Suppression of Epithelial-Mesenchymal Transition in Retinal Pigment Epithelial Cells by an MRTF-A Inhibitor

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PURPOSE. Epithelial-mesenchymal transition (EMT) in retinal pigment epithelial (RPE) cells is related to the pathogenesis of subretinal fibrosis such as that associated with macular degeneration. The role of myocardin-related transcription factor A (MRTF-A) in EMT of RPE cells and subretinal fibrosis was investigated.

METHODS. The migratory activity of human RPE-1 cells in culture was evaluated using a scratch assay. The subcellular distribution of MRTF-A in RPE-1 cells, as well as the extent of subretinal fibrosis in a mouse model, were determined by immunofluorescence analysis. Expression of z-smooth muscle actin (z-SMA), collagen type I (COL1), connective tissue growth factor (CTGF), and paxillin was examined by immunoblot analysis or reverse transcription and quantitative polymerase chain reaction analysis, whereas that of pro-matrix metalloprotease-2 (MMP-2) was assessed by gelatin zymography.

RESULTS. The MRTF-A signaling inhibitor CCG-1423 suppressed RPE-1 cell migration in a concentration-dependent manner. Transforming growth factor-beta (TGF-b2) induced MRTF-A translocation from the cytoplasm to the nucleus of RPE-1 cells, and this effect was attenuated by CCG-1423. TGF-b2 up-regulated the abundance of z-SMA, paxillin, and pro-MMP-2 proteins as well as the amounts of z-SMA, COL1, and CTGF mRNAs in a manner sensitive to inhibition by CCG-1423. Finally, intravitreal injection of CCG-1423 markedly attenuated the development of subretinal fibrosis induced by photocoagulation in vivo.

CONCLUSIONS. Our results implicate MRTF-A in EMT of RPE cells and in the development of subretinal fibrosis in vivo, suggesting that MRTF-A is a potential therapeutic target for retinal diseases characterized by subretinal fibrosis.

Keywords: retinal pigment epithelial cell, myocardin-related transcription factor a, epithelial-mesenchymal transition, fibrosis

Neovascular age-related macular degeneration (nAMD) causes blindness as a result of the development of choroidal neovascularization (CNV). CNV is followed by the development of retinal fibrosis at the end stage of nAMD. Treatments for nAMD include vascular endothelial growth factor (VEGF)-targeted therapy, photodynamic therapy, and laser photocoagulation. Although anti-VEGF therapy has been shown to improve or maintain visual function in individuals with nAMD, it is sometimes followed by the development of subretinal fibrosis. Subretinal fibrosis destroys retinal-choroidal structure and thereby adversely affects visual outcome. The development of subretinal fibrosis subsequent to CNV is associated with multiple biological processes including infiltration of inflammatory cells, epithelial-mesenchymal transition (EMT) of retinal pigment epithelial (RPE) cells, and overproduction or precipitation of extracellular matrix (ECM). EMT is characterized by the loss of epithelial characteristics and the gain of migratory or invasive abilities accompanied by up-regulation of ECM synthesis.

Formation of focal adhesions plays an important role in cell migration, and the activation of paxillin (a focal adhesion protein) contributes to both cell migration and EMT. EMT is also characterized by the expression of z-smooth muscle actin (z-SMA), the ECM proteins collagen type I (COL1) and fibronectin, and connective tissue growth factor (CTGF). Matrix metalloproteinases (MMPs)—including MMP-2 and MMP-9—contribute to EMT, and transforming growth factor-beta (TGF-b), a multifunctional cytokine, induces this process. EMT of RPE cells contributes to the development of fibrosis associated with retinal diseases (e.g., proliferative vitreoretinopathy, proliferative diabetic retinopathy, and uveitis) in addition to that associated with nAMD.
EMT of human RPE cells as well as on the development of subretinal fibrosis in vivo in a mouse model.

**METHODS**

**Materials**

Dulbecco's modified Eagle's medium containing high glucose (DMEM/D6429) as well as fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Rockville, MD, USA). Cell culture dishes and 24-well culture plates were from Corning (Corning, NY, USA). Protease inhibitor cocktail was from Sigma-Aldrich Corp. (St. Louis, MO, USA), bovine serum albumin (BSA) was from Nacalai Tesque (Kyoto, Japan), recombinant human TGF-β2 was from R&D Systems (Minneapolis, MN, USA), and CCG-1423 was from Cayman Chemical (Ann Arbor, MI, USA). Alexa Fluor 488–conjugated isoelectin GS-IB4 was from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibodies to α-SMA and to β-actin were obtained from Sigma-Aldrich; bromodeoxyuridine (BrdU) were from BD Biosciences (Franklin Lakes, NJ, USA); goat polyclonal antibodies to lamin A/C were from Santa Cruz Biotechnology (Dallas, TX, USA); rabbit polyclonal antibodies to MRTF-A (Mkl1) were from Abcam (Cambridge, UK) and Cell Signaling Technology (Danvers, MA, USA) and those to COL1 were from Rockland Immunochemicals (Limerick, PA, USA). Alexa Fluor 488– and Alexa Fluor 568–conjugated secondary antibodies were obtained from Invitrogen. Horseradish peroxidase–conjugated secondary antibodies and ECL Western Blotting Detection Reagents were from GE Healthcare (Little Chalfont, UK); an RNaseasy Mini Kit was from Qiagen (Venlo, The Netherlands); ReverTra Ace qPCR RT Master Mix was from Toyobo (Osaka, Japan); and SYBR Green reagents were from Life Technologies (Carlsbad, CA, USA).

**Cell Culture**

Human retinal pigment epithelial (RPE-1) cells were cultured in DMEM/D6429, supplemented with 10% FBS, as described previously.24 RPE-1 is an immortalized human RPE cell line stably transfected with a vector for the human telomerase reverse transcriptase. The cells are distributed by American Type Culture Collection (Manassas, VA, USA).

**Scratch Assay**

RPE-1 cells were seeded in 24-well plates (4 × 10⁴ cells per well), cultured for 48 hours, and then deprived of serum for 24 hours.
hours before exposure for 30 minutes to CCG-1423 (0 to 3 μM) in DMEM/D6429 supplemented with 0.3% FBS. The cell monolayer was then scratched with a thin rubber tip to inflict a wound ~1 mm in width. The wound was examined immediately and 24 hours after injury with a BZ-X710 fluorescence microscope (Keyence Corp., Osaka, Japan), and the wound area was measured in captured images with the use of ImageJ software version 1.50i (NIH, Bethesda, MD, USA).

Cell Proliferation Assay
RPE-1 cells seeded in 24-well plates (3 × 10⁴ cells per well) were cultured for 24 hours and then deprived of serum for 24 hours before exposure to 10 μM BrdU in DMEM/D6429 with or without 0.3% FBS or 1 μM CCG-1423 for 1 hour. The cells were fixed with 4% paraformaldehyde, exposed to 2 M HCl for 30 minutes at 37°C, neutralized with 0.1 M sodium borate, washed with phosphate-buffered saline (PBS), and exposed to 3% BSA in PBS before incubation overnight at 4°C with antibodies to BrdU (1:100 dilution) in PBS containing 1% BSA, and then washed with PBS, incubated for 1 hour at room temperature with Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (1:200 dilution) in PBS containing 1% BSA, and then mounted in antifade reagents containing DAPI. The cells were examined with a BZ-X710 fluorescence microscope.

Immunocytofluorescence Analysis
Cells grown on 12-mm cover glasses in 24-well plates for 48 hours were deprived of serum for 24 hours and then cultured for 24 hours in serum-free DMEM/D6429 with or without TGF-β2 (10 ng/mL) and with either 1 μM CCG-1423 or 0.03% dimethyl sulfoxide vehicle. The cells were then fixed for 15 minutes at 4°C with 4% paraformaldehyde in PBS, exposed to 0.1% octylphenol ethoxylate for 5 minutes at room temperature, washed twice with PBS, and incubated overnight at 4°C with antibodies to MRTF-A (1:100 dilution) and to α-SMA (1:200 dilution) in PBS containing 1% BSA. The cells were then washed with PBS, incubated for 1 hour at room temperature with Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (1:200 dilution) in PBS containing 1% BSA, and then mounted in antifade reagents containing DAPI. The cells were examined with a BZ-X710 fluorescence microscope.

Immunoblot Analysis
Cells plated at a density of 3 × 10⁵ per 60-mm culture dish were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 100 mM NaF, 1% Nonidet P-40, 0.1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 0.25% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, and a protease inhibitor cocktail. In the extraction of nuclear fractions, cells
plated at a density of $3 \times 10^5$ per 60-mm culture dish were lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagents. The lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% or 10% gel, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific sites of the membrane were blocked by exposure to 5% dried skim milk before incubation with primary antibodies and detection of immune complexes with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents. The intensity of immunoreactive bands was measured with the use of NIH ImageJ software.

**Gelatin Zymography**

Lysates prepared from cells in 100-mm culture dishes were mixed with nonreducing SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, and 0.002% bromophenol blue), and portions (5 μL) of the resulting mixture were subjected to SDS-polyacrylamide gel electrophoresis in the dark at 4°C on a 10% gel containing 0.1% gelatin. The gel was washed with 2.5% Triton X-100 for 1 hour before incubation for 18 hours at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, and 1% Triton X-100. Lastly, the gel was stained with Coomassie brilliant blue.

**RT-qPCR Analysis**

Total RNA was isolated from cells in 60-mm culture dishes with the use of an RNase Mini Kit and subjected to reverse transcription (RT) using ReverTra Ace qPCR RT Master Mix. The resulting cDNA was subjected to quantitative polymerase chain reaction (qPCR) analysis with SYBR Green reagents and a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR primers (forward and reverse, respectively) were 5′-GAATCCTGTGAAGCAGCTCCA-3′ and 5′-CCACGTAGCTGTCTTTTTGTCC-3′ for ACTA2 (NM_001141945), 5′-GAGGGCCAAGACGAAGACATCC-3′ and 5′-CACAGATCACGTCATCGCACAAC-3′ for COL1A1 (NM_000088.3), 5′-GACCTGGAAGAGAACATTAAGAAG-3′ and 5′-CCACAGAATTTAGCTCGGTATG-3′ for CTGF (NM_001901.2), and 5′-AGCCTCAAGATCATCAGCAAT-3′ and 5′-CCTTCCACGGATACCAAGGTGTG-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_002046.5). The amount of each target mRNA was normalized by GAPDH mRNA.
**Induction and Evaluation of CNV and Subretinal Fibrosis**

Female C57BL/6J mice at 8 weeks of age were obtained from Japan SLC (Shizuoka, Japan). All animal experiments were approved by the animal ethics committee of Yamaguchi University Graduate School of Medicine and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A mouse model of CNV and subretinal fibrosis was generated as previously described. The retinas of each mouse were subjected to laser-induced photocoagulation (wavelength, 532 nm; time, 0.1 second; spot size, 75 μm; power, 200 mW), and PBS (1 μL) containing various concentrations of CCG-1423 was injected into the vitreous cavity immediately (for evaluation of CNV) or immediately and after 7 days (for evaluation of subretinal fibrosis). At 1 week (for evaluation of CNV) or 3 weeks (for evaluation of subretinal fibrosis) after photocoagulation, the animals were killed and their eyes were enucleated and fixed with 4% paraformaldehyde in PBS for 2 hours on ice. Choroidal flat-mount preparations were washed with PBS, exposed to 100% methanol at 4°C for 20 minutes, and incubated first for 1 hour at room temperature with 5% dried skim milk in PBS and then for 24 hours at 4°C with Alexa Fluor 488–conjugated isoelectin G5-IB4 (for evaluation of CNV) or with antibodies to COL1 (for evaluation of subretinal fibrosis), each diluted 1:100 in PBS. COL1 immune complexes were detected by additional incubation for 90 minutes at room temperature with Alexa Fluor 568–conjugated secondary antibodies (1:1000 dilution).

**Results**

**Effect of MRTF-A Inhibition on RPE-1 Cell Migration**

We first examined the effect of the MRTF-A signaling inhibitor CCG-1423 on the migration of cultured RPE-1 cells with the use of an in vitro scratch assay. Whereas control cells had largely covered the wound area by 24 hours after the injury, CCG-1423 inhibited migration of the cells into the wound in a concentration-dependent manner, with the effect becoming significant at 1 and 3 μM (Figs. 1A, 1B).
Effect of MRTF-A Inhibition on RPE-1 Cell Proliferation

We next examined the effect of inhibition of MRTF-A signaling on the proliferation of cultured RPE-1 cells by monitoring of BrdU incorporation. The percentages of BrdU incorporation were 0.93% to 1.32%. There was no significant differences among groups (Fig. 1C).

Effects of TGF-β2 and CCG-1423 on the Subcellular Localization of MRTF-A and Expression of EMT Markers in RPE-1 Cells

We examined the subcellular distribution of MRTF-A and α-SMA in RPE-1 cells using immunofluorescence analysis. MRTF-A was localized predominantly to the cytoplasm of unstimulated cells (Fig. 2A). Stimulation of the cells with TGF-β2 (10 ng/mL) for 24 hours induced the translocation of MRTF-A to the nucleus, and this effect was inhibited by 1 μM CCG-1423 (Fig. 2A). Stimulation with TGF-β2 also induced marked up-regulation of the expression of α-SMA in the cytoplasm, indicative of the induction of EMT, and this effect was also attenuated by CCG-1423 (Fig. 3A). These effects of TGF-β2 and CCG-1423 on the nuclear translocation of MRTF-A and the expression of α-SMA in RPE-1 cells were confirmed by immunoblot analysis (Figs. 2, 3). We also examined the expression of genes for EMT-related proteins by RT-qPCR analysis. TGF-β2 increased the amounts of α-SMA (ACTA2), COL1 (COL1A1), and CTGF mRNAs in RPE-1 cells, and these effects were abolished in the presence of CCG-1423 (Fig. 4).

Effects of TGF-β2 and CCG-1423 on Expression of Paxillin and Pro–MMP-2 in RPE-1 Cells

Immunoblot analysis revealed that TGF-β2 increased the abundance of the focal adhesion–associated protein paxillin in RPE-1 cells and that this effect was abolished by CCG-1423 (Fig. 5). Similarly, gelatin zymography showed that TGF-β2 increased the amount of the pro-form of MMP-2 in these cells, and again this effect was prevented in the presence of CCG-1423 (Fig. 6). The active form of MMP-2 was not detected in this assay.
Effects of CCG-1423 in a Mouse Model of CNV and Subretinal Fibrosis

Finally, we examined the effects of CCG-1423 on the development of CNV and subretinal fibrosis in a mouse model that mimics these pathological processes in patients with nAMD and in which TGF-β signaling plays a key role. Fluorescence microscopic analysis of choroidal flat-mount preparations stained with isoelectin GS-IB4 showed that injection of CCG-1423 into the vitreous cavity immediately after photocoagulation had no significant effect on the development of CNV apparent at 7 days (Fig. 7). In contrast, immunohistofluorescence analysis of choroidal flat-mount preparations with antibodies to COL1 showed that injection of CCG-1423 into the vitreous cavity immediately and 7 days after photocoagulation suppressed the development of subretinal fibrosis apparent at 21 days. This effect of CCG-1423 was concentration dependent and was significant when it was injected in a volume of 1 μL at concentrations of 60 or 200 μM (Fig. 8).

DISCUSSION

We have shown here that the MRTF-A signaling inhibitor CCG-1423 suppressed the migratory activity of human RPE cells but had no effects on their proliferation activity. We examined cell migration assay and cell proliferation assay in the similar cultured condition in this study. However, the activity of MRTF-A is involved in proliferation of epithelial cells in a previous report. At present, evaluation of the effects of RPE cells proliferation by CCG-1423 needs further study. Whereas MRTF-A was localized predominantly to the cytoplasm of RPE-1 cells under basal conditions, it underwent translocation to the nucleus in response to cell stimulation with TGF-β. This translocation of MRTF-A was attenuated by CCG-1423. CCG-1423 also inhibited the TGF-β-induced up-regulation of the EMT-related molecules α-SMA, COL1, and CTGF at the mRNA or protein levels. In addition, the expression of paxillin and pro-MMP-2 induced by TGF-β in RPE-1 cells was prevented by CCG-1423. Finally, CCG-1423 attenuated the development of subretinal fibrosis without affecting that of preceding CNV in a mouse model, suggesting that the antifibrotic action of CCG-1423 is direct. These results thus indicate that MRTF-A signaling may play an important role in the development of subretinal fibrosis associated with nAMD.

EMT, the transdifferentiation of epithelial cells into mesenchymal-like cells contributes to many biological processes, including development, wound healing, fibrosis, and cell migration. EMT is triggered by various factors—including TGF-β, CTGF, epidermal growth factor, and fibroblast growth factor—and increases the motility of individual cells, conferring an invasive phenotype.
CCG-1423 suppressed the motility of human RPE cells. RPE cells positive for EMT markers α-SMA and glial fibrillary acidic protein have been detected in retinal fibroblastic tissue of patients with nAMD.35 We found that the TGF-β2–induced expression of α-SMA, COL1, and CTGF in RPE-1 cells was prevented by CCG-1423. CCG-1423 also attenuated the formation of subretinal fibrosis without affecting CNV in an in vivo model, suggesting that the inhibition of subretinal fibrosis by CCG-1423 is direct and not attributable to an inhibitory effect on CNV. Thus, our results indicate that CCG-1423 is highly effective at inhibiting both EMT in human RPE cells and the development of subretinal fibrotic tissue, suggesting that it may attenuate the formation and contraction of subretinal fibrotic tissue associated with the late stage of nAMD.

Tissue remodeling and deposition of ECM proteins are influenced by MMPs.39 We previously showed that TGF-β2 increases MMP activity in RPE cells.4 In the present study, we found that TGF-β2 increased the expression of pro-MMP-2 and that CCG-1423 blocked the TGF-β2–induced expression of COL1 and other EMT markers. These results suggest that MRTF-A may promote ECM production and degradation related to the formation of fibrous subretinal tissue in nAMD.

FIGURE 7. Effect of CCG-1423 in a mouse model of CNV. (A–D) Fluorescence staining with isolectin GS-IB4 in choroidal flat-mount preparations from mice at 7 days after laser photocoagulation. Intravitreal injection (1 μL) either of PBS (A) or of CCG-1423 at 20 μM (B), 60 μM (C), or 200 μM (D) was performed immediately after photocoagulation. Scale bars: 100 μm. (E) Area of CNV determined in the treated mice. Data are means ± SEM (n = 14 to 20 laser spots per group). NS, not significant (Dunnett’s test).
We previously showed that the RAR-\(\gamma\) agonist R667 as well as female sex hormones inhibit EMT in RPE cells induced by TGF-\(\beta_2\).\(^4\,\,40\) The RAR-\(\gamma\) agonist also suppressed subretinal fibrosis in our mouse model.\(^4\) The estrogen 17\(\beta\)-estradiol increases MRTF-A expression in human breast cancer cells.\(^{41}\) Moreover, MRTF-A serves as a downstream effector in retinoic acid–induced neural differentiation of bone marrow–derived mesenchymal stem cells.\(^{42}\) We found that TGF-\(\beta_2\) induced the nuclear translocation of MRTF-A in RPE-1 cells and that this effect was inhibited by CCG-1423 in association with suppression of EMT. These various observations suggest that MRTF-A may interact with nuclear receptors in the control of EMT in RPE cells.

Local administration of an MRTF-A signaling inhibitor suppressed the development of fibrosis at the filtration bleb in an animal model of glaucoma surgery, to an extent similar as with mitomycin C.\(^{23}\) In the present study, we found that intravitreal injection of the MRTF-A signaling inhibitor CCG-1423 also suppressed subretinal fibrosis in a mouse model. Our results suggest that CCG-1423 warrants further investigation as a potential drug for the treatment of fibrosis associated with late-stage nAMD and other proliferative retinal diseases.

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