 6 hours and peaked at 24 hours post stimulation. When compared with 0 and 1 hours, there was a least 17.9 times more full length (38 kDa) CTGF at 24 hours post stimulation ($P < 0.05$). The 21-kDa CTGF fragment was also detected in the conditioned media. The 21-kDa CTGF fragment was detected 6 hours post stimulation and peaked at 24 hours. When compared with 0, 1, 6, and 72 hours there was a least 10.3 times more 21-kDa fragment of CTGF at 24 hours post stimulation ($P < 0.05$). The normalized band intensity of the 21-kDa fragment returned to that of the 0 hour time point at 72 hours.

Immunoprecipitation and Identification of the 21-kDa CTGF Fragment from HCF

The CTGF from the 24 hour cell extract was purified by immunoprecipitation using the US Biological polyclonal antibody. The sample was analyzed by silver stain and Western blot analysis. The full length (38 kDa) and 21-kDa fragment were detected. The 21-kDa band was identified as CTGF by the amino acid sequence, LEDTFGPDPTMIR, which was located in hinge region of CTGF, using tandem mass spectrometry (Fig. 4). These data confirm that the 21-kDa band is a fragment of CTGF and not due to nonspecific binding of the primary antibody.

Identification of the Protease Class that Cleaves CTGF from HCF Extracts

We conducted a processing assay to determine the protease class that cleaves CTGF from the 38-kDa full length form into

FIGURE 4. Western blot analysis of immunoprecipitated CTGF from HCF cells stimulated with TGF- β 1. The 21-kDa fragment (highlighted in the red box) was identified as CTGF from the LEDTFGPDPTMIR sequence located in the hinge region of CTGF.

the 21-kDa fragment in HCF. HCF extracts were incubated with rCTGF for 0 or 1 hour with different protease inhibitors, and the samples were analyzed by Western blot (Fig. 5). The only protease inhibitor that was able to inhibit the processing of CTGF into the 21-kDa fragment was pepstatin, an aspartic acid protease inhibitor. There was no difference in normalized band intensity between the 0 and 1 hour time points ($P = 0.32$). AEBSE, Aprotinin, Bestatin, E-64, Leupeptin, and EDTA did not inhibit appearance of the 21-kDa CTGF fragment when comparing the normalized band intensity of the 0 hour time point with the 1 hour time point ($P < 0.05$). When the difference in the normalized band intensities of the untreated is compared individually with each treatment group, the group treated with pepstatin was significantly reduced ($P < 0.05$).

Identification of a 21-kDa CTGF Fragment in Unwounded Rat, Rabbit, and Mouse Whole Eye Homogenates

To determine if the 21-kDa fragment was unique to the HCF culture system, rabbit, rat, and mouse, whole eyes were homogenized and analyzed by Western blotting using three different antibodies. The US Biological polyclonal, hinge region monoclonal, and C-terminal monoclonal all detected a 25- and 21-kDa fragment in rabbit, rat, and mouse unwounded whole eye homogenates (Fig. 6). Interestingly, there was little 38-kDa full length CTGF detected in any of homogenates from rats, rabbits or mice, but there were ample amounts of the 21-kDa fragment, suggesting that this is the major intraocular form in whole eyes.

Identification of a 21-kDa CTGF Fragment in Ocular Structures in the Rabbit

To analyze what structure(s) in the eye was producing the 21-kDa CTGF fragment, ten rabbit eyes were dissected into individual eye structures (cornea, retina, iris, sclera, lens, and vitreous), the grouped structures homogenized, and then analyzed by Western blot. The cornea, retina, iris, sclera, lens, and vitreous all contained the 21-kDa band and the 25-kDa band (Fig. 7). These 21- and 25-kDa bands were identified by both the US Biological polyclonal and the C-terminal monoclonal antibodies. The lens had at least 5 times more 21-kDa CTGF fragment than any of the other structures, because 6 ug

of total protein from the lens were added as opposed to 30 ug of total protein from the other structures.

5' Rapid Amplification of cDNA Ends of Mouse and Rat RNA

One possible origin of the 21-kDa fragment is alternative RNA transcripts. To determine if the 21- or 25-kDa proteins could be explained by the use of alternative RNA start sites, 5' RACE was performed on mouse and rat RNA (Fig. 8). The only PCR product that was produced was 791 bp corresponding to full length CTGF. No other bands were detected. Therefore, we conclude that only one major RNA start site exists, and that the smaller fragments that are immune reactive to CTGF antibodies are the result of proteolytic processing.

Detection of CTGF during the Course of Corneal Wound Healing in Rats

A time course of CTGF throughout corneal wound healing in rats was performed by ablating normal rat corneas with a laser and collecting the corneas at 0, 1, 6, 12, 18, and 24 hours and 3, 7, 11, 14, and 21 days (Fig. 9). The homogenates of the rat corneas were analyzed by Western blot ($n = 3$). Low levels of full length CTGF (38 kDa) were detected throughout the wound healing process, from 0 hours to 21 days. The normalized band intensity from day 11 post ablation was 1.6 times greater than the 0 hour time point. The lowest normalized band intensity from the 38-kDa full length CTGF occurred at 12 hours post ablation and was 3.7 times less than the day 11 time point. As previously mentioned, a 25-kDa CTGF fragment was detected in uninjured cornea. The greatest normalized band intensity for the 25-kDa CTGF fragment occurred 11 days post ablation and was 2.5 times ($P < 0.05$) greater than the 0 hour time point. The 25-kDa CTGF fragment normalized band intensity at 11 days post ablation was 22.9 times greater than the 12 hour time point which had the lowest band intensity of the 25-kDa CTGF fragment ($P < 0.0001$). A 21-kDa CTGF fragment was detected at the 0 hour time point, and the peak level of detection occurred 11 days post ablation. The normalized band density of the 21-kDa CTGF fragment from day 11 was 32.5 times greater than the 12 hour time point ($P = 0.0051$). There were two other bands detected, of 18 and 13 kDa, that also peaked at 11 days post injury.

FIGURE 5. Western blot analysis of in vitro processing of CTGF into the 21-kDa fragment. (A) Briefly, rCTGF was incubated for 0 or 1 hour with the cell extract from HCF that were stimulated with TGF- β 1. To assess inhibition of processing, the rCTGF protein and cell extract was incubated with different protease inhibitors (Aprotinin, Bestatin, E64, EDTA, Leupeptin, AEBSE, or Pepstatin) or no protease inhibitor. (B) The average relative quantification of the 0 hour and 1 hour time points of the 21-kDa band for each protease inhibitor from three replicates are shown. Significance of $0.01 < P < 0.05$ indicated by * and $P < 0.0001$ indicated by ***. (C) The average difference between the 0 hour and 1 hour normalized band density for each treatment are shown. Three replicates were performed and band intensities were compared by Student *t*-test of the inhibitor treatment compared with the untreated extract. *P* of < 0.05 is indicated by * and < 0.0001 indicated by ***.

FIGURE 6. Western blot analysis of CTGF from unwounded rabbit, rat, and mouse whole eye homogenates. All samples were homogenized in 0.1% triton $\times$ 100 with the exception of the sample labeled 1% SDS Rabbit. Three different antibodies were used to detect the CTGF. The US Biological polyclonal antibody, the hinge region monoclonal antibody and the C-terminal monoclonal antibody all had similar banding patterns identifying a 21- and 25-kDa band.

DISCUSSION

Several studies have reported detecting various size fragments of CTGF in different biological samples, and Duncan et al.¹⁷ showed that N-terminal and C-terminal fragments generated *in vitro* by proteolytic cleavage of recombinant CTGF had different biological activities in cultures of rat kidney fibroblasts.^{10–16,20} The fragmentation patterns of CTGF in normal ocular tissues, and in corneas during healing of excimer laser wounds, in cultures of HCF stimulated by TGF- β 1 have not been determined, nor has the class of protease that generates the CTGF fragments been identified. This study investigates these important biochemical questions.

Recently, Tall et al.¹⁶ analyzed fragmentation patterns of CTGF generated by HCF grown on different extracellular matrix components (collagen, fibronectin, and vitronectin). They detected a major, novel 31-kDa CTGF fragment that lacked the N-terminus in the insoluble fraction of HCF grown on plastic surfaces coated with the three different extracellular matrix proteins (ECM) along with minor bands at 24 kDa and 18 to 20 kDa. The insoluble fraction was defined as the material not solubilized by a detergent solution containing 1% Triton X-100 and 0.05% SDS. This insoluble fraction was collected by centrifugation, and was further solubilized by 2% SDS sample buffer, which presumably contained a reducing agent and was boiled before analyzing by Western blot. The

FIGURE 7. Western blot analysis of CTGF from unwounded rabbit eye structures (cornea, retina, iris, sclera, lens, and vitreous). Two different antibodies were used to detect the CTGF. The US Biological polyclonal antibody and the C-terminal monoclonal antibody all had similar banding patterns identifying a 21- and 25-kDa band in all tissue. * Indicates that six times less total protein from the lens was loaded in the well.

FIGURE 8. Analysis of transcriptional start sites of the CTGF RNA in adult mouse and adult rat eyes using 5' RACE. The full length transcript is 791 bp including the start codon and stop codon.

soluble fraction contained only the full length 38-kDa CTGF, and the 31-, 24-, and 18- to 20-kDa fragments were not detected.

In this study, we detected several CTGF forms (50, 38, 25, 21, 18, and 13 kDa) in the conditioned medium and the soluble extract of HCF cultures grown on plastic stimulated with TGF- β 1, but not coated with ECM proteins. Of these six CTGF forms, only the 21-kDa fragment significantly changed in the soluble extract of HCF in response to stimulation by TGF1 (Fig. 2), while both the 38 and 21 kDa forms significantly increased in conditioned media (Fig. 3). In our experiments, the soluble fraction of HCF was defined as material solubilized in 0.1% Triton X-100. Since the soluble fraction of HCF generated by Tall et al.¹⁶ did not contain the multiple fragments of CTGF that we identified, it is likely that the major differences between the two sets of cell culture experiments are due to the ECM coatings of cell culture dishes.

We also detected a 50-kDa immunoreactive band in Western blots of the conditioned medium of HCF that was not due to nonspecific binding of the secondary antibody. However, the 50-kDa immunoreactive band was not detected in Western blots of soluble extracts (0.1% Triton X-100) of normal rabbit ocular tissues or in soluble extract of HCF cultures. Previously, we produced several CTGF fragments containing the four different modules of CTGF (i.e., CTGF module I, CTGF module I and II, CTGF module I-III, and full length CTGF I-IV), and

analyzed the ability for these different fragments to bind to other growth factors such as Slit3, PDGF-B, VEGF, as well as CTGF and CTGF fragments.²¹ Interestingly, we found that the purified CTGF I-III (25 kDa) fragment produced in CHO cells also generated a band at 50 kDa (dimer) that was not dissociated by boiling in Laemmli sample buffer (4% SDS plus reducing agent). In addition, Abreu et al.²² found that both TGF- β 1 and BMP-4 were able to tightly bind CTGF, with Kds for CTGF binding to BMP-4 of 5 nM and to TGF- β 1 of 30 nM. The BMP-4:CTGF complex was approximately 50 kDa in size. Based on these results, it seems likely that the 50-kDa immunoreactive band we detected in conditioned medium of HCF is a stable aggregate of CTGF fragments either tightly associated to CTGF fragments or to other growth factors. Further experiments are needed to determine the composition of the 50-kDa aggregate that immunoreacts with antibodies specific for CTGF.

Previously, Ball et al.²³ looked at the presence of CTGF fragments during estrous and pregnancy in porcine uterine flushes. They found four fragments of CTGF with sizes of 20, 18, 16, and 10 kDa present in porcine uterine flushes. The full length 38-kDa CTGF, as well as the processed forms of CTGF, were in higher abundance at day 12 of pregnancy than day 12 of the cycle. Using N-terminal sequencing, they determined the 20- and 18-kDa fragments were generated by proteolytic cleavage at Asp186 while the 16-kDa fragment was generated by cleavage at Ala197.²⁰ Using tandem mass spectrometry, we identified that the 21-kDa fragment contained amino acids Leu184 - Arg196, which is located in the hinge region of CTGF (Fig. 4). Because this fragment reacts with the C-terminal specific antibody, we conclude that the cleavage site that produced the 21-kDa band occurred upstream of Leu184.

An important component of the CTGF processing system in HCF is the protease(s) that cuts the full length 38-kDa form of CTGF. We were able to determine that the addition of pepstatin to homogenates of HCF prevented cleavage of rCTGF into the 21-kDa fragment, which establishes that a member of the acid protease superfamily of proteases is responsible for cleavage of CTGF (Fig. 5). None of the other protease inhibitors tested had this effect. Clearly, further investigation is necessary to identify exactly which aspartic acid protease is responsible for cleaving CTGF but renin, cathepsin D and L have all been localized to the cornea.^{24,25} Interestingly, the level of cathepsin D increased in severe burns of the anterior segment of the eye after three weeks.²⁶

Other studies reported the ability of pure proteases to cleave CTGF. For example, Hashimoto et al.²⁷ reported that purified metalloproteinases (MMPs) 1, 3, 7, and 13 were able to cleave CTGF into smaller fragments when CTGF was associated with VEGF, and Guillon-Munos and co-workers²⁸ reported that the kallikrein-related peptidases KLK12 and KLK14, which are secreted serine proteases, cleaved CTGF into smaller fragments. However, both the MMP and kallikrein-related peptidase experiments utilized pure enzymes incubated with CTGF under optimal conditions for cleavage, which may or may not be relevant to physiological conditions.

Our findings in cell culture led us to evaluate the presence of CTGF in pooled (10 eyes) whole eye homogenates of normal mouse, rat, and rabbit from Pel Freeze Biological. Unexpectedly, we found 21- and 25-kDa C-terminal fragments were abundantly present, with relatively low amounts of intact 38-kDa CTGF using both the soluble fraction of the 0.1% Triton X 100 and 1% SDS protein extraction techniques. These findings were replicated in fresh eyes enucleated from rats (data not shown), indicating that the lower molecular weight forms were not due to the degradation of full length (38 kDa) CTGF after processing of the tissue by Pel Freeze Biological. Interestingly, the higher molecular weight forms (50, 38, and 31 kDa) of CTGF were absent from these Western blots of

FIGURE 9. Composite Western blot analysis of CTGF throughout a time course of wound healing after ablation from rat corneal homogenates. (A) A typical Western blot of the time course was performed to look at expression of lower molecular weight forms of CTGF using the US Biological polyclonal antibody. (B) Quantification of individual CTGF bands (38, 25, and 21 kDa) was performed from three replicates. $P < 0.05$ is indicated by *, $0.001 < P < 0.0099$ indicated by **, and $P < 0.0001$ indicated by ***.

whole eye homogenates. We postulate since CTGF has the ability to bind tightly to several growth factors, ECM and itself in cell culture media and extracts (insoluble), these higher molecular weight forms are tightly bound to the insoluble portion of the whole eye homogenates or the full length 38-kDa CTGF synthesized in normal eyes is very effectively processed to the 25- and 21-kDa fragments.

We also examined individual tissues in normal rabbit globes for CTGF forms using Western blots. Once again, we found that the 21-kDa fragment was the predominant form of CTGF in all the structures (Fig. 7), and this 21-kDa fragment was generated from the C-terminal of CTGF based on the binding by the C-terminal specific monoclonal antibody (Fig. 6). These data are consistent with Tikellis et al.²⁹ findings that normal rat retinal tissue contained a greater abundance of a 21-kDa fragment compared with the full length 38-kDa protein, whereas, in the diabetic rat model, they found an increase in the full length 38-kDa protein and a reduced abundance of the 21-kDa fragment, indicating that the full length 38-kDa form is indicative of a diseased phenotype. When comparing the different eye structures, the lens produced the 21-kDa fragment in very high abundance. CTGF expression in the lens was first documented over twenty years ago by Lee et al.,³⁰ but there has been very little published since then. Lee et al.³⁰ analyzed the expression of CTGF mRNA from lens epithelial cells of patients with anterior polar cataracts. They found increased levels of CTGF mRNA in patients who had anterior polar cataracts compared with patients who did not have anterior polar cataracts. In our study of normal ocular tissues, the 25-kDa CTGF fragment was also present in all of the individual eye structures, as identified by the C-terminal monoclonal antibody.

Other possible explanations for smaller forms of CTGF include alternative splicing of CTGF mRNA or alternative transcriptional start sites for translation of CTGF mRNA. Transcripts of other members of the CCN superfamily of proteins, specifically CCN 1, 3, and 4, have been shown to undergo alternative RNA splicing.³¹ However, 5' RACE analysis using RNAs from normal rat and mouse eyes generated only a single amplicons band of the predicted size corresponding to full length mRNA. Thus, we found that the lower molecular weight bands of CTGF found in this study were not due to alternative splicing forms of the CTGF mRNA. Similarly, in 1998, Harding et al.³² also found pig endometrium contained a single CTGF transcript of 2.4 kb and produced a 38-kDa CTGF-immunoreactive protein. Although alternative translation start sites (downstream in-frame AUG codons) are known in eukaryotic genomes, their functional significance remains controversial and are very unlikely to contribute to the large amounts of smaller CTGF proteins identified by Western blot in normal tissues or in cultured cells.³³

To better define the processing of CTGF protein during corneal wound healing, we employed Western blots to assess the expression and processing of CTGF at key time points following excimer laser ablation. The intensities of the CTGF bands in the homogenates of normal whole eyes of mouse, rat, and rabbits (Fig. 6) are much darker than the CTGF bands in homogenates of normal rabbit corneas (Fig. 7) and normal rat corneas (Fig. 9, time 0) because the homogenates of normal whole eyes includes the lens and vitreous, both of which have much higher levels of CTGF protein than found in normal cornea tissue. Similar to the findings of Blalock et al.,⁸ who measured total CTGF protein levels using ELISA, we found very

low levels of all forms of CTGF during the first 18 hours post wounding. The peak expression of all three forms of CTGF (38, 25, and 21 kDa) was 11 days post ablation. In Figure 9, the levels of CTGF are similar to those found in the pooled corneal samples of Figure 7. This reinforces the robust concentration of CTGF protein at day 11 during corneal wound healing.

In summary, processing of CTGF to lower molecular weight fragments has been documented in several tissues and cell culture systems.^{10–16,20} We report the unexpected observation that two lower molecular weight fragments (25 and 21 kDa) are the most abundant forms of CTGF in the uninjured eye. Furthermore, we identified the biologically relevant class of protease that cleaves CTGF as the aspartate acid proteases by incubating recombinant CTGF with extracts of HCE. Finally, we determined the fragmentation pattern of CTGF changes during healing of rat corneal wounds, with a peak of 38, 25, and 21 kDa forms on day 11 post injury. Future studies exploring the biological function of the lower molecular weight fragments of CTGF and the protease that cleaves it will help us determine their role in wound healing and the development of fibrosis in the eye.

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