Suppression of Injury-Induced Conjunctiva Scarring by Peroxisome Proliferator-Activated Receptor γ Gene Transfer in Mice

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Purpose. To examine the effects of introduction of the adenoviral peroxisome proliferator–activated receptor (PPAR)-γ gene on postinjury conjunctival scarring in mice. Its effects on fibrogenic reaction of cultured human subconjunctival fibroblasts (hSCFs) were also evaluated.

Methods. The effects of PPARγ gene introduction on expression of type I collagen, fibronectin, and connective tissue growth factor (CTGF) in hSCFs were examined. A circumferential incision was made in the equatorial conjunctiva of the right eye of generally anesthetized adult C57BL/6 mice (n = 72). PPARγ cDNA-expressing adenoviral vector was topically applied; the control eye received nonfunctioning adenoviral vector. At 2, 5, 7, and 14 days (each, n = 18), the eyes were processed for histologic or immunohistochemical examination to evaluate tissue scarring. Expression of type I collagen and growth factors was evaluated by real-time reverse transcription–polymerase chain reaction in 32 eyes from control and treatment groups.

Results. PPARγ overexpression suppressed type I collagen, fibronectin, and CTGF in cultured hSCFs at the mRNA or protein level. In vivo experiments showed that PPARγ gene introduction suppressed monocyte/macrophage invasion, generation of myofibroblasts, and mRNA upregulation of cytokines/growth factors and collagen Iα2 chain (Col 1A2) in healing conjunctiva.

Conclusions. PPARγ gene transfer suppresses the fibrogenic reaction in hSCFs as well as the injury-induced scarring of conjunctival tissue in mice, suggesting the effectiveness of this strategy in preventing excess scarring after filtration surgery. The mechanism may include suppression of activation of fibroblasts and reduction of macrophage invasion. (*Invest Ophthalmol Vis Sci.* 2009;50:187–193) DOI:10.1167/iovs.08-2282

Conjunctival scarring potentially reduces filtration efficacy after glaucoma filtering surgery. Although a wound-healing reaction is orchestrated by a variety of signals derived from endogenous soluble factors, it is well-established that transforming growth factor (TGF)-β is an active mediator of conjunctival scarring.[1–4] TGFβ family has three isoforms; β1, β2, and β3, each of which has different activities and regulates numerous cell functions, such as proliferation, differentiation, apoptosis, epithelial-mesenchymal transition, and production of extracellular matrix.[5,6] Aqueous humor contains abundant TGFβ2, whereas TGFβ1 and -β2 are expressed in local cells in the filtering bleb tissue.[2,7] Thus, conjunctival scarring may be blocked more effectively by targeting all TGFβ family members rather than each TGFβ isoform. Each TGFβ isoform propagates its signal through a signal transduction network, such as mitogen-activated kinase (MAPK)/Erk, p38MAPK, C-Jun-N-terminal kinase (JNK), and Smad, involving receptor serine/threonine kinases at the cell surface and their substrates.[5,6,8,9] Among these pathways, we have reported that blockage of Smad signaling by adenoviral transfer of the anti-Smad gene (Smad7 or p38) suppresses excess fibrogenic reaction in an injured mouse conjunctiva as well as in cultured human subconjunctival fibroblasts (hSCFs).[10,11]

The peroxisome proliferator–activated receptor (PPAR) family consists of three members, PPARβ, δ and γ, which are involved in modulation of adipose metabolism, inflammatory cell function, and actions of noninflammatory cells such as the fibrogenic reaction and cell proliferation during wound healing.[12–17] PPARγ is a nuclear receptor for ligands of 15-deoxy-δ12, 14-prostaglandin J2 (15d-PGJ2), and thiazolidinedione; 15d-PGJ2 also has PPARγ-independent functions. Although all signaling networks linked to PPARγ have not been completely described, stimulation of this receptor by ligand application results in reduction of the fibrogenic response in cultured mesenchymal cell types.[17–22] Moreover, adenoviral gene transfer of PPARγ exhibits a therapeutic effect in an experimental model of colitis and liver fibrosis.[23,24] As for the eye, we have reported that PPARγ overexpression suppresses the fibrogenic reaction in cultured mouse ocular fibroblasts and macrophages by inhibiting nuclear translocation of the phosphorylated Smads and prevents excess scarring in an alkali-burned mouse cornea.[25]

Based on these findings, we hypothesized that activation of PPARγ signal may suppress injury-induced conjunctival scarring and can be used as a potential therapy for inhibiting excessive bleb scarring in the conjunctiva after glaucoma surgery. Our preliminary experiment showed that an inhibitory effect of overexpression of PPARγ by adenoviral gene introduction on type I collagen protein production was minimally affected by further addition of either 15d-PGJ2 or a PPARγ antagonist, GW9662, in cultured fibroblasts, indicating that overexpressed PPARγ activates its signal(s) independent of its ligands. An overexpression of the PPARγ gene by transfection also reportedly drives PPARγ-related gene expression to such a high level that cannot be reversed by administration of PPARγ antagonist, supporting this notion.[26,27] Moreover, a PPARγ ligand topically administered to the conjunctiva may be easily

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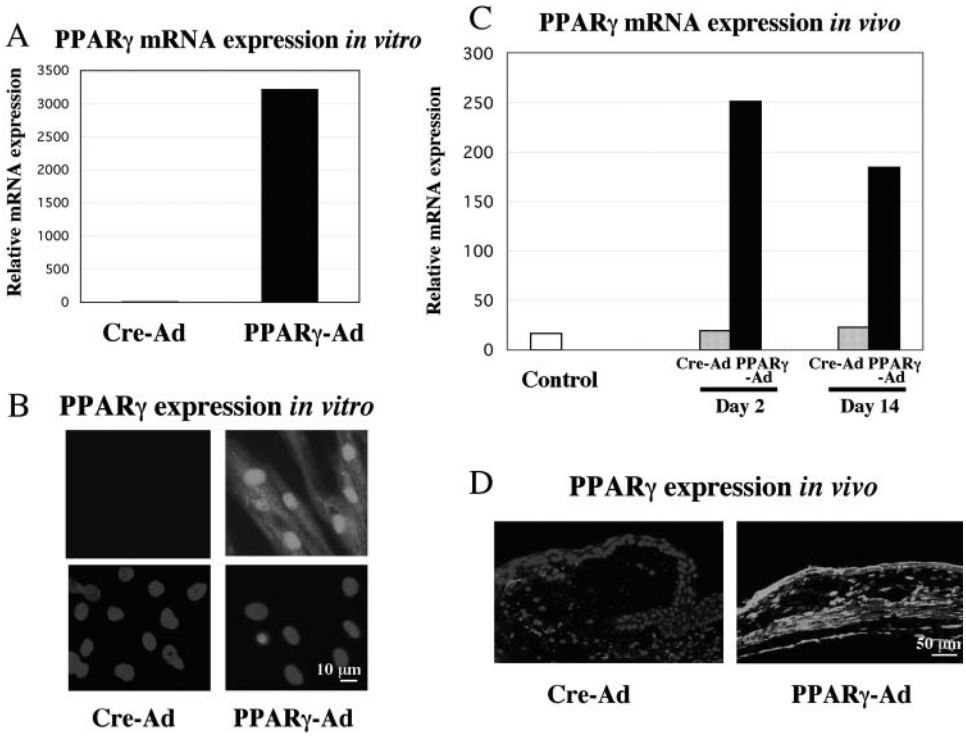

FIGURE 1. Efficacy of viral gene transfer in hSCFs and a mouse model. When the cultured fibroblasts were incubated in a medium containing CAG (cytomegalovirus enhancer, chicken β-actin promoter, and a part of the 3′ UTR of rabbit β-globin) promoter-driven Cre (Cre-Ad) and LNL-mouse peroxisome proliferator-activated receptor γ (PPARγ) cDNA (PPARγ-Ad), mRNA expression of PPARγ was highly enhanced in PPARγ-Ad treated hSCFs (**A**). Marked PPARγ immunoreactivity was also observed on day 2 and later in the nuclei of cultured human SCFs (**B**). In vivo, mRNA expression of PPARγ was highly enhanced in the PPARγ-Ad treated group (**C**). PPARγ protein was detected in the nuclei of conjunctival fibroblasts and epithelium of the wound-healing mice model on day 2 and later (up to day 14) (**D**). Immunolocalization of PPARγ was not detected in eyes treated with CAG-Cre vector only (**D**). Data represent typical results obtained from experiments repeated four times.

washed out from the local tissue. In the present study, we therefore used adenoviral-mediated PPARγ cDNA transfer in place of administration of a PPARγ ligand to activate the PPARγ signal. The anti-fibrogenic effects of PPARγ overexpression by adenoviral gene transfer were evaluated in a mouse model of injury-induced conjunctival scarring and hSCFs.

MATERIALS AND METHODS

All experimental procedures were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki.

Adenovirus Vector Construction and Virus Purification

We used an adenovirus expression set (Cre/LoxP-Regulated Expression Vector Set no. 6151; Takara, Tokyo, Japan) to create recombinant adenovirus-expressing mouse PPARγ, as reported previously.[28] A mixture of recombinant adenoviruses carrying CAG (comprising a cytomegalovirus enhancer, chicken β-actin promoter, and a part of 3′ untranslated region of rabbit β-globin) promoter-driven Cre (Cre-Ad) and LNL-mouse PPARγ cDNA (PPARγ-Ad) was applied to the targets to induce expression of PPARγ protein. The efficacy of gene transfer was confirmed using green fluorescent protein–carrying adenovirus, as reported previously; on observation, almost all cells were positive (data not shown).[10]

Primary Cell Culture of hSCFs and PPARγ Gene Introduction into the Cells

Primary culture of hSCFs was conducted as reported previously in detail.[29] Redundant subconjunctival connective tissue was obtained from patients aged 4 to 10 years during strabismus surgery after informed consent was obtained from the parents of each patient. After two or three passages, the cells were trypsinized for seeding for the experiments. Expression of introduced PPARγ in the cells was evaluated in cultured cells by using real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence microscopy.

The cells (5.0×10^5/mL, 60 × 15-mm culture dish; BD Labware, Franklin Lakes, NJ) or 7.4×10^5 /mL, 16-well chamber slides (Nalge Nunc International, Naperville, IL) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, antibiotics, and an antimycotic (MEM-10) until confluent. They were then incubated for 2 hours in a serum-free medium containing Cre-Ad or both Cre-Ad and PPARγ-Ad at a concentration of 4×10^3 PFU/mL and then incubated for another 48 hours in MEM-10. The cells were then exposed to 5 ng/mL of recombinant human TGFβ1 (R&D Systems, Minneapolis, MN) for 48 hours and were processed for total RNA extraction, enzyme-linked immunosorbent assays (ELISAs), and immunohistochemistry.

Effects of Exogenous PPARγ on mRNA Expression of Collagen Iα2 Chain (Col 1A2) and Connective Tissue Growth Factor (CTGF) by Cultured hSCFs

Total RNA from cultured cells was extracted and processed for semiquantitative real-time RT-PCR for mRNA of human Col 1A2 and CTGF, as reported previously.[10,11,25]

The concentrations of type I collagen and fibronectin proteins were immunoassayed with a commercially available kit (Takara, Tokyo, Japan), as reported previously.[29] Briefly, the cells were seeded into a 24-well cell culture plate (BD Labware) and incubated until reaching confluence. After infection of each adenoviral vector, the cells in each well were treated overnight with Eagle's MEM without serum. Then, the culture medium was harvested, and the concentrations of type I collagen and fibronectin were measured. The medium was supplemented by β-aminopropionitrile fumarate, a lysyl oxidase inhibitor, to prevent collagen peptides from being deposited in the cell layer.

For immunocytochemical analysis, the cells were fixed in cold acetone for 5 minutes. Indirect immunostaining was performed as reported previously.[10] Mouse monoclonal anti-human type I collagen (1:100 in phosphate-buffered saline [PBS]; Fuji Chemical, Toyama,

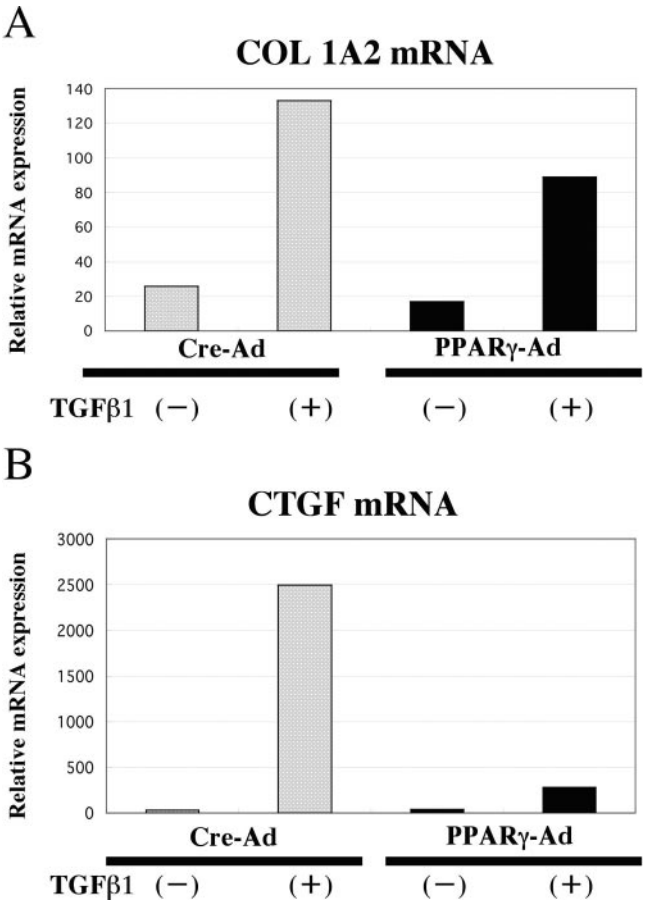

FIGURE 2. Effects of PPARγ gene introduction on expressions of Col 1A2 and CTGF mRNAs by cultured hSCFs. Real-time RT-PCR showed an increase in mRNA expression of Col 1A2 (**A**) and CTGF (**B**) in the presence of exogenous TGFβ1 and its reversal by PPARγ gene introduction. Data represent typical results from experiments repeated four times.

Japan), goat polyclonal anti-CTGF (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) and goat polyclonal anti-fibronectin (1:100 in PBS; Santa Cruz Biotechnology) were used as primary antibodies. Fluorescein isothiocyanate (FITC)-conjugated specific secondary antibodies (1:100 in PBS, ICN-Cappel, Aurora, OH) were used for detection of the primary antibody and 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA) was used for nuclear counterstaining.

Conjunctival Injury in C57BL/6 Mice and PPARγ Gene Transfer to Injured Tissue

We have reported the efficacy of gene transfer as evaluated by coinfection of Cre-Ad and GFP-Ad (data not shown).[10] PPARγ expression in the conjunctival wound-healing mouse model were evaluated by real-time RT-PCR and fluorescence microscopy.

A circumferential incision was made with scissors, in the conjunctiva at the equator in the right eye of generally anesthetized adult C57BL/6 mice (n = 72). A mixture of Cre-Ad and PPARγ-Ad was administered (3 μL, 2 × 10^7 PFU/μL) once after the incision (PPARγ-Ad group). Preliminary experiments showed no obvious difference in the histology or in healing at the microscopic level in mechanically injured mouse eye with CAG/Cre virus (Cre-Ad group) or without application of adenovirus carrying Cre (no vector group). Thus, the eyes of the Cre-Ad group mice were used as the control in the present study. On 2, 5, 7, and 14 days (each n = 18), eyes were enucleated and processed for histologic or immunohistochemical examinations to evaluate tissue scarring.

Histology and Immunohistochemistry

Deparaffinized sections (5 μm thick) were stained with hematoxylin and eosin (HE) or processed for in direct immunofluorescence microscopy, as reported previously.[10] Goat polyclonal anti-CTGF antibody (1:100 dilution in PBS, Santa Cruz Biotechnology), rat monoclonal anti F4/80 macrophage antigen antibody (clone A3-1, 1: 400 dilution in PBS; BMA Biomedicals, Augst, Switzerland) and mouse monoclonal anti-α-smooth muscle actin (αSMA) antibody (1:100 dilution in PBS; Neomarker, Fremont, CA) were used as primary antibodies. Reaction with FITC-conjugated secondary antibodies and DAPI nuclear staining were performed as described earlier.

Expression of mRNA of Col 1A2, CTGF, and Monocyte Chemotactic Protein (MCP)-1

For RNA extraction and real-time RT-PCR, mechanically injured eyes from 32 mice were obtained from each treatment group. Total RNA

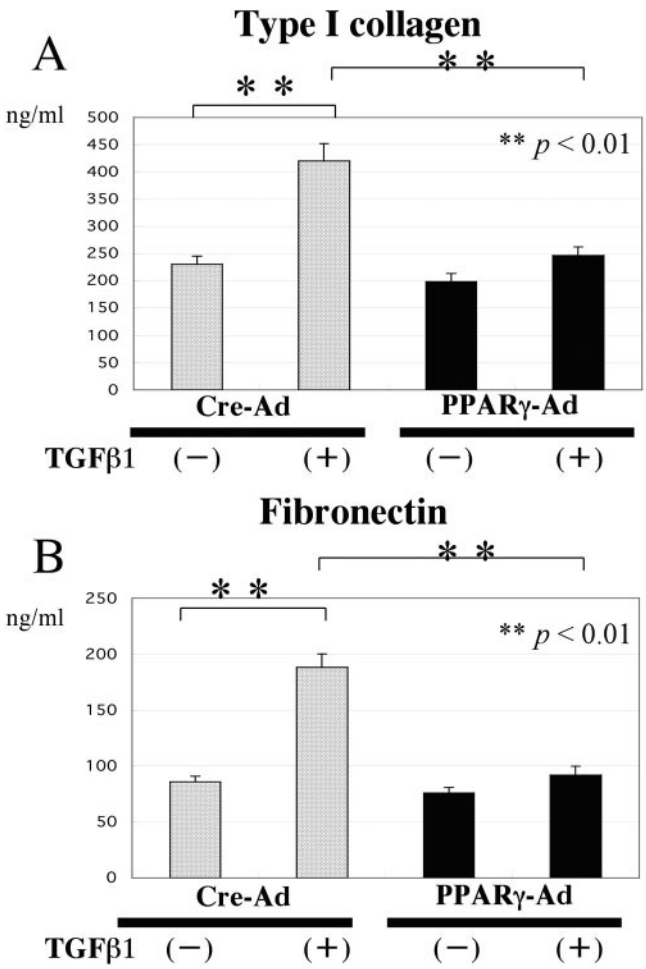

FIGURE 3. Effects of PPARγ gene introduction on productions of type I collagen and fibronectin by cultured hSCFs. ELISA showed an increase in the expression of type I collagen in the presence of exogenous TGFβ1 and its reversal by PPARγ gene introduction. TGFβ1 enhanced the secretion of type I collagen into the culture medium. PPARγ gene transfer reduced the production of type I collagen in the TGFβ1-treated cells to near-control levels (**A**). Similar tendencies were observed in fibronectin productions (**B**).

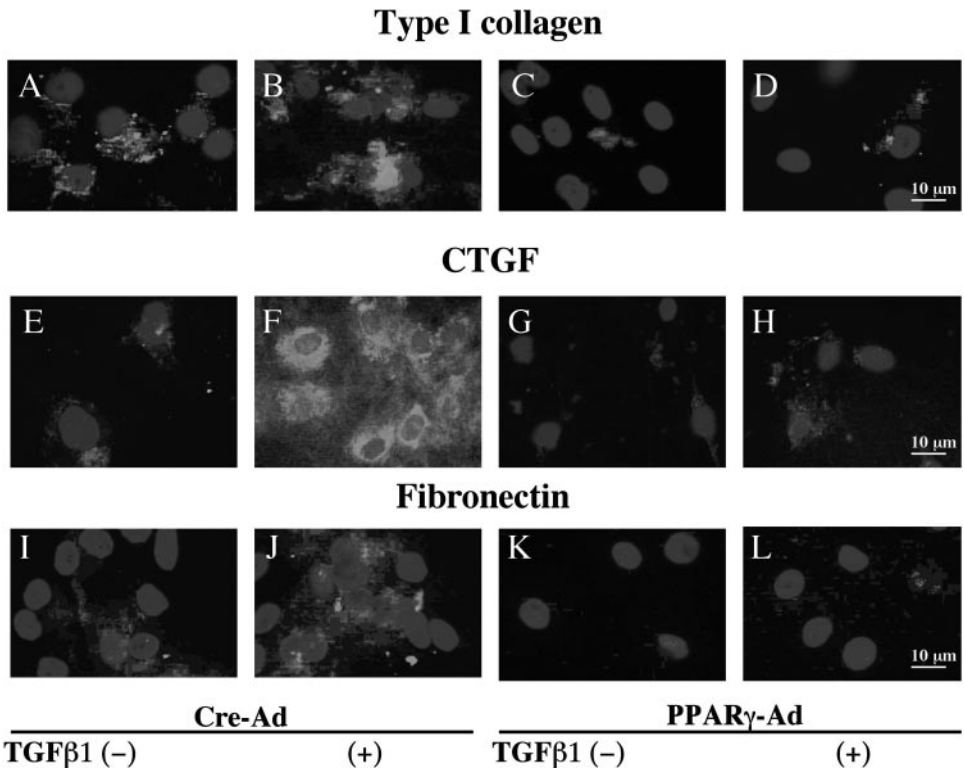

FIGURE 4. Immunolocalization of type I collagen, CTGF and fibronectin after PPARγ gene introduction by cultured hSCFs. Immunoreactivity for type I collagen was detected in most cells in the control culture (**A**), and more intense reactivity was seen in the cells treated with TGFβ1 (**B**). PPARγ gene transfection decreased the immunoreactivity for type I collagen in the cells in the absence (**C**) and presence (**D**) of TGFβ1. Similar tendencies were observed in the expression patterns of CTGF (**E–H**) and fibronectin (**I–L**).

extraction from each eye cup and real-time RT-PCR for mRNAs of mouse Col 1A2, CTGF, or MCP-1 were performed as described earlier. Primers and oligonucleotide probes were designed according to the cDNA sequences in the GenBank database[10,11,25] (Primer Express software; Applied Biosystems, Foster City, CA), as reported previously (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

RESULTS

Efficacy of Viral Gene Transfer into hSCFs and Mouse Model

Real-time RT-PCR (Figs. 1A, 1C) showed upregulated expression of PPARγ mRNA after gene transfer, both in vitro and in vivo. When the cultured fibroblasts were incubated in a medium containing Cre-Ad and PPARγ-Ad, immunoreactivity of PPARγ was observed on day 2 and later in the nucleus of cultured human hSCFs. In hSCFs treated with only Cre-Ad, immunoreactivity of PPARγ was not observed (Fig. 1B).

In case of in vivo gene introduction, immunoreactivity for PPARγ was detected in the nuclei of both conjunctival fibroblasts and epithelium of a wound-healing mice model at day 2 and later (up to 14 days). In eyes with CAG-Cre vector only, immunolocalization of PPARγ was not detected (Fig. 1D). Data represent a typical result obtained from experiments repeated four times.

Effects of PPARγ Gene Introduction on the Expressions of Type I Collagen, CTGF and Fibronectin by Cultured hSCFs

We used TGFβ1-treated SCF cultures to mimic scarring by the tissue fibroblasts in vitro. Real-time RT-PCR (Fig. 2) showed that the addition of TGFβ1 upregulated expression of Col 1A2 and CTGF mRNA. TGFβ1-promoted Col 1A2 mRNA expression was suppressed by PPARγ overexpression. The expression pattern of CTGF mRNA was similar to that of Col 1A2; it was enhanced by adding TGFβ1, and this upregulation was counteracted by PPARγ overexpression. Figure 3 show the concentrations of type I collagen and fibronectin as measured by ELISA. TGFβ1 enhanced the secretion of type I collagen and fibronectin in the culture medium. In the medium, the concentrations of type I collagen and fibronectin in the TGFβ1-treated culture were 182.6% and 218.6% of control cultures, respectively. PPARγ gene transfer reduced the production of type I collagen and fibronectin in the TGFβ1-treated cells to near control levels. The type I collagen and fibronectin concentrations in the medium were 58.6% and 48.9% of the TGFβ1-treated hSCFs, respectively, in both PPARγ-Ad and TGFβ1-treated culture.

Immunohistochemical analysis detected expression of type I collagen, CTGF, and fibronectin in the hSCF culture. Immunoreactivity for type I collagen was detected in most of the cells in the control culture (Fig. 4A), and more intense reactivity was seen in the cells treated with TGFβ1 (Fig. 4B), whereas it was suppressed in the cells with PPARγ overexpression both in the presence and absence of exogenous TGFβ1 (Figs. 4C, 4D).

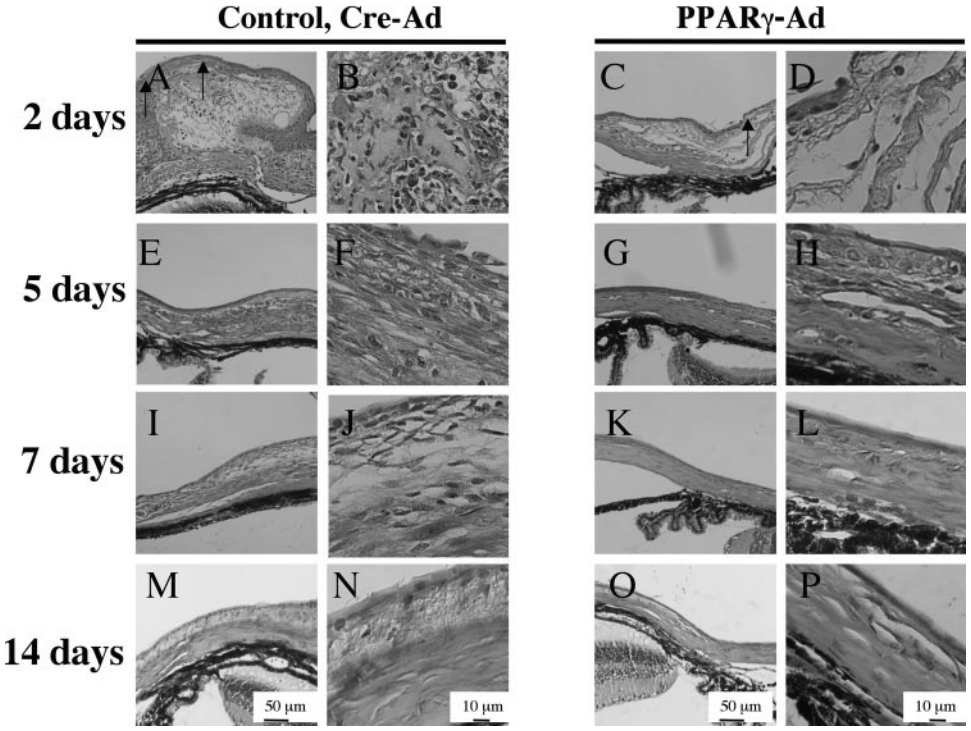

FIGURE 5. Histology of injured conjunctiva. HE staining showed that the defect of conjunctival epithelia remained on day 2 in the control group (**A**, **B**), while such defect was minimal in the PPARγ-Ad group (**C**, **D**). Arrows indicate the interruption of healing epithelium. On day 5, epithelial defect was closed in both the groups (**E–H**). The point of the original injury was not identified with regenerated epithelia. On day 5 (**E–H**) and day 7 (**I–L**), PPARγ gene introduction seemed to suppress the degree of edema and inflammatory cell infiltration in subconjunctival connective tissue, but not on day 14. On day 14, a histologic difference was not marked between both treatment and control groups; both showed reduced cell population and reduction in the thickness of subconjunctival matrix (**M–P**).

Expression patterns of CTGF (Figs. 4E–H) and fibronectin (Figs. 4I–L) demonstrated similar tendencies.

Histology and Immunohistochemistry

HE staining revealed the distribution of inflammatory cells to be more marked in the control group than in the PPARγ-Ad group until day 7 of the observations (Fig. 5). The defect in the conjunctival epithelia remained unclosed on day 2 in the control group (Figs. 5A, 5B), whereas such defects were minimal in the PPARγ-Ad group (Figs. 5C, 5D). On day 5 and later, the epithelial defects were closed in both the groups (Figs. 5E–L). The point of the original injury was not identified with regenerated epithelia. On day 14, the histologic difference was not marked between the treatment and control groups; both showed reduced cell population and reduction in the thickness of subconjunctival matrix (Figs. 5M–P).

To further characterize the effects of PPARγ gene transfer on conjunctival healing, we performed immunohistochemical analysis. Fibrogenic reaction, macrophage invasion, and generation of myofibroblasts were examined by immunodetection of CTGF, F4/80 antigen, and αSMA, respectively (Fig. 6). αSMA is the hallmark of myofibroblast generation and development of fibrotic tissue. Immunohistochemical analysis of αSMA showed that many fibroblasts in the subconjunctiva were labeled with anti-αSMA antibody in the control Cre-Ad specimens, but only a few fibroblasts in PPARγ-Ad specimens were labeled on day 5 (Figs. 6A, 6C). On day 7, a few cells were still positive in the control, but not in the PPARγ-Ad group (Figs. 6B, 6D). Invasion of F4/80-labeled macrophages and protein expression of CTGF were all decreased in the PPARγ-Ad group compared with the Cre-Ad group (Figs. 6E–L). PPARγ overexpression suppressed protein expression of CTGF, invasion of macrophages into the healing subconjunctival tissue, and generation of myofibroblasts.

Expression of mRNA of Col 1A2, CTGF, and MCP-1

Real-time RT-PCR was performed to further evaluate the fibrogenic reaction in the conjunctiva. Expressions of Col 1A2, CTGF, and MCP-1 mRNAs were higher in the control Cre-Ad group than in the PPARγ-Ad group at day 5 and 14 (Fig. 7).

DISCUSSION

In the present study, we first demonstrated that the adenoviral gene transfer of PPARγ suppresses fibrogenic behavior in hSCFs in vitro. PPARγ gene transfer suppressed expression of Col 1A2 and CTGF in hSCFs both in the presence as well as absence of TGFβ1, the major growth factor involved in conjunctival scarring/fibrosis. These findings suggest that PPARγ overexpression may have an antifibrosis effect in injured conjunctiva in vivo. Therefore, we subsequently evaluated its beneficial effect by using a mouse model of mechanical conjunctival injury. Similar therapeutic potential of PPARγ gene introduction via adenoviral vector has been reported in an experimental colitis and liver fibrosis model as well as in a corneal alkali burn mouse model.[23,24] As previously reported, overexpression of the PPARγ gene exhibited its effects without further addition of its ligands in the injured conjunctiva.[25–27]

Conversion of fibroblasts to myofibroblasts, as characterized by αSMA expression, is an important element in subconjunctival tissue scarring and is associated with upregulation of matrix components.[30–33] In the present study, immunohistochemical analysis showed that treatment with PPARγ-Ad suppressed myofibroblast generation in tissue. Real-time RT-PCR further showed that PPARγ over-expression suppressed expression of CTGF and MCP-1 mRNAs and potent monocyte/macrophage chemoattractant proteins, further confirming the antifibrogenic and anti-inflammatory effects of PPARγ signaling. Inflammatory cells such as macrophages also have important roles in the development of tissue scarring during the wound-healing process.[34–36] It is believed that invasive macrophages are one of the main sources of cytokines, including TGFβ, that activate local fibroblasts and induce their transformation into myofibroblasts. Our previous study showed that PPARγ overexpression suppressed activation of monocytes/macrophages in response to exogenous TGFβ1 exposure in

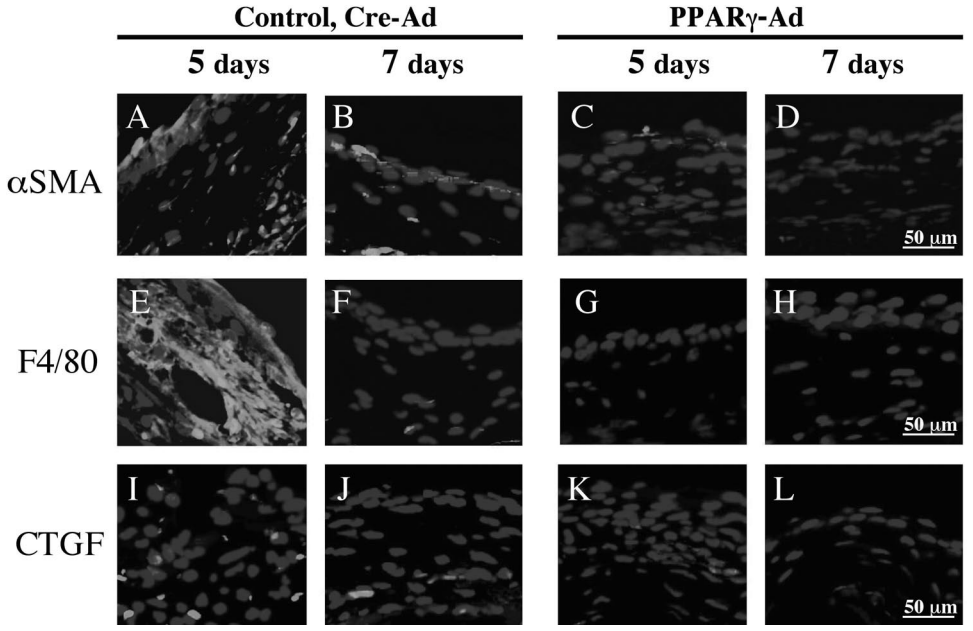

FIGURE 6. Immunohistochemical analysis of injured conjunctiva. CTGF invasion (**I–L**) of F4/80-labeled macrophages (**E–H**) as well as αSMA (**A–D**) expression by fibroblasts was decreased in the PPARγ-Ad-treated group compared with the control group.

vitro. The present study demonstrates that a similar effect is observed in in vivo conjunctiva. PPARγ-Ad treatment also suppressed recruitment of monocytes/macrophages to the healing subconjunctival tissue. Suppression of MCP-1 may account for the reduction of monocytes/macrophages in the healing tissue.

Modulation of cytoplasmic signaling by PPARγ is not fully understood and requires further investigation. Nevertheless, cross-talk between PPARγ-linked signaling and other signal(s) derived from various ligands/receptors have been partially clarified. For example, PPARγ receptor–derived signal activates AP-1, MAP kinase/Erk, and p38 MAP kinase, which in turn

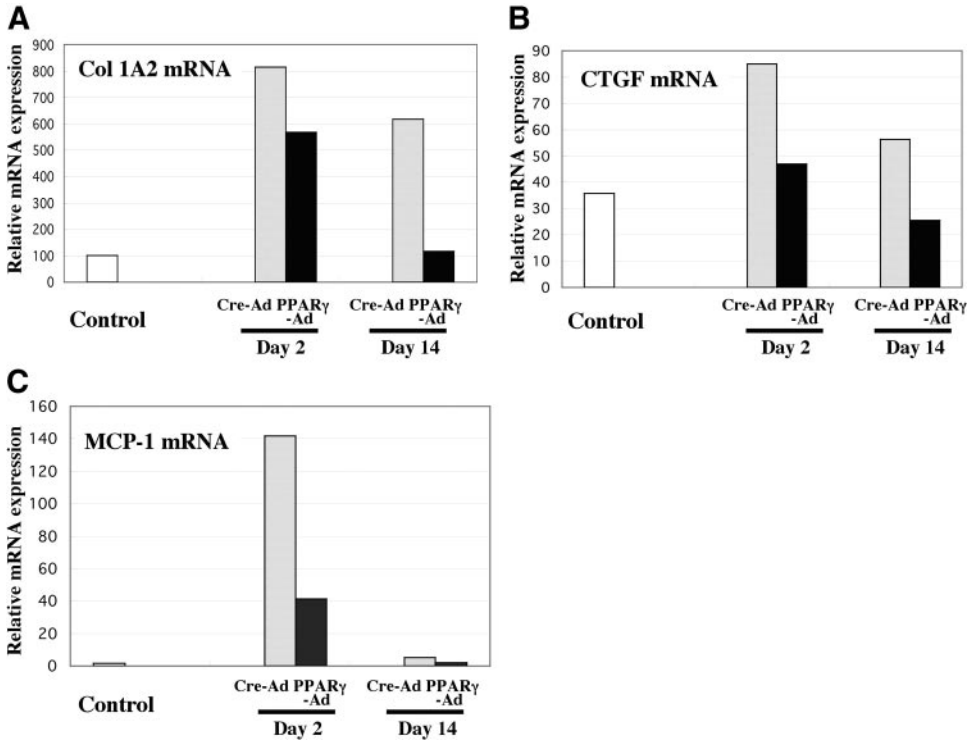

FIGURE 7. Expression of mRNA of Col 1A2, CTGF, and MCP-1. Expressions of mRNA of Col 1A2 (**A**), CTGF (**B**), and MCP-1 (**C**) were much higher in the control group at day 5 and 14 than that in the PPARγ-Ad–treated group. Data represent typical results from experiments repeated four times.

exhibit complex cross-talk.[12–22,37] We and others have reported that PPARγ signaling inhibits TGFβ/Smad signaling, which plays a significant role in the fibrogenic reaction in conjunctiva/subconjunctiva, in fibroblasts, and in cultured macrophages in vitro.[25,26]

It has been reported that activation of PPARγ signaling is of a therapeutic value in fibrotic diseases in other organs, such as the liver.[17–19,22,24] Our present study indicates that such a strategy of activation of PPARγ signal may be effective in suppression of excess scarring in posttrabeculectomy conjunctiva, regardless of the method of activation, whether by PPARγ ligand administration or overexpression of the receptor.

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