Vascular Endothelial Growth Factor Reduced and Connective Tissue Growth Factor Induced by Triamcinolone in ARPE19 Cells under Oxidative Stress

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PURPOSE. To investigate whether triamcinolone acetonide (TA) affects the expression of vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) in retinal pigment epithelial (RPE) cells exposed to oxidative stress.

METHODS. TA (10 nM, 1 μM, or 100 μM) was added to ARPE19 cells exposed to oxidative stress induced by hypoxia-reoxygenation and paraquat. Cellular expression of VEGF, CTGF, and an inducer of both growth factors, transforming growth factor (TGF)-β was investigated with real-time reverse transcription–polymerase chain reaction and Western blot analysis. Tube-forming assays were conducted with human umbilical vein endothelial cells (HUVECs) in conditioned medium from RPE cells exposed to oxidative stress, with or without TA treatment.

RESULTS. Oxidative stress induced mRNA expression of VEGF, CTGF, and TGF-β by RPE cells. TA reduced upregulation of VEGF and TGF-β in a concentration-dependent manner. In contrast, upregulation of CTGF by oxidative stress was accelerated by TA concentrations of 10 nM and 1 μM. Tube formation by HUVECs was strongly inhibited by exposure to conditioned medium from oxidative stress–stimulated ARPE19 cells treated with 1 μM TA compared with cells not treated with TA.

CONCLUSIONS. TA reduced VEGF expression and induced CTGF expression in ARPE19 cells exposed to oxidative stress, and conditioned medium from these cells inhibited tube formation by HUVECs. Because VEGF is a major cytokine involved in angiogenesis, and CTGF is a main cytokine related to fibrosis, these results suggest that changes in their expression may be important mechanisms underlying the decreased choroidal neovascularization and fibrosis after administration of TA.

Triamcinolone acetonide (TA) is a corticosteroid suspension that has long been injected periocularly to treat inflammatory ocular diseases. Recently, more diseases are being treated with TA—for example, macular edema due to diabetic retinopathy,1–5 retinal vein occlusion,1–6 proliferative vitreoretinopathy,7,8 and exudative age-related macular degeneration (AMD).9,10

Exudative AMD leads to severe deterioration of central vision in elderly individuals as the result of the development of choroidal neovascularization (CNV) in the macular region.11,12 In animal studies, intravitreal administration of TA effectively reduced the incidence of experimental neovascularization.13 Penfold et al.14 first used intravitreal TA in patients with exudative AMD and found that it significantly reduced severe visual loss and stabilized CNV membranes. The effect of TA on CNV has been confirmed by other studies,9,10,15,16 and a recent prospective, randomized clinical trial involving single-dose intravitreal injection of TA also showed a significant decrease in the size of CNV in the treated group 5 months after injection.17 Although these experimental and clinical data reveal that steroids can suppress CNV, the mechanism of action of TA on CNV has not yet been determined.

Oxidative stress is considered to be a risk factor for AMD,18–20 and our in vitro experiments have shown that it can induce vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) in retinal pigment epithelial (RPE) cells (Gomi F, unpublished data, 2004). VEGF plays an important role in conditions that involve ocular angiogenesis, including CNV.21–23 CTGF is considered to play a major role in the pathogenesis of fibrotic disease.24–28 CTGF is expressed in excised CNV tissue, which suggests a relationship to the pathogenesis of CNV.29

Based on the clinical and experimental results of TA administration for CNV,9,10,14–16 TA may alter the expression of VEGF and CTGF by RPE cells to induce suppression and the fibrosis associated with CNV. Therefore, we investigated whether the expression of VEGF and CTGF in RPEs after oxidative stress would be affected by administration of TA. Because the concentration of TA that suppresses CNV has not been determined, we used different concentrations. Moreover, to confirm whether changes in the expression of these growth factors affects in vitro angiogenesis, tube-forming assays were performed by using conditioned medium harvested from RPE cultures after exposure of the cells to oxidative stress in the presence or absence of TA.

MATERIALS AND METHODS

Cell Culture

ARPE19 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium/F-12 human amniotic membrane nutrient mixture (DMEM/F-12; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a humidified incubator at 37°C in an atmosphere of 5% CO2. The medium was changed every 3 days.

Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo Industries, Ltd. (Osaka, Japan) and grown in complete medium with supplements (Humedia-Eg2; Kurabo). Cells from passages 5 to 6 were used for the experiments.

Preparation of TA

TA (Sigma-Aldrich) was dissolved in methanol (Sigma-Aldrich) at a concentration of 1000 times 10 nM, 1 μM, and 100 μM to produce...
stock solutions. These solutions were serially diluted to 1:1000 with medium to obtain 10 nM, 1 μM, and 100 μM TA-containing culture medium (10 nM, 1 μM, and 100 μM correspond to 4.3 ng/mL, 0.43 μg/mL, and 43 μg/mL, respectively). To evaluate the effect of methanol, medium containing only methanol (1:1000 of total volume) was also prepared for each study.

**Cell Proliferation Assay**

Subcultured ARPE19 cells were transferred into 24-well plates (Nalg Nunc International, Rochester, NY) containing DMEM/F-12 medium supplemented with 10% FBS at a density of an equal number of cells per well (1 × 10⁵). After 24 hours, the original medium was removed, and medium containing 10 nM, 1 μM, or 100 μM TA was added to the cells. As a control, ARPE19 cells were cultured with normal medium or with normal medium containing only methanol. Each culture medium was replaced every 2 days. Four days after treatment with TA, phospho-micrographs were taken, the culture medium was removed, and each well was washed with phosphate-buffered saline (PBS) to expel the floating cells. The adherent cells were detached with trypsin-EDTA and the total number of cells was counted. Each assay was performed in triplicate.

**Oxidative Stress and Exposure to TA**

**Hypoxia-Reoxygenation.** Hypoxia was created using an incubator attached to a low-oxygen chamber (Coy Laboratory Products, Ann Arbor, MI) that maintained a humidified atmosphere at a low oxygen tension (PO₂: 12–14 mm Hg). Confluent ARPE19 cells were incubated in the chamber for 24 hours and then returned to a normal atmosphere (reoxygenation), whereas the conditioned medium was rapidly exchanged for fresh medium containing TA (0, 10 nM, 1 μM, or 100 μM). Cells were harvested 1 hour after reoxygenation for real-time reverse transcription-polymerase chain reaction (RT-PCR) and at 2 hours for Western blot analysis, since the expression of VEGF and CTGF were at their maximum at these times after reoxygenation (Matsuda S, unpublished data, 2004).

**Paraquat.** Confluent ARPE19 cells were cultured in DMEM/F-12 containing 500 μM paraquat (Sigma-Aldrich) and different concentrations of TA. The medium was renewed every 24 hours for continuous exposure to paraquat and cells were harvested 24 hours after exposure to five doses.

To evaluate the levels of the two forms of oxidative stress, real-time RT-PCR for heme oxygenase (HO)-1 was performed.

**Real-Time RT-PCR**

RNA was prepared from ARPE19 cells with or without exposure to oxidative stress (RNasey Mini Kit; Qiagen, Tokyo, Japan) and quantified by ultraviolet spectrometry at 260 nm. RT was then performed (First-Strand cDNA Synthesis Kit; Amersham Biosciences, Piscataway, NJ) to obtain cDNA for PCR. Real-time PCR was performed on a thermocycler (Light Cycler; Roche Diagnostics, Burgdorf, Switzerland) with nuclear stain (SYBR Green; Applied Biosystems, Inc., Foster City, CA) reagents according to the manufacturer’s instructions. Amplification of PCR products was quantified by measurement of fluorescence associated with the SYBR green dye in the reaction mixture. The sequences of the primers used in this study were as follows: human CTGF forward primer, 5'-CGGCTTTACCGACTGGAA-GAC-3', and reverse primer, 5'-GTCGTTAGATCTCCAAAGCAG-3'; human VEGF forward primer, 5'-CGACGCACTGCTGCGTCAATC-3'; human HO-1 forward primer, 5'-GGTAGTGGAAAGCTGGTGA-TGCTCA-3'; and reverse primer, 5'-GGATGACCGTGAAGCTGGTGA-TGCTCA-3'; human HO-1 forward primer, 5'-GGATGACCGTGAAGCTGGTGA-TGCTCA-3'; human β-actin forward primer, 5'-CTCTCCCTGGTGGAGGA-GAGGCTA-3', and reverse primer, 5'-CTCTCCCTGGTGGAGGA-GAGGCTA-3' as the internal control.

This assay allows accurate and specific quantification of amplified PCR products by measuring the fluorescence produced by fluororescence resonance energy transfer after the simultaneous hybridization of two gene-specific fluorophore-labeled DNA oligonucleotides to adjacent sequences within the amplified product. After an initial denaturation step of 95°C for 1 minute, PCR involved 45 cycles at 95°C for 10 seconds, 60°C for 1 second, and 72°C for 10 seconds.

**Western Blot Analysis**

Cell extracts for Western blot analysis were prepared by washing cells three times with PBS, followed by lysis in sample buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% SDS, 100 mM NaCl, 0.5% NP-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were boiled for 5 minutes before 20 μL aliquots were subjected to electrophoresis on 7.5%, 10%, and 12% polyacrylamide gels. After the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA), the membrane was incubated in blocking buffer containing 1% BSA and 5% powdered skim milk for 1 hour at room temperature and then probed overnight at 4°C with the primary antibody in blocking buffer (1:500). The membrane was then washed three times in PBS containing 0.05% Tween-20, probed for 1 hour at room temperature with the secondary antibody in blocking buffer (1:5000), and washed again in PBS containing 0.05% Tween-20. Visualization of signals was achieved with an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences). The following were used as primary antibodies: anti-CTGF goat polyclonal, anti-VEGF polyclonal, and TGF-β polyclonal (all from Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies were horse-radish peroxidase (HRP)-labeled anti-goat IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) and HRP-labeled anti-rabbit IgG (Cell Signaling Technology, Beverly, MA). Protein levels were quantitated by densitometry and normalized to the β-actin levels.

**Synthetic Matrix Tube-Forming Assay**

Twenty-four-well culture plates (Nalg Nunc International) were coated with 0.4 μl low-growth-factor synthetic matrix (Matrigel; BD Bioscience, San Jose, CA) and incubated for 30 minutes at 37°C. HUVECs starved of serum for 4 hours were treated with trypsin-EDTA and suspended in the culture medium for 20 minutes. The cells were then seeded at a density of 40,000 cells per well on polymerized matrix with each medium, (described later). HUVECs were incubated for 6 hours at 37°C, and micrographs were taken under a phase contrast light microscope (Olympus, Tokyo, Japan). Tube formation was then quantified by counting the number of joined cells in five randomly selected fields at ×200 magnification, and that number was divided by the total number of cells in the same field, as described previously.

To study the cytotoxicity of TA in HUVECs, we performed the tube-formation assay in the HUVEC culture medium with supplements (except hydrocortisone) and 10 nM, 1 μM, or 100 μM TA. The HUVECs were then tested with the conditioned medium (DMEM/F-12, 1% FBS) collected from ARPE19 cells exposed to hypoxia-reoxygenation (2 hours after reoxygenation) in the presence or absence of TA.

**Results**

**Effect of TA Concentration on RPE Cell Proliferation**

As shown in Figure 1, TA had a biphasic effect on RPE cell proliferation. It stimulated cell growth at 10 nM and 1 μM after 4 days of culture but inhibited cell growth at 100 μM. RPE cell proliferation was promoted significantly by 1 μM TA compared with control cultures (P < 0.05; Figs. 1A, 1D, 1F). Methanol (the vehicle for TA) had little effect on cell proliferation compared with the control culture (Figs. 1A, 1B, 1F).

**Level of Oxidative Stress and Each Molecule Expression**

To examine the level of oxidative stress, real-time RT-PCR for HO-1 was performed and showed that HO-1 mRNA was exclu-
Effect of TA on Expression of CTGF after Oxidative Stress

To examine the effect of TA on CTGF after RPE cells were exposed to oxidative stress, and real-time RT-PCR, and Western blot analysis were performed. Real-time RT-PCR revealed that CTGF mRNA was upregulated by oxidative stress, and there was significantly more upregulation in cells cultured with 1 μM TA than in cells cultured without TA, whereas it was downregulated in the presence of 100 μM TA (Figs. 4A, 4C), which was probably the result of the cytotoxic effect of TA on RPE cells. Western blot analysis showed that production of CTGF protein was increased in the presence of 1 μM TA (Fig. 4B).

Effect of TA on Expression of TGF-β after Oxidative Stress

Because the results showed that TA inhibited VEGF expression but stimulated CTGF expression, the effect of TA on expression of TGF-β, the inducer of CTGF, as well as VEGF, was examined by real-time RT-PCR and Western blot analysis. Real-time RT-PCR showed that the upregulated expression of TGF-β mRNA by oxidative stress decreased in the presence of TA in a concentration-dependent manner (Fig. 5A). Western blot analysis showed that the production of TGF-β protein also was reduced by 1 μM TA (Fig. 5B). These results suggest that TA reduces VEGF expression by inhibiting TGF-β expression but stimulates CTGF expression through other signaling pathways.

Effect of TA on Tube Formation by HUVECs

From the clinical and experimental data on the effect of TA on CNV, it seems likely that TA suppresses CNV. To confirm whether TA reduces angiogenesis, a tube-forming assay was performed. Because cytotoxic effects of TA on HUVECs was seen in cultures of control medium with 100 μM TA (Fig. 6D), but not with 10 nM (Fig. 6B) and 1 μM (Fig. 6C) TA, we did not study the effect of 100 μM TA. As shown in Figure 6, tube formation was strongly induced by conditioned medium from ARPE19 cells exposed to hypoxia-reoxygenation when compared with conditioned medium from normal cells (Fig. 6E, 6F). The effect of each TA concentration on tube formation was then investigated, and the assay showed that conditioned medium from cells treated with 1 μM TA significantly reduced tube formation (Figs. 6H, 6I). These results indicate that TA inhibits angiogenesis induced by factors released from ARPE19 cells under oxidative stress.

DISCUSSION

Recently, intravitreal injection of TA has been reported as a therapeutic modality for exudative age-related macular degeneration (AMD). In some cases, regression of CNV occurred after administration of TA. However, the molecular mechanism of TA for CNV has not been clarified sufficiently.

In the present study, TA inhibited upregulation of VEGF expression, whereas it promoted upregulation of CTGF expression after oxidative stress. Oxidative stress on the RPE has been reported to be one of the most important causes of AMD, and our in vitro experiments showed that oxidative stress caused upregulation of VEGF and CTGF expression in RPE cells. Many reports have suggested that VEGF may play a major role in the development and growth of CNV, and clinical trials of anti-VEGF therapy, such as anti-VEGF aptamer and anti-VEGF antibody fragment, have also shown its efficacy on CNV in AMD. CTGF is believed to be associated with the pathogenesis of fibrotic disease. Although there are conflicting data on CTGF and angiogenesis, that CTGF both promotes and inhibits angiogenesis, CTGF was ex-
pressed in surgically excised choroidal neovascular membranes, there is a possible relationship between CTGF and CNV. In the present study, we confirmed that tube formation by HUVECs was accelerated in the conditioned medium from ARPE19 cells subjected to oxidative stress in which the expression of both VEGF and CTGF increased. Therefore, besides...
upregulation of VEGF, the increased expression of CTGF may be involved in the formation of CNV.

TA inhibited upregulation of VEGF expression in a concentration-dependent manner. Downregulation of VEGF expression by glucocorticoids has been demonstrated in human vascular smooth muscle cells, growth plate chondrocytes, and porcine brain endothelial cells. However, Gao et al. showed that intravitreal TA injection did not cause significant changes in VEGF mRNA expression in the rat retina, and they suggested that TA may not affect VEGF expression in normal tissue. In the present study, we confirmed that TA reduces VEGF expression in RPE cells subjected to oxidative stress, which is more similar to the state of pathologic RPE in AMD.

Taken together, TA may reduce increased VEGF expression in abnormal RPE cells. TA also inhibited upregulation of TGF-β, which induces VEGF; therefore, TA may suppress VEGF by downregulating TGF-β.

Contrary to VEGF, TA promoted upregulation of CTGF expression. Several studies have reported that glucocorticoids may induce CTGF. The expression of TGF-β (a potent inducer of CTGF as well as VEGF) was downregulated by TA in a concentration-dependent manner. Dammeier et al. also reported that the induction of CTGF by dexamethasone was not through TGF-β in fibroblasts. Kubota et al. reported that dexamethasone activates the CTGF gene promoter in NIH3T3 cells, so TA may act on the CTGF promoter and induce its expression.

FIGURE 4. The change of CTGF expression by TA in ARPE-19 cells after oxidative stress, hypoxia-reoxygenation (A, B) and paraquat exposure (C). (A, C) Real-time RT-PCR; (B) Western blot and densitometric analyses. Results are expressed as the mean ± SD (n = 3). *P < 0.05 for comparison with no TA treatment (oxidative stress only) by Student’s t-test.

FIGURE 5. Downregulation of TGF-β expression by TA in ARPE-19 cells after oxidative stress. (A) Real-time RT-PCR; (B) Western blot and densitometric analyses. Results are expressed as the mean ± SD (n = 3). *P < 0.05 for comparison with no TA treatment (oxidative stress only) by Student’s t-test.
expression in ARPE19 cells through a pathway not mediated by TGF-β.

These data suggest several mechanisms of TA inhibition of CNV. First, TA may downregulate VEGF in abnormal RPE cells around the CNV and thus diminish the extent of the CNV. Second, TA may upregulate CTGF, after which secreted CTGF may promote the fibrosis associated with CNV. Moreover, TA may accelerate RPE proliferation to enclose the areas of CNV.

First, TA may downregulate VEGF in abnormal RPE cells. When 4 mg of TA is administered intravitreally in humans, the vitreous concentration of TA is 1 mg/mL. With this type of administration, it is unclear what concentration of TA acts on RPE and choroids. Further in vivo studies are needed to determine the clinically adequate dose of TA for treatment of CNV.

References

16. Navajas EV, Costa RA, Farah ME, Cardillo JA, Bonomo PP. Indocyanine green-mediated photothermolysis combined with intravitreal factor-induced migration and tube formation in choroidal microvascular endothelial cells. An important role of macrophages has been suggested for the pathogenesis of AMD; therefore, it is possible that TA may change the expression of many other cytokines that lead to the inhibition of VEGF expression.

Anecortave acetate, an angiostatic steroid that also has been used clinically to treat AMD, may exert an inhibitory effect on endothelial cell growth in vitro by increasing the synthesis of plasminogen activator inhibitor type 1. Further detailed studies of the mechanism of steroids on CNV are needed.


