A Role for Connective Tissue Growth Factor in the Pathogenesis of Choroidal Neovascularization

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Objective: To evaluate the expression of connective tissue growth factor (CTGF) in choroidal neovascular membranes from patients with age-related macular degeneration and the effect of CTGF on choroidal endothelial cell (CEC) function.

Methods: Using immunohistochemical methods, we analyzed CTGF expression in 13 surgically excised choroidal neovascular membranes related to age-related macular degeneration. The expression of CTGF in retinal pigment epithelial and CEC cultures was determined by means of reverse transcriptase polymerase chain reaction and Western blot, and its regulation by vascular endothelial growth factor and transforming growth factor β was determined. The effects of CTGF on bovine CEC proliferation, attachment, migration, and tube formation were measured.

Results: Vascularized human choroidal neovascular membranes showed strong CTGF immunoreactivity. Double staining disclosed colocalization of CTGF with retinal pigment epithelial cells and CECs. The CTGF induced a significant increase in attachment and migration of CECs; however, it did not stimulate CEC proliferation. The CTGF protein was up-regulated in retinal pigment epithelial cells and CECs by stimulation with transforming growth factor β and vascular endothelial growth factor, respectively.

Conclusions: The expression of CTGF in choroidal neovascular membranes, its regulation by angiogenic growth factors, and its proangiogenic effects on CEC function suggest that CTGF may play a role in the pathogenesis of choroidal neovascularization.

Clinical Relevance: Multiple growth factors are involved in the pathogenesis of choroidal neovascularization in age-related macular degeneration.

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cally study CECs, since endothelial cell heterogeneity is well described in vivo and in vitro, and there are marked functional differences between CECs and endothelial cells from other regions.22-25

In the present study, we show that CTGF expression is present in CNVMs of patients with age-related macular degeneration. We then demonstrate that CTGF mRNA is expressed in RPE cells and CECs and that the production of CTGF protein is up-regulated in RPE cells and CECs by stimulation with TGF-β and VEGF, respectively. Furthermore, we show that CTGF induces proangiogenic responses in CECs in vitro.

**METHODS**

**IMMUNOHISTOCHEMICAL STAINING**

Thirteen surgically excised subfoveal CNVMs from patients with age-related macular degeneration (aged 66-85 years) and 3 normal adult postmortem donor retinas (from the Lions Doheny Eye Bank, aged 55-97 years) were prepared for immunostaining. The study was approved by the institutional review board of the University of Southern California, Los Angeles. Five of the membranes were predominantly vascular, 3 were predominantly fibrotic, and 3 were mixed in appearance. Tissues were snap frozen and sectioned at 6 µm with the cryostat. Thawed tissue sections were air dried, rehydrated with phosphate-buffered saline (pH 7.4), and blocked with 3% normal goat serum for 15 minutes. Sections were incubated with anti-CTGF polyclonal antibody (FibroGen, Inc, South San Francisco, Calif) for 60 minutes and then treated with biotinylated secondary anti-rabbit antibody (1:400; Vector Laboratories, Burlingame, Calif) followed by streptavidin peroxidase. The red color was developed with an aminoethyl carbazole kit (Zymed Laboratories, Inc, South San Francisco). After each step, sections were given three 5-minute washes with phosphate-buffered saline. Slides were counterstained with hematoxylin and mounted with glycerin-gelatin medium. Controls for the immunoperoxidase studies included (1) staining of adjacent tissue sections by means of an identical protocol except that the primary antibody was omitted and (2) staining of adjacent tissue sections with an identical protocol with the use of antiserum preadsorbed with recombinant CTGF. In both controls, negligible background or non-specific staining was found (Figure 1 C and F).

**DOUBLE IMMUNOPEROXIDASE AND ALKALINE PHOSPHATASE STAINING**

Thawed tissue sections were fixed for 10 minutes with 10% neutral buffered formalin (Polysciences, Inc, Warrington, Pa), given three 5-minute washes with Tris hydrochloride buffer (pH 7.4), and blocked with 3% goat serum for 15 minutes. After cells were immunostained for CTGF as described above, the slides were washed with the Tris buffer 3 times. Primary antibodies were added to cover the tissue, and the slides were incubated for 1 hour at room temperature. The sections were washed 3 times with Tris buffer, and secondary alkaline phosphatase antibody (Vector Laboratories) was added for 30 minutes. After another triple wash with Tris buffer, an alkaline phosphatase chromogen substrate (Vector Blue; Vector Laboratories) was added to the slides for 15 minutes for color development.

**ISOLATION OF RPE CELLS AND CECs**

Human RPE cells were obtained from human fetal eyes (18-26 weeks of gestation; Advanced Bioscience Resources, Inc, Alameda, Calif).26 Second- through fourth-passage cells were used for all experiments. The RPE cells were cultured in Dulbecco modified Eagle medium (Fisher Scientific, Pittsburgh, Pa) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md), 2mM glutamine, 100-µg/mL streptomycin, and 100-U/mL penicillin (Sigma-Aldrich Corp, St Louis, Mo). The purity of cultured RPE cells was confirmed by means of immunocytochemical staining; more than 95% of cells were positive for cytokeratin (DAKO, Carpinteria, Calif), while no cells were positive for the endothelial cell antigen von Willebrand factor (DAKO) or macrophage antigen C11c (DAKO). Magnetic beads carrying the specific endothelial marker Lycopersicon esculentum (Sigma-Aldrich Corp) were used to isolate CECs from bovine eyes, as previously described.27 Positive immunostaining for von Willebrand factor and binding of diacetylated low-density lipoprotein confirmed that the CECs were vascular endothelial cells. Although it is likely that the distinct functional characteristics of CECs may be lost after prolonged passage, previous work has shown that low-passage endothelial cells of diverse origin retain functional and structural differences.28 In our experiments, all CECs were in passage 2 or 3.

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION**

PolyA (A)+RNA was isolated by means of a kit (Fast Track; Invitrogen Corp, Carlsbad, Calif). First-strand complementary DNA was synthesized at 42°C with Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The polymerase chain reaction was initiated in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, Conn) with CTGF primer pairs (human CTGF: GCCATCGGTACTCTTAAATC [antisense] and CTGTCTCCATACCTCCGGGT [sense]; bovine CTGF: GATCATAGGACGGTATTAGTG [sense] and CTGACTTGAAGAGCAACCTTG [antisense]). After 30 cycles of polymerase chain reaction, the products were resolved on a 1.2% agarose gel and stained with ethidium bromide, and the gel was photographed under ultraviolet illumination.

**WESTERN BLOT ANALYSIS**

The RPE cells and CECs were grown in 6-well plates and starved for 24 hours in Dulbecco modified Eagle medium or essential growth media (BioWhittaker, Walkersville, Md) with 0.1% bovine serum albumin. The medium was then removed and replaced with fresh medium. For stimulation studies, the CEC replacement medium included 1% fetal bovine serum and recombinant VEGF (10-50 ng/mL); for RPE cells, the medium was serum-free and included TGF-β recombinant VEGF (1-30 ng/mL). Lysed cells were collected after 48 hours of additional incubation; proteins were resolved on Tris hydrochloride 10% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Hercules, Calif) at 120 V, with 8 µg of protein added to each lane. The proteins were transferred to a polyvinylidene fluoride blotting membrane (Millipore, Billerica, Mass); these membranes were then probed with polyclonal anti-CTGF antibody (FibroGen, Inc), followed by horseradish peroxidase–conjugated goat anti-rabbit antibody (Vector Laboratories) for 30 minutes at room temperature. Images were developed by adding chemiluminescence detection solution (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

**ATTACHMENT AND MIGRATION ASSAYS**

The RPE cells or CECs (10⁴/mL) were trypsinized and resuspended in cell culture medium with 0.4% fetal bovine serum. After a 48-hour treatment with CTGF, 100 mL of cell suspension (10⁴ cells) was added to each well of a 96-well fibronectin-coated plate (Becton, Dickinson Labware, Bedford, Mass), and cells were allowed to attach for 60 minutes.26 The cells were gently washed twice with phosphate-buffered saline, and fresh

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medium (150 mL) was added to each well with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 5 mg/mL, 20 mL (Sigma-Aldrich Corp). After a 5-hour incubation, the supernatants were decanted; the formazan precipitates were solubilized by adding 150 mL of 100% dimethyl sulfoxide (Sigma-Aldrich Corp) and placed on a plate shaker for 10 minutes. Absorbance at 550 nm was determined on a microplate reader (Benchmark Microplate Reader; Bio-Rad Laboratories).

Migration of RPE cells and CECs was measured by means of a modified Boyden chamber assay in 24-well plates with fibronectin-coated inserts. Recombinant CTGF, 10 to 50 ng/mL (FibroGen, Inc), was added to the lower chamber as the stimulant. After a 5-hour incubation, the inserts were washed 3 times with phosphate-buffered saline, fixed with cold (4°C) methanol for 10 minutes, and counterstained with hematoxylin for 20 minutes. The number of migrated cells was counted by means of phase-contrast microscopy (×320). Four randomly chosen fields were counted per insert.

CELL PROLIFERATION ASSAYS
Two methods of measuring cell proliferation were used. Subconfluent CECs grown in 6-well plates were treated with recombinant CTGF (0, 1, 10, 50, and 100 ng/mL) for 48 hours. Cell proliferation was measured by cell counting of representative triplicate samples with a hemocytometer, and by tritiated thymidine uptake assay as previously described.

CEC TUBE FORMATION
The CECs were subcultured on fibronectin-coated, 6-well plates. The original medium was replaced with essential growth media containing 1% fetal bovine serum and 20-ng/mL CTGF, and cells were incubated for 9 days. Tube formation was monitored every day by phase-contrast microscopy.

RESULTS
CTGF EXPRESSION IN HUMAN CNVM
All 13 CNVMs stained positively for CTGF, with the most prominent staining found in the vascularized regions of the membranes (Figure 1A). Portions of the intact RPE monolayer were found in 5 membranes; occasional RPE in these regions stained strongly positive for CTGF.
Cultures of both human RPE and bovine CECs expressed CTGF (Figure 3B). In CECs, VEGF stimulation had no effect on CTGF expression (Figure 3A), with the greatest effect at 1 and 20 ng/mL. Stimulation of CECs with CTGF (10-100 ng/mL) for 48 hours also increased attachment of the cells to fibronectin (Figure 3D). In contrast, culture of CECs with TGF-β2 did not stimulate expression of CTGF in CECs (Figure 3C).

**EFFECT OF CTGF ON CEC FUNCTION**

The migration of CECs in the modified Boyden chamber assay was stimulated by recombinant CTFG (Figure 4). The stimulation was concentration dependent, with the maximal response to CTGF at a concentration of 30 ng/mL in the medium (P < .05). Interestingly, the maximal CTGF response was as effective as VEGF (10 ng/mL) in stimulating CEC migration. The dose of VEGF shown is submaximal and the CTGF effect is significantly less than a maximal VEGF stimulation. Stimulation of CEC with CTGF (10-100 ng/mL) for 48 hours also increased attachment of the cells to fibronectin (P < .05) (Figure 5). The effect was dose dependent, with a maximal effect at 50 to 100 ng/mL. Stimulation of CECs with CTFG (1-100 ng/mL) for 48 hours produced no proliferative response as measured by thymidine uptake or by cell counting (results not shown). When CECs were plated on fibronectin for 9 days in the presence of 1% serum, there was minimal, if any, tube formation (Figure 6A). In the presence of CTGF, 20 ng/mL, the attached endothelial cells extended cell processes, formed cell-cell contact, and established a branched network of tubelike structures (Figure 6B).

**COMMENT**

Growth factors play an important role in the development, progression, and regression of pathological angiogenesis. In choroidal neovascularization, studies have demonstrated the presence of a complex mixture of angiogenic and antiangiogenic molecules, whose interactions likely result in the neovascular response. In this study, we demonstrate that our series of CNVMs uniformly express CTGF and that this
expression is most prominent in the vascular regions of the membrane. The expression of CTGF is localized to stromal RPE and CECs as well as RPE in the residual monolayer. The pattern of immunoreactivity is similar to that seen previously for VEGF and provides support for the central role of RPE and their secreted growth factors in the pathogenesis of the disorder.9 No CTGF immunoreactivity was found in the RPE or CECs of normal adult retinas, consistent with previous reports that CTGF is undetectable in normal vessels,30,31 but high expression levels are found in endothelial cells of atherosclerotic lesions and other angiogenic sites.32 The presence of CTGF in the residual RPE monolayer of CNVMs suggests the possibility that CTGF has the potential to activate normal underlying choroidal endothelium.

This article demonstrates for the first time, to our knowledge, that RPE and CECs each express CTGF mRNA and protein in vitro. There is considerable evidence that CTGF is a major downstream effector of TGF-β action in many cell types.15,16,33-34 and TGF-β clearly up-regulates CTGF protein expression in RPE. The effect of TGF-β on endothelial cell subtypes, however, is more controversial. The expression of CTGF is up-regulated by TGF-β in peri-aortic, omental, and retinal endothelial cells, but not pulmonary or aortic endothelial cells.33 In this study, we saw no increase in CTGF protein when bovine CECs were stimulated with TGF-β. The mechanism of the differential response to TGF-β in various cell types has not been defined; however, induction of CTGF by TGF-β in fibroblasts is largely at the level of transcription initiation.36

The regulation of CTGF by VEGF in endothelial cells is also controversial. Although VEGF up-regulates CTGF in bovine retinal endothelial cells, there was no such up-regulation in retinal vascular endothelial cells from Macaca mulatta.20,22 Our results clearly show that VEGF stimulation of bovine CEC results in increased CTGF protein expression. The possibility that other factors play a role in stimulating CTGF expression should also be considered, since CTGF has also been shown to be regulated by advanced glycosylation end products, mechanical stress, or hypoxia.37-39

In this report, we show that CTGF stimulates CEC migration, adhesion to fibronectin, and tube formation. One of the characteristic features of CTGF is its induction of a fibrotic response, typified by the increased pro-
duction of extracellular matrix molecules by fibroblasts.\textsuperscript{33} In support of this possibility, CTGF mRNA was recently demonstrated by in situ hybridization in transdifferentiated RPE cells in association with type 1 collagen in an epiretinal fibrovascular membrane.\textsuperscript{40}

Our results demonstrate that CTGF is expressed by RPE and CECs, and that CTGF has the potential to stimulate choroidal angiogenesis through regulation of CEC function. These results, together with the observation that CTGF is prominently expressed in CNVM, suggest that CTGF may participate in the pathogenesis of CNVM in age-related macular degeneration.

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