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CTGF Is Increased in Basal Deposits and Regulates Matrix Production through the ERK (p42/p44^{mapk}) MAPK and the p38 MAPK Signaling Pathways

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Abstract

Purpose—Matrix expansion is an early change in age-related maculopathy. The aim of this study was to determine whether connective tissue growth factor (CTGF) regulates the production of extracellular matrix components by retinal pigmented epithelial (RPE) cells.

Methods—ARPE-19 cells were treated with CTGF and analyzed for fibronectin, laminin, and MMP-2 by RT-qPCR, Western blot analysis, or zymography. Cells were also pretreated with an MEK-1/2 inhibitor (PD98059) or a p38 inhibitor (SB203580) and an anti-CTGF antibody to analyze the signaling contributing to fibronectin, laminin, and MMP-2 production. Human maculas were analyzed for mRNA using laser capture microdissected RPE cells and by immunohistochemistry for the topographic distribution of CTGF.

Results—CTGF induced fibronectin mRNA ($P = 0.006$) and protein ($P = 0.006$), and laminin mRNA ($P = 0.006$) and protein ($P = 0.02$) by ARPE-19 cells. CTGF also induced MMP-2 mRNA ($P = 0.002$) and protein secretion ($P = 0.04$). Using zymography, CTGF increased the latent and active forms of MMP-2 compared to controls ($P = 0.02$). An anti-CTGF antibody inhibited fibronectin, laminin, and MMP-2 after CTGF stimulation. CTGF increased the phosphorylation of p38 and ERK1/2. Fibronectin and MMP-2 mRNA and protein were suppressed by a MEK-1/2 inhibitor, but not with a p38 inhibitor. Laminin expression was suppressed by both inhibitors. RT-qPCR analysis showed that macular RPE cells from human donors express CTGF. Immunohistochemistry of human maculas showed strong labeling of CTGF in Bruch membrane, including basal deposits and drusen.

Conclusions—CTGF is increased in basal deposits and drusen of AMD specimens, and it induces matrix protein production in ARPE-19 cells through the ERK (p42/p44^{mapk}) and p38^{mapk} signaling pathways.

Age-related macular degeneration (AMD) is the most common cause of blindness in the United States.¹ The most prominent histopathological changes involve the retinal pigmented epithelium (RPE) and Bruch membrane. With aging, Bruch membrane thickens and accumulates basal deposits. The earliest basal deposits consist mainly of normal basement membrane proteins such as laminin and fibronectin.² Thickening basal deposits are associated with AMD.³ The RPE is believed to play a primary role in the formation of basal deposits. For example, oxidative stress to the RPE increases the production of extracellular matrix components.⁴ Interestingly, RPE cells from AMD donors secrete twofold to threefold more fibronectin and MMP-2 than RPE cells from age-matched healthy donors.⁵ Understanding how

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the earliest basal deposits form before they become associated with disease represents a potential therapeutic target.

Our laboratory previously identified advanced glycation endproducts (AGEs) and oxidized apolipoprotein B100 lipoproteins in Bruch membrane of early AMD samples.^{6,7} In addition, we found that connective tissue growth factor (CTGF) and transforming growth factor beta (TGF- β) were upregulated in RPE cells exposed to either AGEs or oxidized apolipoprotein B100 lipoproteins.^{7,8} The relationship between these changes in Bruch membrane and matrix expansion during early basal deposit formation is unknown at this time. The upregulation of these cytokines is intriguing, because TGF- β appears to promote profibrotic signals through CTGF.⁹

CTGF is a 38-KDa cysteine-rich polypeptide that was originally identified from conditioned medium of human umbilical vein endothelial cells.¹⁰ The biological role of CTGF is pleiotropic and cell-type specific. In the present study, we address the hypotheses that CTGF promotes altered matrix regulation by RPE cells, and that CTGF can be localized to Bruch membrane during early AMD. We have targeted the response by RPE cells from CTGF stimulation to fibronectin, laminin, and MMP-2 production, since they are significant components of Bruch membrane.

Materials and Methods

Cell Culture and Stimulation with Recombinant CTGF

The human retinal pigment epithelial line ARPE-19 was used for experiments. ARPE-19 cells were seeded in 6-well plates and maintained in minimal essential medium (MEM; Sigma-Aldrich, Inc., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C in 5% CO₂. When cultures achieved confluence, the medium was removed and replaced with serum-free MEM containing 1% bovine serum albumin (BSA). After 36 hours of serum starvation, various concentrations of CTGF (Cell Sciences, Canton, MA) were added to the medium, and the cultures were incubated for another 24 hours for protein analyses or zymography. ARPE-19 cells were also treated with a CTGF-specific monoclonal antibody (FG-3019; a kind gift from William Usinger, FibroGen Inc., San Francisco, CA) or control human IgG1 preincubated cells for 2 hours before treatment with or without exogenous CTGF. FG-3019 is a full human IgG1 MAb recognizing domain two of human and rodent CTGF. FG-3019 was purified and formulated in a 25 mM histidine buffer (pH 6.0). To analyze downstream signaling of CTGF, cells were preincubated with a MEK-1 inhibitor (PD98059; Calbiochem, Inc., San Diego, CA), and a p38 MAPK inhibitor (SB203580; Calbiochem, Inc.), for 2 hours before treatment with exogenous CTGF.

Real-Time RT-qPCR

Total RNA was extracted using a purification kit (RNeasy Mini Kit; Qiagen Inc., Valencia, CA) from ARPE-19 cells and reverse transcribed using a cDNA synthesis kit (SuperScript II; Invitrogen, Carlsbad, CA). First strand cDNA was assayed using a real-time PCR analysis system (LightCycler; Roche Diagnostics, Nutley, NJ). Primers (QuantiTect Primer Assay; Qiagen, Valencia, CA) were used for fibronectin, laminin, and MMP-2. Primer sequences and the expected size of amplified cDNA fragments of GAPDH were 5'-CGA CCA CTT TGT CAA GCT CA-3' (sense) and 5'-AGG GGT CTA CAT GGC AAC TG-3' (antisense); 228 bp). The standard curve consisted of PCR products for the gene of interest using serial dilutions of 5×10^{-5} – 5×10^{-9} ng/ μ L. Thermo-cycling of each reaction was performed in a final volume of 20 μ L (SYBR Green PCR Master Mix; 10 μ L; Qiagen), Primer A and B (10 μ M each), and 2 μ L template DNA in a concentration of 2.5 mM MgCl₂. The cDNA was denatured at 95°C for 15 minutes followed by PCR settings of 94°C for 15 seconds, Tm-5°C for 20 seconds, and 72°C

C for x sec where x = PCR product length (bp/20). PCR products were quantified using the second derivative maximum values calculated (LightCycler analysis software; Roche Applied Science, Indianapolis, IN). Expression levels of all genes were normalized to GAPDH mRNA levels. All PCR products were checked by melting point analysis. For each sample, the experiment was repeated twice using different samples. Negative controls without template were produced for each run. The non-parametric Wilcoxon test was used to compare the differential gene expression. $P < 0.05$ was considered significant.

Western Blot Analysis

After reaching confluence, ARPE-19 cells were grown in MEM containing 1% BSA for 24 hours. After 30 minutes incubation with CTGF, the cell lysate was processed for Western blot analysis for p38 and ERK1/2 MAP kinases. After 36 hours incubation with CTGF, the supernatant for Western blot analyses of fibronectin and MMP-2 and cell lysates for laminin were collected. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed on ice with buffer (RIPA; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) containing a protease (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich). Cell extracts were then centrifuged at 15,000g for 5 minutes at 4°C, supernatants were collected, and protein content was determined using an assay reagent kit (Bradford protein assay reagent; Bio-Rad, Hercules, CA). The lysate was centrifuged and the supernatant was collected. Each sample containing 30 μ g of total protein of cell lysates or 20 μ L of supernatant was separated by SDS-PAGE and electroblotted to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). After blocking nonspecific binding with 5% skim milk, the membranes were incubated with a mouse monoclonal antibody against fibronectin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), laminin (1:200), MMP-2 (1:100), or a rabbit polyclonal antibody against p38 MAPK (1:1000), ERK1/2 (1:1000), phosphorylated forms of p38 MAPK or ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA), followed by incubation with a horseradish peroxidase-conjugated goat antibody against rabbit IgG (GE Healthcare, Buckinghamshire, UK). The signals were visualized with a kit (ECL kit; GE Healthcare) according to the manufacturer's protocol. Signals were normalized with protein concentration of cell lysates for experiments using supernatant, and to α -tubulin for experiments testing cell lysates.

Analysis of Proteinase Function by Zymography

ARPE-19 cells were seeded at a confluent density in 6-well plates and grown in MEM containing 1% BSA for 24 hours. Cells were treated with CTGF (10 or 40 ng/mL) with or without FG-3019 (10 μ g/mL) in serum free medium. After 36 hours of treatment, 500 μ L of conditioned medium was concentrated tenfold using 30K spinning devices (Millipore, Billerica, MA) for 10 minutes at 14,000g. The concentrated conditioned medium was used for zymography. Samples were electrophoresed on a 10% tris-glycine SDS-polyacrylamide gel with 1% gelatin (Novex, San Diego, CA). After electrophoresis, the gel was incubated in buffer (Novex Zymogram Renaturing Buffer; Novex) with gentle agitation for 30 minutes at room temperature. Another buffer (Zymogram Developing Buffer; Novex) was used after the renaturation, and the gel was incubated overnight at 37°C in this buffer. Gels were stained with Coomassie blue.

Human Tissue Processing

The protocol in this study adhered to the tenets of the Declaration for Helsinki for research involving human tissue. Globes from 42- to 95-year-old donors were obtained from NDRI (Philadelphia, PA) within 6 hours of death, and were on life support for <24 hours (Table 1). Macular calottes were fixed for 1 hour in 2% paraformaldehyde and then cryoprotected by progressive infiltration in 10% and 20% sucrose in PBS (w/v) before freezing in 2:1 sucrose

20% (w/v):OCT compound at -80°C . Sections used for laser capture microdissection were not fixed, but were instead cryoprotected with sucrose infiltration, and then frozen. AMD was defined by a clinical history of AMD and the presence of basal deposits thicker than the normal height of an RPE cell (i.e., $>8\text{ }\mu\text{m}$) in macular sections, using our previously published criteria that is based on the work of Sarks.^{11,12}

Laser Capture Microdissection

Cells of interest on $7\text{ }\mu\text{m}$ cryosections were dissected with a laser capture microdissector (Arcturus PixCell II; Arcturus Engineering, Inc., Mountain View, CA) using transfer film (Cap-Sure TF-100; Arcturus Engineering) according to our previously published protocol.¹³ Morphologically normal macular RPE cells were defined using the criteria established by Curcio et al.¹⁴ and Sarks.¹¹ Specifically, normal macular RPE were defined as having regular cuboidal-columnar cell shape, homogeneous melanin pigmentation, a height estimated at 10 to $15\text{ }\mu\text{m}$ (using the $7.5\text{ }\mu\text{m}$ and $15\text{ }\mu\text{m}$ spot size of the laser aiming beam), and attached to non-thickened Bruch membrane, defined as $<1/4$ RPE cell height.¹³ After dissection, the transfer cap was inspected with the microscope for contaminating tissue, which verified a cleavage plane at the RPE-Bruch membrane junction, before being placed in $70\text{ }\mu\text{L}$ denaturing buffer that contained 4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarcosyl, and $2\text{ }\mu\text{L}$ β -mercaptoethanol (14.5 M; Qiagen).

For RT-qPCR analysis, total RNA was extracted from laser captured RPE cells (RNeasy Micro Kit) and treated with DNase I (both from Qiagen) during RNA purification. A sample of RPE cells was obtained from a peripheral calotte that was not used for RT-qPCR analysis from each donor which showed preserved 28S and 18S rRNA bands. RNA quality was also assessed by the expression of GAPDH from 100 cells using real time RTPCR with primers designed at the 5' end of the gene, as we have previously described.¹³ Total RNA from the equivalent of 200 laser captured RPE cells was reverse transcribed using a cDNA synthesis kit (SuperScript II; Invitrogen) in the presence of protein (T4gene32 protein; Ambion Inc., Austin, TX) to enhance first strand cDNA production.¹⁵ First strand cDNA was assayed (LightCycler apparatus; Roche Diagnostics) as described above. The primer sequences and the expected size of amplified cDNA fragments of CTGF were 5'-GCA GGC TAG AGA AGC AGA GC-3' (sense) and 5'-ATG TCT TCA TGC TGG TGC AG-3' (antisense; 153 bp).

Immunohistochemistry

Immunohistochemistry was performed as previously reported.¹⁶ In brief, $7\text{ }\mu\text{m}$ cryosections were blocked with 2% normal goat serum and an avidin-biotin complex (ABC) blocking kit (Vector Laboratories, Inc., Burlingame, CA), incubated overnight at 4°C with a polyclonal rabbit anti-CTGF antibody (a gift from FibroGen, Inc.) or rabbit IgG. Sections were incubated for 30 minutes at room temperature with rat biotinylated secondary antibody (1:1000), streptavidin APase (1:500), and APase activity was developed with a 5-bromo-4-chloro-3-indoyl phosphate (BCIP)-NBT kit (Vector Laboratories, Inc.), which gives a violet-blue color.

Results

Stimulation of Fibronectin and Laminin by CTGF in ARPE-19 Cells

CTGF has an established role in regulating the extracellular matrix. Fibronectin and laminin have been identified in early basal deposits.^{2,5,17,18} To determine whether CTGF activates fibronectin and laminin, ARPE-19 cells were treated with various concentrations (1, 10, 100 ng/mL) of CTGF for up to 24 hours to analyze the production of fibronectin and laminin by RT-qPCR and Western blot analyses. CTGF (10 ng/mL) induced fibronectin mRNA expression by 2.3-fold ($P = 0.006$), while laminin was induced 1.9-fold ($P = 0.006$) after 3 hours of exposure (Fig. 1A). Figure 1C shows that CTGF treatment also increased fibronectin

protein secreted by ARPE-19 cells after 24 hours. Similar results were seen for CTGF induction of laminin mRNA (Fig. 1B; $P = 0.006$ for 10 ng/mL, $P = 0.03$ for 100 ng/mL) and protein (Fig. 1D; $P = 0.02$). We next treated ARPE-19 cells with CTGF and a CTGF-specific monoclonal antibody FG-3019 or control human IgG1. Blockade of CTGF by FG-3019 inhibited CTGF-induced mRNA expression (Fig. 2A, 2B) and protein production (Fig. 2C, 2D) of fibronectin (Fig. 2A, $P = 0.02$; Fig. 2C, $P = 0.02$) and laminin (Fig. 2B, $P = 0.02$; Fig. 2D, $P = 0.03$).

Stimulation of MMP-2 by CTGF in ARPE-19 Cells

MMP-2 is a known target of CTGF in other cell types,^{19,20} and has been identified as protease important for regulating Bruch membrane.²¹ To examine whether MMP-2 is induced, ARPE-19 cells were treated with CTGF and examined for mRNA and protein induction. CTGF (10, 100 ng/mL) induced MMP-2 mRNA expression by 1.5- and 1.8-fold, respectively ($P = 0.007$, 0.002; Fig. 3A). MMP-2 protein secreted into the supernatant by ARPE-19 cells, as assessed by Western blot analysis, was also significantly increased by CTGF treatment ($P = 0.04$ for 10 ng/mL; $P = 0.02$ for 100 ng/mL; Fig. 3B). FG-3019 inhibited MMP-2 expression after stimulation with CTGF (Fig. 3C, $P = 0.02$; Fig. 3D, $P = 0.004$). Since TIMP-2 is an inhibitor of MMP-2,²² its expression was examined after CTGF stimulation. Using the identical conditions as described above, there was no significant change in the mRNA expression of TIMP-2 (data not shown).

To determine whether secreted MMP-2 is functionally active, we performed gelatin zymography. ARPE-19 cells grown under serum free conditions showed two proteins with a molecular weight of 65 and 58 kDa, corresponding to the latent and active form of MMP-2. Stimulation with CTGF at doses of 10 and 40 ng/mL showed a significant increase in both bands in comparison to vehicle controls (Fig. 4; $P = 0.02$).

Activation of p38 MAPK and ERK1/2 by CTGF in ARPE-19 Cells

Some of the biological effects of CTGF are mediated by activation of the MAPK signaling pathway in certain cell types. The signaling pathway of CTGF in RPE cells is unknown. We examined the levels of p38 MAPK and ERK1/2 MAPK proteins in ARPE-19 cells treated with CTGF (10 or 40 ng/mL) from 5 to 90 minutes of stimulation, by Western analyses using anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK1/2, and antiphospho-ERK1/2 antibodies. No significant differences at these chosen time points were seen (data not shown). ERK1/2 activation is typically seen at 30 minutes in other cell types,^{23,24} and TGF- β , an upstream molecule of CTGF, activates ERK1/2 and p38 30 minutes after stimulation.²⁵ We therefore evaluated MAPK signaling 30 minutes after CTGF stimulation. Exogenous CTGF at these doses increased the phosphorylation of p38 (Fig. 5A; $P = 0.02$) and ERK1/2 (Fig. 5B; $P = 0.02$) 30 minutes after treatment.

Effect of MAPK Inhibitors on Fibronectin, Laminin, and MMP-2 Production

To determine whether the MAPK pathway is involved in mediating CTGF-induced fibronectin, laminin, and MMP-2 expression, ARPE-19 cells were treated with CTGF and a specific inhibitor of p38 MAPK (SB203580; 20 μ M) or ERK1/2 (PD098059; 20 μ M). Figure 6 shows that at these doses, the inhibitors were specific. mRNA (Fig. 7A, 7C) and protein (Fig. 7D, 7F) expression of fibronectin (Fig. 7A, $P = 0.02$; Fig. 7D, $P = 0.02$) and MMP-2 (Fig. 7C, $P = 0.02$; Fig. 7F, $P = 0.004$) were suppressed by the MEK-1/2 inhibitor, but not by the p38 MAPK inhibitor. Laminin expression was suppressed by both the MEK-1 inhibitor (Fig. 7B, $P = 0.006$; Fig. 7E, $P = 0.02$) and the p38 MAPK inhibitor (Fig. 7B, $P = 0.04$; Fig. 7E, $P = 0.04$).

Immunohistochemical Evidence of CTGF in Human Bruch Membrane of Early AMD

CTGF is a secreted growth factor. To determine whether CTGF localizes to Bruch membrane, postmortem eyes without AMD ($n = 17$) including seven from donors under 52 years old, and with early AMD ($n = 13$), were used to evaluate the topographic distribution of CTGF in the RPE-Bruch membrane-choroid. In the donors under 52 years old, light staining was seen in Bruch membrane and the choroid (Fig. 8). The staining intensity for CTGF appeared relatively stronger in the older than younger age set. In eyes with AMD, CTGF staining was prominent in Bruch membrane and the choroid. Staining was seen in the RPE basement membrane, basal deposits, drusen, and the choriocapillaris basement membrane of all eyes with AMD (Fig. 8).

Expression of CTGF by RPE Cells In Vivo

To provide evidence that RPE cells in situ express CTGF, the RPE from macular samples of three donors (Table 1) were laser capture microdissected and evaluated by RT-qPCR for CTGF mRNA expression. RPE cells were selected based on their normal cuboidal morphology and attachment to unthickened Bruch membrane. All three samples expressed CTGF. Relative to GAPDH, the relative expression was 0.002 ± 0.002 arb units.

Discussion

Changes to Bruch membrane with aging and early AMD include increased thickness due to net matrix expansion and the development of basal deposits. Previous histopathologic studies have delineated a progression from matrix expansion of Bruch membrane components to an accumulation of heterogeneous material, known as basal deposits in early disease.^{2,5,17,18} Some of the changes that are important during this progression are the accumulation of advanced glycation endproducts and apolipoprotein B100 lipoproteins.^{6,26–28} Our laboratory also has identified oxidized apolipoprotein B100 lipoproteins in basal deposits during early AMD.⁷ In several cell types, low density lipoproteins, which appear to have similarity to the lipoproteins recovered from Bruch membrane, induce CTGF.^{29,30} In RPE cells, we have shown that TGF- β and CTGF are increased after exposure to either AGEs or oxidized apoB100 lipoproteins.^{7,8} Our immunohistochemical investigation here shows that CTGF accumulates in basal deposits and Bruch membrane of early AMD. Furthermore, our laser capture microdissection experiments show that RPE cells make CTGF transcripts. We therefore conducted a series of experiments to determine whether increased CTGF has any functional consequences related to the RPE cell's influence on its extracellular matrix.

Our in vitro results start to unravel the signaling pathways after CTGF stimulation. Our experiments were conducted in the established ARPE-19 cell line. Although this is a popular cell line due to its ability to simulate the RPE in vivo,^{31–33} we acknowledge that the results obtained here may not be generalizable to all RPE cells or to the in vivo environment. In this study, we show that CTGF is linked to induction of the major matrix components fibronectin and laminin, and the protease MMP-2 by ARPE-19 cells. Interestingly, CTGF did not affect the expression of TIMP-2, a major regulator of MMP-2. Furthermore, the increased production induced by CTGF was abrogated by neutralizing CTGF antibodies. Both fibronectin and laminin have been found in early basal laminar deposits.^{2,5,17,18} At present, it is unclear whether the increased deposition results from increased matrix protein synthesis or faulty degradation. The induction of fibronectin and laminin by CTGF suggests increased production. The increased synthesis and activity of MMP-2 suggests increased matrix turnover. We are unsure how this finding translates to the in vivo environment. Given increased oxidative- and AGE-cross-linking of Bruch membrane with aging, it is possible that MMP-2 is increased and enzymatically active, but unable to degrade aging-related modifications to matrix proteins. This suggestion is supported by Mott et al., who have shown that AGE-modification to collagen IV inhibits MMP3 and MMP9.³⁴ The net result would be matrix expansion.

Increased MMP-2 can have another potential undesired effect on the RPE. Liu et al. have shown that CTGF-induced increased MMP-2 causes deadhesion and apoptosis through the mitochondrial intrinsic pathway of retinal pericytes.³⁵ These authors cited three potential mechanisms for cell death, including MMP-2-induced degradation of cell survival fragments contained in matrix proteins, the release of pro-apoptotic fragments, or reduced adhesion resulting from matrix deposition by MMP-2. With the defined role of RPE cell apoptosis in early AMD,^{36,37} investigating the role that CTGF and MMP-2 have on RPE cell survival appears warranted in the future.

Although a specific CTGF receptor has not yet been identified, CTGF appears to perform many of its functions through integrins, heparin sulfate-containing proteoglycans, and the low-density lipoprotein receptor-related protein (LRP).³⁸ In chondrocytes, for example, CTGF regulates extracellular matrix, and both the expression and signaling properties of integrin $\alpha 5$ via ERK1/2 signaling.³⁹ CTGF activates the MAPK signaling pathway after binding to the lipoprotein receptor-related protein (LRP) via a heparan sulfate proteoglycan dependent process.^{40,41} In renal myofibroblasts, CTGF induces tyrosine phosphorylation of the cytoplasmic domain of LRP and activates the ERK1/2 signaling pathway.^{24,42} The MAPK are a family of serine-threonine protein kinases that are activated by a number of extracellular stimuli. ERK (p42/44^{mapk}), p38^{mapk}, and JNK are three major subfamilies of MAPK that mediate downstream effects such as cellular proliferation, differentiation, and apoptosis through activation of appropriate transcription factors.^{43,44} Since activation of the MAPK pathways are dependent in part on the cell type, we performed experiments to determine whether any of these pathways were involved in matrix regulation resulting from CTGF stimulation of ARPE-19 cells by either inhibiting CTGF with either a neutralizing anti-CTGF antibody or validated blocking agents of these pathways after CTGF stimulation, and evaluating the production of fibronectin, laminin, and MMP-2 as a functional outcome. Our experiments support a role for both the ERK (p42/p44^{mapk}) and p38^{mapk} signaling pathways since we demonstrated phosphorylation of ERK1/2 and p38^{mapk} by Western blot analysis after exogenous CTGF stimulation of ARPE-19 cells. In addition, the specific pathway was clarified using specific inhibitors of the MAPK pathway. After stimulation with CTGF, the expression of fibronectin and MMP-2 were suppressed by the ERK1/2 inhibitor, PD98059, but not by the p38^{mapk} inhibitor, SB203580. These results suggest that CTGF induces signaling through the ERK1/2 pathway to increase fibronectin and MMP-2 production. On the other hand, laminin appears to be regulated through both the ERK1/2 and p38^{mapk} pathways since induction after CTGF stimulation was suppressed by both inhibitors. These results indicate crosstalk between these two signaling pathways. Yosimichi et al. have found that inhibition of one pathway can induce the induction of the other pathway.⁴⁵

As our understanding of genetic susceptibility of AMD increases, it will be important to integrate functional changes with these genetic abnormalities. In this regard, the association of the polymorphism in the serine protease HTRA1 with AMD is of interest.^{46–48} HTRA1 is known to degrade fibronectin.⁴⁹ In addition, HTRA1 binds to and inhibits TGF- β .⁴⁹ Thus, a sequence variation in HTRA1 could either inhibit fibronectin breakdown and promote matrix accumulation directly, or increase TGF- β and its downstream modulator CTGF, thereby increasing the matrix.

Besides its profibrotic activity, CTGF was also thought to initiate angiogenesis because recent studies have found CTGF overexpression in CNV of AMD patients.^{50,51} However, Kuiper et al. recently found that CTGF played a negligible role in CNV development using a CTGF knockout mouse.⁵² The data from our work suggest a role for CTGF as a profibrotic mediator through activation of the ERK and p38^{mapk} signaling pathways, to induce matrix proteins such as fibronectin and laminin, as well as MMP-2. Further work clarifying its exact role in matrix expansion might lead to a new therapeutic approach to preventing the onset of early AMD.

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Figure 1.

Stimulation of fibronectin and laminin expression in ARPE-19 cells by CTGF. (**A**, **B**) RT-qPCR analyses show significant mRNA increase of fibronectin and laminin expression in CTGF-treated ARPE-19 cells. Statistically significant differences compared with the control are indicated. (**C**, *top*) Representative Western blot showing CTGF significantly increased protein levels of fibronectin in the supernatant of ARPE-19 cells. Fibronectin signal was normalized to cell lysate protein. (**D**) Representative Western blot showing increased laminin in cell lysates of ARPE-19 cells treated with CTGF. Production was normalized to α-tubulin. Statistically significant differences from controls are indicated.

Figure 2. Blockade of CTGF by FG-3019 inhibited CTGF-induced mRNA expression (**A, B**) and protein production (**C, D**) of fibronectin (**A, C**) and laminin (**B, D**). Statistically significant differences compared with the control (IgG1) are indicated.

Figure 3.

Stimulation of MMP-2 expression in ARPE-19 cells by CTGF. (A) RT-qPCR analyses show significant increase of MMP-2 expression in CTGF-treated ARPE-19 cells. Statistically significant differences, compared with the control. (B, top) Western blot from a representative experiment. CTGF significantly increased protein levels of MMP-2 in the supernatants of ARPE-19 cells. (C, D) Blockade of CTGF by FG-3019 inhibited CTGF-induced mRNA expression (C) and protein production (D) of MMP-2.

Figure 4. Stimulation of MMP-2 activation in ARPE-19 cells by CTGF. MMP-2 protein activity was evaluated by zymography in presence of 10 or 40 ng/mL CTGF for 36 hours. *Top*: gelatin zymogram from a representative experiment. *Bottom*: statistically significant differences ($P = 0.02$), compared with the control.

Figure 5.

Activation of p38 (**A**) and ERK1/2 (**B**) MAP kinases by CTGF in ARPE-19 cells. *Top*: Western blot for phosphorylated and total levels of p38 and ERK1/2 in the ARPE-19 cells. *Bottom*: the graphs show the ratios of phosphorylated to total p38 (**A**) and ERK1/2 (**B**). p38 and ERK1/2 were significantly activated by CTGF. Statistically significant differences ($P = 0.02$) are seen, compared with controls.

Figure 6. Representative Western blot showing specific inhibition by (A) p38 MAPK (SB203580; 20 μ M, designated SB) of phosphorylated p38 (p-p38), and (B) ERK1/2 (PD098059; 20 μ M, designated PD) inhibition of phosphorylated ERK1/2 (p-ERK1/2) of ARPE-19 cells stimulated with CTGF.

Figure 7.

Inhibition of fibronectin (**A**, **D**), laminin (**B**, **E**), and MMP-2 (**C**, **F**) expression by an ERK1/2 inhibitor. (**A–C**) RT-qPCR analyses show significant suppression of fibronectin (**A**), laminin (**B**), and MMP-2 (**C**) expression by the MEK-1 inhibitor, PD98059. (**D–F**) *Top*: Western blot from a representative experiment. *Bottom*: graphical representation of differences compared with the vehicle control. Statistically significant differences are indicated. PD, ERK1/2 inhibitor PD098059; SB, p38 MAPK inhibitor SB203580.

Figure 8.

Immunohistochemistry of CTGF in human maculas. (A, B) A 78-year-old female with AMD shows strong labeling for CTGF throughout Bruch membrane (BrM). Strong immunolabeling is seen in the RPE basement membrane (*long arrows*), basal deposits (*short arrows*), a druse (*), and the choriocapillaris basement membrane. The choroid also shows staining for CTGF. (C) A 42-year-old female with no history of AMD shows light immunostaining of Bruch membrane and the choroid. (D) IgG control of the 78-year-old female shown in (A, B). Scale bar, 15 μ m.

Table 1

Donor Characteristics

Patient	AMD	Age (years)	Sex	Death to Enucleation (hours)	Cause of Death
<i>Immunohistochemistry</i>					
1	No	42	F	4:05	Encephalopathy
2	No	46	M	4:12	Cardiac arrest
3	No	46	M	4:10	Motor vehicle accident
4	No	47	F	5:31	Metastatic rectal cancer
5	No	49	F	5:08	Ovarian cancer
6	No	50	M	7:55	Congestive heart failure
7	No	51	M	5:53	Aneurism
8	No	67	M	4:05	Unknown
9	No	80	M	2:44	Pneumonia
10	No	80	F	4:54	Dysphagia
11	No	83	M	3:38	Lung cancer
12	No	86	M	4:26	Stroke
13	No	86	F	3:22	Lung cancer
14	No	89	M	2:55	Prostate cancer
15	No	90	M	2:45	Stroke
16	No	95	M	4:42	Leukemia
17	No	95	M	5:05	Congestive heart failure
18	Yes	70	F	6:10	Lung cancer
19	Yes	77	M	2:58	Myocardial infarction
20	Yes	78	F	3:55	Cardiomyopathy
21	Yes	81	M	2:52	Stroke
22	Yes	81	M	3:00	Pleural effusion
23	Yes	85	F	2:28	Multiorgan failure
24	Yes	85	M	3:25	Respiratory failure
25	Yes	87	F	3:16	Myocardial infarction
26	Yes	87	F	5:43	Metabolic acidosis
27	Yes	90	F	2:15	Stroke
28	Yes	93	F	5:46	Renal failure

Patient	AMD	Age (years)	Sex	Death to Enucleation (hours)	Cause of Death
29	Yes	94	F	5:35	Cardiac arrest
30	Yes	95	F	5:01	Unknown
<i>Laser Capture Microdissection and RT-qPCR</i>					
1	No	74	M	3:12	Emphysema
2	No	82	M	3:00	Malignant melanoma
3	No	82	M	2:40	Pneumonia