Inhibition by All-Trans-Retinoic Acid of Transforming Growth Factor-β–Induced Collagen Gel Contraction Mediated by Human Tenon Fibroblasts

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PURPOSE. Excessive wound contraction can lead to scar formation in the conjunctiva. The effects of all-trans-retinoic acid (ATRA) on the contractility of human Tenon fibroblasts (HTFs) cultured in three-dimensional (3D) collagen gels were investigated.

METHODS. Human Tenon fibroblasts were cultured in 3D gels of type I collagen and in the absence or presence of TGF-β, ATRA, or various inhibitors. Collagen gel contraction was evaluated by measurement of gel diameter. Phosphorylation of various signaling molecules was examined by immunoblot analysis. The formation of actin stress fibers and focal adhesions was detected by laser confocal microscopy.

RESULTS. All-trans-retinoic acid inhibited TGF-β–induced collagen gel contraction mediated by HTFs in a concentration- and time-dependent manner. The TGF-β–induced phosphorylation of focal adhesion kinase (FAK) and formation of stress fibers and focal adhesions in HTFs were attenuated by ATRA. All-trans-retinoic acid also inhibited the TGF-β–induced phosphorylation of the mitogen-activated protein kinases (MAPKs) extracellular signal–regulated kinase (ERK), p38, and c-Jun NH₂-terminal kinase (JNK) as well as that of c-Jun and Smad2/3. Furthermore, TGF-β–induced collagen gel contraction was blocked by inhibitors of ERK, p38, or JNK signaling.

CONCLUSIONS. All-trans-retinoic acid inhibited TGF-β–induced collagen gel contraction mediated by HTFs, most likely by attenuating the formation of actin stress fibers and focal adhesions as well as signaling by MAPKs, c-Jun, and Smads. All-trans-retinoic acid may therefore prove effective for inhibition of conjunctival scarring through attenuation of the contractility of Tenon fibroblasts.

Keywords: Tenon fibroblasts, all-trans-retinoic acid, transforming growth factor-β, wound healing

 Conjunctival scarring is a final common pathway for a wide spectrum of ocular diseases including cicatricial conditions such as trachoma and pemphigoid as well as for traumatic injuries such as chemical burns. Such scarring is also important in conditions in which the results of treatment depend on the healing response after surgery, such as pterygia and strabismus. Wound healing in the conjunctiva is characterized by inflammation followed by re-epithelialization, synthesis of new extracellular matrix, wound contraction, and the formation of a subconjunctival fibrous scar.¹ Excessive contraction of subconjunctival tissue at the wound site can result in excessive scarring. The cytokine TGF-β is thought to be a key factor in the regulation of wound healing and a major driving force of scar formation.² ³ The binding of TGF-β to its heterodimeric receptor activates intracellular signaling cascades, such as the canonical Smad and mitogen-activated protein kinase (MAPK) pathways, and such signaling molecules are major targets of evolving antifibrotic strategies.

All-trans-retinoic acid (ATRA), a derivative of vitamin A, is a potent regulator of the growth and differentiation of as well as matrix production by various cell types.⁴ ⁵ All-trans-retinoic acid possesses not only anti-inflammatory properties as a result of its specific inhibition of nuclear factor-κB (NF-κB) signaling,⁶ but also antifibrotic potential as a result of its attenuation of TGF-β actions.⁷ It was found to inhibit signaling by bone morphogenetic protein, a member of the TGF-β superfamily of proteins, by promoting the ubiquitin-dependent degradation of phosphorylated Smad1.⁸ A derivative of ATRA was shown to inhibit TGF-β–induced liver fibrosis via suppression of promoter activity at the collagen 1A2 gene.⁹ 9-Cis-retinoic acid, an isomer of ATRA, attenuates the TGF-β–induced production of several profibrotic molecules including fibronectin and plasminogen activator inhibitor-1 (PAI-1) in cultured human mesangial cells.⁷ These observations suggested the possibility that ATRA might modulate subconjunctival wound healing by targeting TGF-β signaling.

Human Tenon fibroblasts (HTFs) play an essential role in subconjunctival wound healing. Topical application of retinoic acid has been shown to attenuate squamous metaplasia and keratinization of the conjunctiva.¹⁰ ¹¹ However, such treatment did not inhibit the chemotactic effect of fibronectin on rabbit Tenon fibroblasts.¹² Contraction of collagen gels...
Effects of ATRA on HTFs

Media and reagents

Pharmacia Biotech (Uppsala, Sweden). All media and reagents were obtained from Amersham (Arlington Heights, IL, USA). Nitrocellulose membranes and an enhanced chemiluminescence (ECL) kit were obtained from Amersham (Arlington Heights, IL, USA). Latanoprost was obtained from Pharmacia Biotech (Uppsala, Sweden) and was reconstituted in saline. Latanoprost was freshly prepared before each determination. The antiglaucoma drug latanoprost induced collagen gel contraction mediated by HTFs. With the use of this model system, we have now examined the effect of ATRA on TGF-β-induced collagen gel contraction mediated by HTFs. The effects of ATRA on phosphorylation of the MAPKs extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK) and of c-Jun, Smads, focal adhesion kinase (FAK), and paxillin as well as on the formation of actin stress fibers and focal adhesions in HTFs were also investigated.

Methods

Materials

Eagle's minimum essential medium (MEM), 10% MEM, and fetal bovine serum (FBS) were obtained from In Vitrogen-Gibco (Rockville, MD, USA), and 24-well culture plates and cell culture flasks were from Corning (Corning, NY, USA). Native porcine type I collagen (acid solubilized) and reconstitution buffer were obtained from Nitta Gelatin (Osaka, Japan); bovine serum albumin (BSA) was from Nacalai Tesque (Kyoto, Japan); and ATRA, a product of the inhibitor cocktail, collagenase, and antibodies to β-actin and to α-smooth muscle actin (α-SMA) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Antibodies to ERK1 or -2 (ERK1/2), to phosphorylated ERK1/2, to p38, to phospho-p38, to JNK, to phospho-JNK, to Smad2, to phospho-Smad2, to Smad3, to phospho-Smad3, to c-Jun, to phospho-c-Jun, to phospho-FAK (phospho-Tyr576/577), and to phospho-paxillin (phospho-Tyr118) were obtained from Cell Signaling (Beverly, MA, USA); those to FAK and to paxillin were from BD Biosciences (San Jose, CA, USA); and those to ERK1 or -2 (ERK1/2), to phosphorylated ERK1/2, to p38, to phospho-p38, to JNK, to phospho-JNK, to Smad2, to phospho-Smad2, to Smad3, to phospho-Smad3, to c-Jun, to phospho-c-Jun, to phospho-FAK (phospho-Tyr576/577), and to phospho-paxillin (phospho-Tyr118) were obtained from Cell Signaling (Beverly, MA, USA).

Isolation and Culture of HTFs

Human subconjunctival Tenon fibroblasts were obtained with informed consent from individuals undergoing strabismus surgery. The human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. The patients had no history of systemic or conjunctival diseases and did not take any topical ocular medications. The subconjunctival tissue was digested for 1 hour at 37°C with collagenase (2 mg/mL) to provide a suspension of Tenon fibroblasts. The cells from each patient were cultured separately in MEM supplemented with 10% FBS, and they were used for the present study after three to eight passages.

Assay of Collagen Gel Contraction

Collagen gels were prepared as described previously. In brief, type I collagen (3 mg/mL), 10% MEM, reconstitution buffer, and HTF suspension (1.1 × 10^6 cells/mL in MEM) were mixed on ice in the volume ratio of 3:1:1:2. A portion (0.5 mL) of the mixture was added to each well of a 24-well culture plate that had been coated with 1% BSA for 1 hour at 37°C. The mixture was allowed to solidify, and the collagen gels were then freed from the sides of the wells with a micropatula. Serum-free MEM (0.5 mL) containing TGF-β, ATRA, or various inhibitors were then added on top of each gel. The diameter of each gel was measured three times with a ruler, and the mean value was calculated. The diameter of the gels was measured daily, and the extent of gel contraction was calculated by subtraction of the diameter at each time point from the initial diameter. A minimum of three gels was assayed for each experimental condition, and all experiments were repeated at least three times.

Immunoblot Analysis

Immunoblot analysis was performed as described previously. For immunoblot analysis of FAK, paxillin, and α-SMA, HTFs cultured in collagen gels were lysed with an ice-cold lysis buffer. For immunoblot analysis of MAPKs, Smads, and c-Jun, HTFs (5 × 10^5 cells) were cultured in 60-mm dishes for 24 hours in MEM supplemented with 0.5% FBS and then for an additional 24 hours in serum-free medium. They were then treated with ATRA (1 μM) for 12 hours before exposure to TGF-β (1 ng/mL) for various times at 37°C followed by cell lysis. All cell lysates were subjected to SDS-PAGE on a 10% gel. The separated proteins were transferred electrophoretically to a nitrocellulose membrane, nonspecific sites of which were blocked before incubation with primary antibodies (each at a 1:1000 dilution). Immune complexes were then detected with the use of horseradish peroxidase–conjugated secondary antibodies and ECL reagents.

Fluorescence Microscopy

Staining of F-actin and phosphotyrosine in HTFs was performed as described previously. In brief, cells in collagen gels were fixed for 30 minutes at room temperature with 1% paraformaldehyde in PBS, allowed to dry, and then permeabilized for 30 minutes with 1% Triton X-100 in PBS. After nonspecific sites were blocked with 1% BSA in PBS (PBS-BSA), the cells were incubated at room temperature first for 1 hour with antibodies to phosphotyrosine (1:200 dilution in PBS-BSA) and then for 30 minutes with Alexa Fluor 488–conjugated secondary antibodies (1:1000 dilution in PBS-BSA). Finally, the cells were incubated for 30 minutes with Alexa Fluor 568–conjugated phalloidin (1:200 dilution in PBS-BSA) to stain F-actin and then for 10 minutes with TOTO-3 iodide (3:1000 dilution in PBS-BSA) to stain nuclei. The cells were examined with a laser-scanning confocal microscope (Axiovert 200M; Carl Zeiss, Tokyo, Japan).

Statistical Analysis

Data are presented as means ± SD. All experiments were performed at least in triplicate and were repeated at least three times. Gel contraction data were subjected to two-way analysis of variance followed by the Tukey-Kramer test with the use of statistical analysis software (StatView for Windows, ver. 5.0; SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Effect of ATRA on TGF-β-Induced Collagen Gel Contraction Mediated by HTFs

We first examined the effect of ATRA on TGF-β-induced collagen gel contraction mediated by HTFs. Incubation of the
cells for 3 days with various concentrations of ATRA (0.01–1 μM) revealed that the drug inhibited TGF-β-induced gel contraction in a concentration-dependent manner (Fig. 1A); the effect was statistically significant at 0.01 μM and maximal at 1 μM. The effect of ATRA (1 μM) on TGF-β-induced gel contraction mediated by HTFs was also dependent on the time of incubation (Fig. 1B), becoming statistically significant at 2 days.

Effects of ATRA on Phosphorylation of FAK and Paxillin and α-SMA Expression in HTFs Cultured in Collagen Gels

We next examined whether ATRA might affect the phosphorylation of FAK and paxillin as well as the expression of α-SMA in HTFs cultured in collagen gels. Immunoblot analysis revealed that TGF-β (1 ng/mL) induced the phosphorylation of FAK and paxillin in HTFs, and that this effect on FAK phosphorylation, but not that on paxillin phosphorylation, was inhibited by ATRA in a concentration-dependent manner (Fig. 2A). The abundance of FAK or paxillin was not affected by TGF-β or ATRA. Culture of the cells for 3 days in the presence of TGF-β (1 ng/mL) and various concentrations of ATRA had no effect on the abundance of α-SMA (Fig. 2B).

Effects of ATRA on TGF-β-Induced Formation of Stress Fibers and Focal Adhesions in HTFs Cultured in Collagen Gels

With the use of fluorescence microscopy, we next examined the effects of ATRA on the formation of stress fibers and of focal adhesions containing phosphotyrosine in HTFs (Fig. 3). Cells cultured in collagen gels for 3 days without TGF-β exhibited a dendritic morphology with no prominent stress fibers and few fluorescence signals specific for phosphotyrosine. Cells cultured in the presence of TGF-β (1 ng/mL) manifested pronounced actin stress fibers and a pronounced punctate pattern of phosphotyrosine immunofluorescence. Merging of the F-actin and phosphotyrosine fluorescence images suggested that phosphotyrosine was associated with sites of stress fiber formation. In contrast, cells exposed to TGF-
β in the presence of ATRA (1 μM) exhibited a distorted morphology with fewer stress fibers as well as a reduced extent both of phosphotyrosine staining and of focal adhesion formation (Fig. 3).

Effects of ATRA on TGF-β–Induced MAPK and c-Jun Phosphorylation in HTFs

To investigate the possible effects of ATRA on TGF-β–dependent signal transduction in HTFs, we first examined the levels of MAPK and c-Jun phosphorylation (activation; Fig. 4). Immunoblot analysis revealed that TGF-β (1 ng/mL) induced the rapid and transient phosphorylation of ERK1/2 and JNK, and that these effects were inhibited by the presence of 1 μM ATRA. The effect of TGF-β on the phosphorylation of p38 was maximal at approximately 1 hour after the onset of stimulation and persisted for up to 24 hours. The activation of p38 from 6 to 24 hours was attenuated in the presence of ATRA. Transforming growth factor–β also induced the activation of c-Jun in a time-dependent manner, with the maximal effect being apparent at 24 hours. This activation of c-Jun was greatly inhibited in the presence of ATRA. The abundance of ERK1/2, p38, JNK, or c-Jun was not affected by TGF-β or ATRA.

Effects of MAPK Inhibitors on TGF-β–Induced Collagen Gel Contraction Mediated by HTFs

We next examined whether MAPK inhibitors affected TGF-β–induced collagen gel contraction mediated by HTFs. The cells were incubated with 30 μM PD98059 (ERK signaling inhibitor), 10 μM SB203580 (p38 inhibitor), or 10 μM JNK inhibitor II for 1 hour before exposure to TGF-β (1 ng/mL) for 3 days. The stimulatory effect of TGF-β on collagen gel contraction was inhibited by 28%, 55%, or 72% in the presence of PD98059, SB203580, or JNK inhibitor II, respectively (Fig. 5). These MAPK inhibitors had no effect on HTF-mediated collagen gel contraction in the absence of TGF-β.

Effects of ATRA on TGF-β–Induced Smad2 and Smad3 Phosphorylation in HTFs

Finally, we examined whether ATRA might affect the phosphorylation of Smad2 and Smad3 induced by TGF-β in HTFs (Fig. 6). Immunoblot analysis revealed that TGF-β (1 ng/mL) induced the phosphorylation (activation) of Smad2 and Smad3 in a time-dependent manner. The activation of Smad2 was first apparent at 30 minutes, was maximal at 1 hour, and persisted for up to 24 hours after the onset of stimulation, and this effect of TGF-β was attenuated in the presence of 1 μM ATRA. The activation of Smad3 was first apparent at 30 minutes after the onset of TGF-β stimulation and persisted for up to 24 hours. The activation of Smad3 by TGF-β was inhibited by ATRA at later time points (6–24 hours). The abundance of Smad2 or Smad3 was not affected by either TGF-β or ATRA.

DISCUSSION

Prevention or modulation of conjunctival scarring remains a clinical challenge in ophthalmology. Recent studies into alternative methods for preventing such tissue fibrosis have focused on the regulation of HTFs by various growth factors, on fibroblast proliferation, or on inflammatory processes. Transforming growth factor–β has become a major target for modulation of the wound healing response. The vitamin A derivative ATRA was recently shown to suppress the contractility of cultured retinal pigment epithelial cells. In the present study, ATRA inhibited TGF-β–induced collagen gel contraction mediated by HTFs in a concentration- and time-dependent manner, consistent with previous studies showing that it inhibited collagen gel contraction mediated by dermal fibroblasts or human gingival fibroblasts. The formation of actin stress fibers and focal adhesions contributes to TGF-β–induced cell contractility. Consistent with previous observations, we found that TGF-β induced cell spreading as well as the formation both of stress fibers and

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effects of ATRA on TGF-β–induced formation of stress fibers and of focal adhesions containing phosphotyrosine in HTFs cultured in collagen gels. Cells embedded in collagen gels were incubated first for 12 hours in the absence or presence of 1 μM ATRA and then for 3 days in the additional absence or presence of TGF-β (1 ng/mL). The cells were then stained with Alexa Fluor 568–labeled phalloidin (for F-actin, red) as well as with antibodies to phosphotyrosine and Alexa Fluor 488–conjugated secondary antibodies (green). Nuclei were stained blue with TOTO-3. Scale bars: 50 (low magnification) or 20 μm (high magnification). Data are representative of three independent experiments.
FIGURE 4. Effects of ATRA on the time course of MAPK or c-Jun phosphorylation induced by TGF-β in HTFs. Cells were cultured for 24 hours in MEM supplemented with 0.5% FBS and then for an additional 24 hours in serum-free medium. They were then incubated first with or without ATRA (1 μM) for 12 hours and then in the additional presence of TGF-β (1 ng/mL) for the indicated times. Cell lysates were then subjected to immunoblot analysis with antibodies to total or phosphorylated (p-) forms of ERK, p38, JNK, or c-Jun. Data are representative of three independent experiments.

FIGURE 5. Effects of MAPK inhibitors on TGF-β–induced collagen gel contraction mediated by HTFs. Cells were incubated first for 1 hour in the absence or presence of 30 μM PD98059, 10 μM SB203580, or 10 μM JNK inhibitor II and then for 3 days in the additional absence or presence of TGF-β (1 ng/mL), after which the change in diameter of each gel was measured. Data are means ± SD from four independent experiments. *P < 0.01 versus the corresponding value for cells cultured without TGF-β; †P < 0.05 versus the corresponding value for cells cultured with TGF-β alone (Tukey-Kramer test).
of focal adhesions containing phosphotyrosine in HTFs. All-trans-retinoic acid inhibited these effects of TGF-β, with such inhibition likely playing an important role in its attenuation of TGF-β-induced contraction in these cells. The activation of tyrosine kinases in fibroblasts contributes to signaling events that underlie the formation of focal adhesions induced by TGF-β. Focal adhesion kinase and paxillin are components of focal adhesions and undergo tyrosine phosphorylation in response to TGF-β stimulation. We have now shown that ATRA inhibited the TGF-β-induced phosphorylation of FAK, but not that of paxillin, in HTFs in a concentration-dependent manner. During wound contraction of granulation tissue, fibroblasts undergo a phenotypic transformation into myofibroblasts, which are characterized by their expression of the contractile protein α-SMA. The mechanism of matrix contraction appears to differ depending on whether the matrix is mechanically floating or stressed at the time contraction is initiated, and tension generated within the matrix is thought to be required for the induction of α-SMA expression in cells. Transforming growth factor-β was thus found to upregulate α-SMA expression in fibroblasts cultured in a stressed collagen gel but not in those seeded in a floating collagen lattice. Consistent with these observations, we found that α-SMA expression in HTFs cultured in a floating collagen gel was not affected by TGF-β or ATRA.

In addition to the classical Smad cascade, TGF-β activates other signaling cascades, including MAPK signaling pathways. We have now shown that TGF-β induced a rapid and transient activation of ERK and JNK signaling pathways as well as a sustained activation of p38 and Smad2/3 signaling in HTFs. Inhibitors of p38 were previously shown to prevent collagen gel contraction mediated by HTFs. In the present study, TGF-β-induced collagen gel contraction mediated by HTFs was markedly suppressed by the MAPK signaling inhibitors PD98059 (a specific inhibitor of ERK1/2 activation), SB203580 (a selective p38 inhibitor), or JNK inhibitor II, suggesting that MAPKs play an important role in this effect of TGF-β. Both MAPK and Smad signaling pathways have been implicated in TGF-β-induced stress fiber formation and epithelial-to-mesenchymal transdifferentiation. A derivative of ATRA was previously shown to inhibit TGF-β-induced collagen expression by blocking Smad2/3 phosphorylation in hepatic stellate cells. Our findings that ATRA attenuated TGF-β-induced activation of MAPK and Smad cascades in HTFs suggest that these effects contribute to the inhibition by this agent of TGF-β-induced collagen gel contraction mediated by HTFs. The mechanisms of MAPK activation by TGF-β remain poorly characterized. Many molecules including Ras as well as various MAPK kinase kinases and MAPK kinases contribute to the rapid activation of MAPKs, whereas MAPK activation with slower kinetics may result from Smad-dependent transcriptional responses. The differences in the time courses of TGF-β-induced MAPK phosphorylation and the associated effects of ATRA in HTFs may reflect this complexity. Further studies are necessary to clarify the mechanisms of ATRA action in our model system.

The activation of various transcription factors, including activator protein–1 (AP-1), contributes to the regulation of cell function by TGF-β. Activator protein–1 is a heterodimer composed of members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) protein families, and the expression of these proteins is induced by a variety of stress signals and contributes to subsequent stress responses, including cell proliferation, inflammation, and wound healing. Inhibition of AP-1 signaling abrogates TGF-β-induced stress fiber formation in dermal fibroblasts and prevents experimental fibrosis in mice. In addition, AP-1 has been implicated in the regulation of smooth muscle contractility through effects on both myosin and the actin cytoskeleton. We found that the activation of c-Jun induced by TGF-β in HTFs was inhibited by ATRA, with this effect also likely contributing to the inhibition by ATRA of HTF contractility.

In summary, our study is the first to show that ATRA inhibits the TGF-β-induced contractility of HTFs cultured in a three-dimensional collagen gel. This action of ATRA is likely mediated by inhibition both of the formation of actin stress fibers and focal adhesions as well as of signaling by MAPKs, c-Jun, and Smads in these cells. All-trans-retinoic acid might thus prove effective for inhibiting scar formation during subconjunctival wound healing through attenuation of the contractility of Tenon fibroblasts. Further characterization of the effects of ATRA on Tenon fibroblasts and on wound healing in the conjunctiva in vivo is warranted.
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**References**