Thyroid eye disease is characterized by the infiltration of leukocytes and accumulation of hyaluronan (HA) in orbital tissue. Inflamed orbital tissue expands in size due to excessive HA and to the formation of scar tissue (fibrosis) and/or adipose accumulation. Transforming growth factor-β (TGF-β) acts as a key inducer of fibrosis by enhancing extracellular matrix production. Treatment of primary human orbital fibroblasts with TGF-β led to significant increases in both HA synthesis and secretion. TGF-β also strongly induced hyaluronan synthase 1 (HAS1) and HAS2 mRNA levels, which increased 50- and 6-fold, respectively. Remarkably, the addition of the peroxisome proliferator-activated receptor (PPARγ) ligands pioglitazone (Pio) or rosiglitazone (Rosi) to TGF-β-treated orbital fibroblasts attenuated HA synthesis and reduced HAS1 and HAS2 mRNA levels.

The attenuation of TGF-β function by Pio and Rosi was independent of PPARγ activity. Furthermore, Pio and Rosi treatment inhibited TGF-β-induced T cell adhesion to orbital fibroblasts. Our findings demonstrate that TGF-β plays an important role in HA synthesis and in the inflammatory response by enhancing or facilitating inflammatory cell infiltration and adhesion to orbital tissue. Pio and Rosi exhibit anti-fibrotic and anti-inflammatory activity and may be useful in treating thyroid eye disease.

Thyroid eye disease (TED) is a debilitating and sight-threatening disorder that commonly occurs in patients with hyperthyroidism (1, 2). TED is an autoimmune disease in which activated T cells infiltrate the orbital soft tissue (periorbital space) leading to drastic tissue remodeling. Enlargement of orbital tissue mass occurs through the accumulation of extracellular matrix (ECM), scar-forming myofibroblasts, and/or fat (3–5).

Patients develop periorbital swelling, protrusion of the eyes (exophthalmos), and extraocular-muscle dysfunction. In severe cases the increase in orbital mass and scarring leads to double vision, corneal exposure, and neuropathy, affecting visual functions (6–8).

The earliest stages of TED involve infiltration of the orbital tissue by T cells (5, 9, 10). Recent studies have uncovered the importance of intercellular communication between T cells and orbital fibroblasts in the onset of disease. T cell-fibroblast interactions are mediated through cytokines, adhesion molecules, and other co-stimulatory molecules (11–13). These interactions stimulate fibroblasts to deposit ECM (14, 15), proliferate (16, 17), and/or differentiate into myofibroblasts or adipocytes (12, 18, 19). For example, T cells can secrete the proadipogenic prostaglandin, 15-deoxy-D12,14-prostaglandin J2, to drive orbital fibroblast differentiation into adipocytes (12). Likewise, when orbital fibroblasts are activated, they release T cell chemoattractants and initiate interactions in which both cell types are further galvanized.

One of the key events initiating TED is considered to be excess synthesis of ECM by orbital fibroblasts (20). Hyaluronan (HA) is the major ECM glycosaminoglycan (GAG) in inflamed orbital tissue. HA can be produced by three different HA synthases (HAS) at the plasma membrane (21). The newly synthesized HA polymer can be secreted out of the cell or anchored to the cell surface through the activity of HAS enzymes. HA can also bind its cognate cell surface receptors CD44 or RHAMM (receptor for hyaluronic acid mediated motility) to form a pericellular coat (22). HA synthesis is implicated in the increase in orbital tissue volume due to its remarkable hydrophilic characteristics, resulting in severe swelling and vision impairment presented in TED (23). In addition to its structural functions, HA also mediates a variety of signaling events, including effects on cell behavior and cell-cell interactions (24). The interaction between HA and its primary cell surface receptor CD44, which is present on most circulating leukocytes (including T cells and monocytes), is important for recruitment of immune cells to sites of inflammation and cytokine release (25–28). The mechanism how HA mediates its biologic functions in TED is poorly understood. Here we hypothesize that in inflamed orbital tissues the accumulation of HA may affect numerous cellular functions involved in the pathogenesis of TED, including regulation of inflammatory cell adhesion to orbital connective tissue.

*This work was supported, in whole or in part, by National Institutes of Health Grant EY017123 from NEI. This work was also supported by a Research to Prevent Blindness Foundation grant.

1 Supported by NIEHS, National Institutes of Health Training Grant ES07026.

2 To whom correspondence should be addressed: Dept. of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Box 850, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-275-8326; Fax: 585-506-0239; E-mail: Richard_Phipps@urmc.rochester.edu.

3 The abbreviations used are: TED, thyroid eye disease; ECM, extracellular matrix; GAG, glycosaminoglycan; HABP, hyaluronan-binding protein; HA, hyaluronan; HAS, HA synthase; qRT, quantitative reverse transcriptase; PPARγ, peroxisome proliferator-activated receptor γ; Pio, pioglitazone; Rosi, rosiglitazone; SC, scrambled; TZD, thiazolidinedione; HA’ase, hyaluronidase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.
Increased transforming growth factor β (TGF-β) mRNA levels have been observed in orbital tissue from patients with TED (29). TGF-β is a key cytokine that regulates ECM production, inflammation, fibrosis, and tissue remodeling. Extracellular matrix molecules such as collagen, fibronectin, proteoglycans, matrix metalloproteases, and tissue transglutaminase are targets of TGF-β (30–32). HAS enzymes are also TGF-β targets (21, 33), and TGF-β-mediated HA synthesis has been reported in human orbital fibroblasts (15, 34). Hence, we speculated that blockage of TGF-β activity provides a therapeutic mechanism to inhibit fibrosis and HA production associated with periorbital edema in TED. Increasing evidence demonstrates that peroxisome proliferator-activated receptor γ (PPARγ) acts as a negative regulator of TGF-β (35). PPARγ is a ligand-activated transcription factor that modifies numerous pathways including promoting adipogenesis and insulin sensitivity while suppressing inflammation and fibrosis (36). PPARγ ligands, such as the thiazolidinedione (TZD) class of anti-diabetic drugs (e.g. pioglitazone, rosiglitazone, ciglitazone, and troglitazone), inhibit the expression of various inflammatory proteins like inducible nitric-oxide synthase, tumor necrosis factor-α (TNFα), and matrix metallopeptidase (MMP9) in macrophages (37) and suppress the fibrosis of lung fibroblasts (35, 38, 39), dermal fibroblasts (40), and retinal pigment epithelial cells (41). The mechanism of action of PPARγ ligands is under investigation but involves both PPARγ-dependent and PPARγ-independent pathways (35). Here we report the new finding that the PPARγ ligands pioglitazone (Pio) and rosiglitazone (Rosi) suppress TGF-β-induced HA production and HAS activation in human orbital fibroblasts through PPARγ-independent pathways. Pio and Rosi also attenuate TGF-β-mediated T cell adhesion to orbital fibroblasts by decreasing HA synthesis.

EXPERIMENTAL PROCEDURES

Materials

Rosiglitazone and GW9662 were purchased from Cayman Chemical (Ann Arbor, MI). Pioglitazone HCl was purchased from ChemPacific (Baltimore, MD). Recombinant human TGF-β1 was purchased from Calbiochem (EMD Bioscience, La Jolla, CA). [3H]Glucosamine hydrochloride was purchased from PerkinElmer Life Sciences. Hyaluronic acid potassium salt from human umbilical cord and Streptomyces hyaluronidase (HA’ase) were purchased from Sigma. Unlike other HA’ases, this enzyme is specific for HA and is not active with chondroitin and chondroitin sulfate substrates (42).

Tissue Collection and Orbital Fibroblast Cell Culture

Orbital Fibroblasts—Primary orbital fibroblasts were isolated from TED patients undergoing orbital decompression surgery. The protocol for orbital biopsy and blood sample isolation (see below) was approved by the Internal Review Board and informed; written consent was obtained from all patients. The primary fibroblasts were established by standard explant techniques (43, 44) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan UT), 2-mercaptoethanol (Eastman Kodak Co.), 1-glutamine (Invitrogen), HEPES (U. S. Biochemical Corp.), and nonessential amino acids, sodium pyruvate, and gentamicin (Invitrogen). Fibroblasts were characterized by their adherent morphology, expression of vimentin and collagen (types I and III), and the absence of CD45, factor VIII, and cytokeratin. Fibroblasts were used at the earliest passage possible (between passages 4–10).

Human Peripheral Blood T Cells—One unit of blood was obtained from healthy donors as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection. Peripheral blood mononuclear cells were obtained by density-gradient centrifugation of buffy coat using Ficoll-Paque Plus (Amersham Biosciences). Peripheral blood mononuclear cells were washed in PBS, and T cells were enriched using CD3/CD28 T cell Expander beads (Dynal Inc., Brown Deer, WI). Specifically, 5 × 10⁶ peripheral blood mononuclear cells were incubated with CD3/CD28 beads at ratio 1:1 in RPMI 1640 with 10% FBS medium at 37 °C for 2 days. After that, 50 units of recombinant IL-2/ml was added to the culture and incubated for another 2 or 3 days. On day 5, cells were diluted to 0.5 × 10⁶/ml in culture medium containing 50 units/ml recombinant IL-2 and incubated for another 3–7 days according to the cell number. After T cell expansion, the cells were examined for purity by staining with an anti-CD3 phycoerythrin-labeled antibody (BD Biosciences). The T cell purity was >95%.

Cell Viability Assay

Cell viability was assayed using the colorimetric XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) assay kit (Roche Applied Science). Briefly, orbital fibroblasts were seeded in a 96-well plate and cultured with various treatments. Twenty hours after treatment, 50 µl of XTT labeling mixture was added, and cells were incubated at 37 °C for 4 h. The amount of cleaved XTT product generated by metabolically active cells was assayed by measuring absorbance using an ELISA plate reader at 480 nm with a reference wavelength at 650 nm. Cell viability results were also confirmed by trypan blue staining.

Flow Cytometry

Enriched human peripheral blood T lymphocytes were surface-stained for CD3 (BD Biosciences) or CD44 (clone IM7, Biologend, San Diego, CA) for 20 min at 4 °C, washed in staining buffer (PBS with 0.3% BSA), and pelleted by centrifugation. Samples were run on a FACSCalibur (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star).

Quantitation of HA

Confluent monolayers of orbital fibroblasts were serum-starved in 0.5% FBS RPMI 1640 medium for 3 days and pretreated with different concentrations of Pio or Rosi for 1 h and then treated with or without 2 ng/ml TGF-β1 for 24 h. After treatments, an aliquot of culture medium (secreted HA) was removed and centrifuged at 8000 × g at 4 °C for 5 min, and the supernatant was saved for later analysis. The cells were washed twice with PBS and treated with 0.25% trypsin, EDTA solution (Invitrogen) at 37 °C for 5 min, the reaction was stopped by addition of culture medium, and cell number was determined using a hemocytometer. The cells were then centrifuged, and...
the supernatant was collected and incubated at 100 °C for 10 min to inactivate trypsin activity. This supernatant was saved for pericellular HA detection. The cell pellet was washed with PBS once and digested with 120 μg/ml proteinase K in 0.1% SDS, 0.1 M Tris-HCl (pH 7.6) at 37 °C for 1 h, and proteinase K was inactivated by incubation at 100 °C for 10 min. The cell lysate was centrifuged, and supernatant was analyzed for intra-cellular HA.

The amount of HA in each extract (secreted HA, pericellular HA, and intracellular HA) was measured by ELISA based on the specific interaction of HA with HA-binding protein (HABP). The HA detection kit was purchased from R&D Systems (Minneapolis, MN). Briefly diluted samples were incubated in HABP-coated microwells, allowing HA present in samples to react with the immobilized HABP. After extensive washing, biotinylated HABP was added to the microwells to form complexes with bound HA. After another round of washing, streptavidin-conjugated horseradish peroxidase was added. After a brief incubation period and washing, chromogenic substrate was added, and HA levels were determined using a Varioskan Flash plate reader (Thermo Fisher Scientific, Milford, MA). A fraction of the samples were pretreated with HA’ase (2 units/ml) before being subjected to the ELISA assay as a negative control. Furthermore, an additional 50 ng/ml (final concentration) of free soluble HA was added to a fraction of the cell lysate samples to confirm that there are no inhibitory factors present and that the assays show the expected additivity when a known amount of exogenous HA is included.


Confluent monolayers of orbital fibroblasts were serum-starved for 3 days and treated with or without 2 ng/ml TGF-β1 for 24 h. One hour after TGF-β1 treatment, [^3]H]glucosamine hydrochloride (PerkinElmer Life Sciences) was added to the medium to a final concentration of 20 μCi/ml. After labeling, the medium, trypsin extract solution, and cell lysate were collected as described above. Radiolabeled macromolecules in each extract were concentrated using a Vivaspin 10-kDa cut-off ultrafiltration spin column (Sartorius Stedim Biotech, Goettingen, Germany) and washed with PBS twice. An equivalent radiolabeled aliquot was digested with HA’ase (2 units/ml) at 37 °C overnight before concentration. The concentrated solution was transferred to scintillation vials, and ^3H incorporation was determined with a microplate scintillation counter (TopCount; PerkinElmer). Incorporation of[^3]H]glucosamine into HA was calculated by subtracting the counts from the HA’ase-digested fraction.

**T Cell Adhesion Assay**

Preparation of Peripheral Blood Mononuclear T Cells—Enriched T cells (1 × 10^7 cells/ml) were fluorescently labeled by incubation with calcein-AM (10 μg/ml, Molecular Probes) for 45 min in RPMI 1640 medium without phenol red and washed twice in RPMI 1640 medium with 0.5% FBS. After labeling, some T cells were incubated with monoclonal CD44 antibody (clone IM7, Biolegend) or isotype (rat IgG2b) at a dilution of 1:25 (final concentration is 40 μg/ml) at 37 °C for 15 min.

Preparation of Orbital Fibroblasts—Confluent orbital fibroblasts in a 96-well plate were serum-starved and incubated with different concentrations of Pio or Rosi treated with or without 2 ng/ml TGF1 for 24 h. In some cultures, orbital fibroblasts were treated with HA’ase (100 milliunits/ml at 37 °C for 1 h) before the adhesion assay to deplete endogenous HA.

Adhesion Test—Immediately before the adhesion assay, the conditioned medium of orbital fibroblasts was removed to eliminate treatments and secreted ECM. 1 × 10^5/well of calcine-AM-labeled T cells were added to a 96-well plate with a confluent orbital fibroblast monolayer and allowed to adhere for 90 min at 4 °C as previously described (45, 46). Plates were washed 3 times with the addition of 200 μl of PBS followed by plate inversion and gentle tapping to remove the wash solution. Fluorescence was measured with an excitation of 485 nm and detection of 535 nm in a Varioskan Flash plate reader (Thermo Electron Corp.). There is a positive correlation between the labeled T cell number and fluorescence intensity (r^2 is >0.998); thus, the number of T cells bound per well was calculated from the fluorescence intensity of the well.

**Immunohistology**

Orbital fibroblasts were cultured in 8-chamber slides (BD Biosciences), fixed in room temperature 3% paraformaldehyde in PBS, and stained with 1 μg/ml biotinylated HABP (Seikagaku, Cape Cod, MA) followed by Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). F-actin was stained with phalloidin (Molecular Probes). In some cultures, orbital fibroblasts were treated with HA’ase before staining. T cells were stained with an anti-CD3 monoclonal antibody (clone UCHT, SouthernBiotech, Birmingham, AL) followed by a goat anti-mouse antibody labeled with Alexa-488 (Molecular Probes). DAPI (10 μg/ml, Anaspec, San Jose, CA) was used for nuclear staining.

Orbital adipose tissues were obtained from TED patients undergoing orbital decompression surgery. The tissues were fixed in 3% paraformaldehyde in PBS at 4 °C for 1 h and washed with PBS. Tissues were then saturated in 20% sucrose, PBS at 37 °C for 2 h, embedded in OCT compound (Sakura Tissue Tek), and frozen in dry ice. Ten-μm-thick sections of frozen tissue were first incubated with biotinylated HABP, anti-CD44 and anti-CD3 monoclonal antibodies and the stained with Cy3-conjugated streptavidin, Alexa-488 conjugated donkey anti-rat antibody, and Alexa-647-conjugated goat anti-mouse antibody. Some sections were pretreated with 2 units/ml HA’ase to digest endogenous HA before staining to determine that staining was specific for HA. As an additional negative control, free soluble HA (200 μg/ml) was added to the HABP probe to block binding to endogenous HA. Fat was stained with Bodipy 493/503 dye (Molecular Probes). Slides were photographed using a Carl Zeiss Axio Imager M1 Microscope.

Reverse Transcriptase PCR (RT-PCR) and Quantitative RT-PCR (qRT-PCR)

RNA was isolated from orbital fibroblast strains using the RNeasy Mini kit according to the manufacturer’s protocol (Qiagen, Valencia, CA) and reverse-transcribed to cDNA.
PPARγ Ligands Inhibit T Cell Adhesion to Orbital Fibroblasts

PPARγ ligands were shown to inhibit TGF-β1-induced HAS1 and HAS2 mRNA expression—Increased synthesis of HA mediated by an induction of HAS gene expression in orbital fibroblasts has been described previously (29). To determine whether the PPARγ ligands Pio and Rosi could inhibit TGF-β1-induced increases in HAS mRNA levels, fibroblasts were treated with 2 ng/ml TGF-β1 with or without Pio or Rosi for 6 h, RNA was isolated, and HAS expression was determined by real-time PCR. Fig. 2 shows that TGF-β1 strongly induced HAS1 and HAS2 mRNA levels, which increased about 50- and 6-fold, respectively, whereas HAS3 mRNA levels remained unchanged. Pio treatment significantly inhibited TGF-β1-induced HAS1 mRNA expression in a dose-dependent manner, whereas Rosi treatment inhibited HAS1 mRNA expression by ~50%. 10 μM Rosi had similar effects on HAS1 and HAS2 mRNA levels (Fig. 2).

Pio and Rosi Do Not Influence Orbital Fibroblast Viability—To rule out the possibility that the reduction in HA synthesis is a result of toxicity by Pio and Rosi, viability of human orbital fibroblasts treated with the PPARγ ligands was measured by the XTT assay. Viable cells actively cleave the XTT reagent and form a water-soluble orange formazan dye, the appearance of which is proportionate to the number of viable cells. As Fig. 3

mRNA Knockdown Using Small Interfering RNA (siRNA)

Orbital fibroblasts were cultured to 80–90% confluence and transfected with PPARγ SMARTpool siRNA and scramble control (SC) siRNA (Dharmacon). Final concentration of siRNA was 80 nM using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Forty-eight hours after transfection, the cells were serum-starved for 48 h, and the medium was replaced with fresh medium containing 2 ng/ml TGF-β1 with or without 10 μM Pio or 10 μM Rosi. After 24 h of incubation, the medium was collected, and HA levels were analyzed by ELISA (see above). For PPARγ mRNA detection, 4 days after transfection, RNA was prepared as above, and PPARγ1 and PPARγ2 mRNA levels were analyzed using real-time PCR. HAS1 and HAS2 siRNAs (Santa Cruz Biotechnol-ogy) were also used to knock down HAS1 and HAS2 mRNA expression, respectively, as described previously (47). After 24 h of transfection, the cells were serum-starved and treated with TGF-β1 for 24 h before further experiment.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). For comparison between groups of three or more, an analysis of variance with a Newman-Keuls multiple comparison test was used to determine differences between treatments. Error bars represent the S.D. of triplicate samples. A p value <0.05 is considered significant. All experiments were performed at least three times.

RESULTS

TGF-β1 Induces HA Synthesis, and Pio and Rosi Inhibit TGF-β1-induced HA Biosynthesis in Human Orbital Fibroblasts—To determine whether the PPARγ ligands Pio and Rosi might block TGF-β1-induced HA synthesis, we selected several representative primary human orbital fibroblast strains and treated them with 2 ng/ml TGF-β1 in the presence or absence of the PPARγ ligands Pio or Rosi at varying concentrations. Representative results from two fibroblast strains are shown.

There was 2.31 ± 0.36-fold more secreted HA compared with pericellular HA in fibroblasts treated with TGF-β1. Strain #2 showed similar results (Fig. 1A). Therefore, about 70% of the TGF-β1-mediated, newly synthesized HA was secreted into the ECM. The remaining newly synthesized HA was localized pericellularly on the cell surface. Fig. 1A also demonstrates that Pio or Rosi treatment alone has no significant effect on HA synthesis. However, treatment with Pio and Rosi substantially diminished the induction of secreted HA and pericellular HA by TGF-β1 in a dose-response manner. 10 μM Pio inhibited TGF-β1-mediated secretion and pericellular accumulation of HA by a 52 ± 2.2% and 40 ± 2.2%, respectively. Experiments using 10 μM Rosi instead of 10 μM Pio showed similar results (Fig. 1A). However, 20 μM Rosi significantly reduced intracellular HA levels in both strains.

To confirm the HA ELISA results, [3H]glucosamine, which is incorporated into newly synthesized HA, was used to compare HA levels between cell-associated and secreted HA levels. The results showed similar increases in HA accumulation (data not shown).

Immunofluorescence was also used to analyze HA expres-sion. Fig. 1B shows untreated cells (top row) with HA staining (green) mainly around the nucleus. In TGF-β1 treated cells (middle row), HA staining is more pronounced and forms microvillus-like protrusions (49). Cells treated with Streptomycines HA’ase before immunostaining (bottom row) did not show HA staining and furthermore did not affect stress fiber F-actin staining (red) or nuclear staining (blue), confirming specificity of the experiment. TGF-β1 treatment did not significantly alter F-actin levels or affect cell structure in this system as cells were only treated with TGF-β1 for 24 h.

Pio and Rosi Inhibit TGF-β1-induced HAS1 and HAS2 mRNA Expression—Increased synthesis of HA mediated by an induction of HAS gene expression in orbital fibroblasts has been described previously (29). To determine whether the PPARγ ligands Pio and Rosi could inhibit TGF-β1-induced increases in HAS mRNA levels, fibroblasts were treated with 2 ng/ml TGF-β1 with or without Pio or Rosi for 6 h, RNA was isolated, and HAS expression was determined by real-time PCR. Fig. 2 shows that TGF-β1 strongly induced HAS1 and HAS2 mRNA levels, which increased about 50- and 6-fold, respectively, whereas HAS3 mRNA levels remained unchanged. Pio treatment significantly inhibited TGF-β1-induced HAS1 mRNA expression in a dose-dependent manner, whereas Rosi treatment inhibited HAS1 mRNA expression similar to both doses used (Fig. 2, top graph). Both Pio and Rosi treatment significantly inhibited TGF-β1-induced HAS2 mRNA expression in a dose-dependent manner (Fig. 2, middle graph). 10 μM Pio inhibited TGF-β1-mediated HAS1 and HAS2 mRNA induction by ~50%. 10 μM Rosi had similar effects on HAS1 and HAS2 mRNA levels (Fig. 2).

Pio and Rosi Do Not Influence Orbital Fibroblast Viability—To rule out the possibility that the reduction in HA synthesis is a result of toxicity by Pio and Rosi, viability of human orbital fibroblasts treated with the PPARγ ligands was measured by the XTT assay. Viable cells actively cleave the XTT reagent and form a water-soluble orange formazan dye, the appearance of which is proportionate to the number of viable cells. As Fig. 3

using iScript cDNA synthesis kit (Bio-Rad). Primers for human HAS1, -2, - and 3 and 7 S and the qRT-PCR method to detect relative abundance of mRNA were previously described (47). Primers for human PPARγ1 (NM138711) and PPARγ2 (NM015869) mRNA were 5’-AAGAAGCCGACCATAAACC-3’ (sense) and 5’-CTTCCATTACGGAGAGTACCC-3’ (antisense) and 5’-GCCATTCCTTCATGACTAC-3’ (sense) and 5’-CTTCCATTACGGAGAGTACCC-3’ (antisense), respectively.
demonstrates, after 24 h of treatment, there was no significant difference in cell viability among treatment conditions with or without the addition of TGF-β1. Furthermore, in the experiment for Fig. 1, cells were counted after each treatment to normalize HA production per cell. No significant changes in cell number were detected from cells treated with Pio or Rosi (data not shown). Thus, there was no evidence of cell toxicity in orbital fibroblasts exposed to Pio and Rosi at the concentrations used.

**FIGURE 1.** PPARγ ligands Pio and Rosi inhibit TGF-β1-induced HA production in human orbital fibroblasts. **A**, confluent strains of human orbital fibroblasts were cultured in RPMI 1640 with 0.5% FBS for 3 days before treatment with different concentrations of Pio or Rosi with or without 2 ng/ml TGF-β1 for 24 h. The culture medium (secreted HA), cell trypsin solution (pericellular HA), and cell lysate (cellular HA) were assayed by an HA ELISA as described under “Experimental Procedures.” TGF-β1-treated samples show a robust induction of secreted and pericellular HA levels. There was no significant change in intracellular HA levels. The experiment was performed in triplicate. *, p < 0.05; ***, p < 0.001 (compared with vehicle control (V)); †, p < 0.05; †††, p < 0.001, compared with TGF-β1 treatment; ^, p < 0.05; ^^^, p < 0.001 (5 μM Pio versus 10 μM Pio or Rosi versus 20 μM Rosi). Samples were run in duplicate utilizing three separate human orbital fibroblast strains (representative results are shown). Results are expressed as the mean ± S.D. **B**, confluent orbital fibroblasts were cultured in reduced serum for 3 days and treated with 2 ng/ml TGF-β1 for 24 h. Cells were stained with biotinylated HABP (for HA; green, a, d, and g), phalloidin (for F-actin; red, b, e, and h), and DAPI (for nucleus; blue). a–c, untreated cells; d–f, TGF-β1-treated cells; g–i, orbital fibroblasts were treated with HA’ase before fixation; c, f, and i, merged fluorescence with DAPI staining.

PPARγ Ligands Inhibit T Cell Adhesion to Orbital Fibroblasts
PPARγ Ligands Inhibit T Cell Adhesion to Orbital Fibroblasts

![Graph showing mRNA expression of HAS1, HAS2, and HAS3](image)

**FIGURE 2.** Pio and Rosi inhibit TGF-β1-induced HAS1 and HAS2 mRNA expression in human orbital fibroblasts. Total RNA from orbital fibroblasts treated with different concentrations of Pio or Rosi with or without TGF-β1 for 6 h was analyzed by qRT-PCR as described under “Experimental Procedures.” White bar, no TGF-β1; black bars, 2 ng/ml TGF-β1. There was a significant increase in HAS1 and HAS2 mRNA levels (**p < 0.001 compared with vehicle control) after TGF-β1 treatment. HAS3 had no significant change. This increase in HAS1 or HAS2 induced by TGF-β1 was significantly inhibited by Pio or Rosi (##p < 0.001). Results are expressed as the mean ± S.D. of triplicate samples performed on triplicate cultures.

![Graph showing TGF-β1 treatment](image)

**FIGURE 3.** Pio and Rosi do not influence human orbital fibroblast viability. Confluent strains of human orbital fibroblasts were cultured in RPMI 1640 with 0.5% FBS for 3 days before treatment with different concentrations of Pio or Rosi with or without 2 ng/ml TGF-β1 for 24 h. Viability was measured by XTT assay. Results shown are representative of three independent experiments and are the mean ± S.E. (n = 8). No significant differences were observed with any treatment (analysis of variance). V, vehicle control.

PPARγ, permanently altering its ability to bind its ligands (50). Previously, we reported that PPARγ ligand-driven adipogenesis is PPARγ-dependent and is completely inhibited by GW9662 (12). However, the addition of GW9662 did not significantly reduce the ability of either Pio or Rosi to inhibit TGF-β1-induced HA synthesis (Fig. 4A), suggesting that Pio and Rosi function through molecular pathways independent of PPARγ activation.

To confirm this result using a genetic approach, PPARγ-specific siRNAs were introduced into orbital fibroblasts to down-regulate PPARγ expression. Real-time PCR demonstrated that PPARγ1 and PPARγ2 mRNA levels were decreased by more than 90% in PPARγ siRNA-treated samples compared with control siRNA treated samples (Fig. 4B). PPARγ siRNA also inhibited orbital fibroblast adipogenesis driven by PPARγ ligands as demonstrated by oil red O staining (data not shown). However, PPARγ siRNA did not influence HA production in orbital fibroblasts treated with or without TGF-β1 compared with control siRNA and did not prevent the inhibition of TGF-β1-mediated HA production by Pio and Rosi (Fig. 4C). These data provide evidence that Pio- and Rosi-mediated effects on HA production are PPARγ-independent.

**TGF-β1-treated Orbital Fibroblasts Bind Activated Human T Cells through HA-CD44 Interaction**—One of the hallmarks of TED is the infiltration of leukocytes (particularly T cells) into orbital tissue. Extracellular HA binds the cell surface receptor CD44 and promotes lymphocyte rolling and adhesion to sites of inflammation. Therefore, increased production of HA mediated by TGF-β1 led us to investigate the possibility that TGF-β1 promotes T cell adhesion to orbital fibroblasts. Human peripheral blood T cells were activated using IL-2 and enriched by CD3/CD28 beads. The expression of CD44 and CD3 (T cell marker) were detected by flow cytometry. Fig. 5A demonstrates that about 99% of the enriched human T cells are CD44 and CD3 positive, suggesting that T cells have the potential to bind HA produced by orbital fibroblasts.

Next, the T cell adhesion assay was performed with human orbital fibroblasts. Orbital fibroblasts were treated with or without TGF-β1 for 24 h. To eliminate the influence of TGF-β1 or other factors in conditioned medium, fresh medium was added to fibroblast cultures immediately before the addition of T cells. To determine whether HA is associated with the adhesion of T cells, a fraction of TGF-β1-treated fibroblast cultures were incubated with Streptomyces HA’ase to digest extracellular HA, and a fraction of TGF-β1-treated fibroblast cultures were incubated with Streptomyces HA’ase to digest extracellular HA, and a fraction of T cells were incubated with a monoclonal CD44 antibody or an isotype control to block the CD44 binding site. T cells were pre-labeled with calcine-AM and added to fibroblast cultures and allowed to incubate at 4 °C for 90 min. Fig. 5B shows that adhesion of T cells to orbital fibroblasts treated with TGF-β1 significantly increased compared with untreated control fibroblasts. T cells preincubated with CD44 antibody adhered less to fibroblasts than did T cells preincubated with isotype control antibody (Fig. 5B, third bar, p < 0.01). As another control, T cells were incubated with exogenous HA at 37 °C for 1 h before being added to orbital fibroblasts. As expected, the addition of 100 or 500 μg/ml exogenous HA significantly reduced T cell adhesion to orbital fibroblasts (data not shown). Furthermore, pretreatment of fibroblast cultures with HA’ase, which completely digests ligands, and PPARγ acts as a negative regulator of TGF-β (35). GW9662 is a highly specific PPARγ antagonist that covalently binds to a cysteine residue within the ligand binding domain of
extracellular HA, abolished TGF-β1-induced T cell adhesion (Fig. 5B, fifth bar, p < 0.01). These data indicate that newly synthesized pericellular HA is required for cell-cell adhesion and that the HA-CD44 interaction plays an important role in T cell adhesion to orbital tissue. A non-fibroblast control was used to confirm that the binding is through cell-cell adhesion, not a cell-substratum adhesion.

**TGF-β1-induced Adhesion of Orbital Fibroblasts and Activated Human T Cells Is Attenuated by HAS2 mRNA Knockdown**—We recently demonstrated that HAS2 is the dominant isoform responsible for increased HA synthesis by orbital fibroblasts in response to prostaglandin D2 (47). To test the involvement of HAS enzymes in the TGF-β-mediated response, siRNA was used to selectively knock down HAS1 or HAS2 mRNA levels in fibroblasts. Fig. 6A shows that siRNA directed against HAS1 (open bars) or HAS2 (shaded bars) selectively and significantly reduced (by ~65–80% of mRNA levels) TGF-β-induced HAS1 and HAS2 compared with the scrambled control (SC) siRNA (black bars). In addition, the marked up-regulation of secreted HA and pericellular HA fraction (pHA) was collected, and HA levels were analyzed by ELISA. Results shown are the mean ± S.D. for three independent experiments with duplicate cultures in each experiment.

**FIGURE 4. Neither the irreversible PPARγ antagonist GW9662 nor PPARγ siRNA inhibits Pio- and Rosi-mediated suppression of HA synthesis.**

**A.** Primary orbital fibroblasts were pretreated with 1 μM GW9662 (GW) for 1 h or left untreated and were then treated with 2 ng/ml TGF-β1 and either 10 μM Pio or 10 μM Rosi for 24 h. HA synthesis was analyzed by ELISA. GW9662 did not restore TGF-β-stimulated HA synthesis in cells treated with Pio or Rosi. Results shown are representative of three independent experiments. **, p < 0.01; ***, p < 0.001 (compared with TGF-β1 treatment). **B.** Orbital fibroblast cultures were transfected with PPARγ SMARTpool siRNAs or nonspecific control siRNA. Forty-eight hours after transfection, the medium was changed, and culture was continued for 2 days. Total RNA was collected and PPARγ1, and PPARγ2 mRNA levels were analyzed by qRT-PCR and normalized to 7 S RNA. Results shown are the mean ± S.D. for two independent experiments with triplicate cultures in each experiment. ***, p < 0.001, compared with scramble siRNA. **C.** PPARγ siRNA transfected orbital fibroblast cultures were serum-starved and then treated with TGF-β1 with or without 10 μM Pio or 10 μM Rosi. Twenty-four hours after treatment the conditioned medium or secreted HA fraction (sHA) and cell trypsin solution or pericellular HA fraction (pHA) were collected, and HA levels were analyzed by ELISA. Results shown are the mean ± S.D. for three independent experiments with duplicate cultures in each experiment.

extracellular HA, abolished TGF-β1-induced T cell adhesion (Fig. 5B, fifth bar, p < 0.01). These data indicate that newly synthesized pericellular HA is required for cell-cell adhesion and that the HA-CD44 interaction plays an important role in T cell adhesion to orbital tissue. A non-fibroblast control was used to confirm that the binding is through cell-cell adhesion, not a cell-substratum adhesion.

**TGF-β1-induced Adhesion of Orbital Fibroblasts and Activated Human T Cells Is Attenuated by HAS2 mRNA Knockdown**—We recently demonstrated that HAS2 is the dominant isoform responsible for increased HA synthesis by orbital fibroblasts in response to prostaglandin D2 (47). To test the involvement of HAS enzymes in the TGF-β-mediated response, siRNA was used to selectively knock down HAS1 or HAS2 mRNA levels in fibroblasts. Fig. 6A shows that siRNA directed against HAS1 (open bars) or HAS2 (shaded bars) selectively and significantly reduced (by ~65–80% of mRNA levels) TGF-β-induced HAS1 and HAS2 compared with the scrambled control (SC) siRNA (black bars). In addition, the marked up-regulation of secreted HA and pericellular HA fraction (pHA) was collected, and HA levels were analyzed by ELISA. Results shown are the mean ± S.D. for three independent experiments with duplicate cultures in each experiment.
Pio and Rosi Inhibit TGF-β1-induced T Cell Adhesion to Orbital Fibroblasts—Because Pio and Rosi attenuate TGF-β1-induced HA synthesis and HA mediates fibroblast and T cell adhesion, we hypothesized that Pio and Rosi might prevent TGF-β1-induced fibroblast-T cell adhesion. Fibroblasts were treated with different concentrations of Pio or Rosi together with TGF-β1. After 24 h of treatment, the conditioned medium was removed to eliminate the influence of TGF-β1 and drugs on T cells, and fresh medium was added along with the T cell suspension. As predicted, Fig. 6A demonstrates that fibroblasts pretreated with Pio or Rosi had a significantly reduced ability to adhere to T cells compared with fibroblasts treated with TGF-β1 only. Immunostaining was used to confirm the adhesion assay results. T cells stained with the T cell marker CD3 (green) colocalize with fibroblasts and are associated with HA stained with biotinylated HABP (red) (Fig. 7B). TGF-β1-treated fibroblasts adhere to a greater number of T cells (Fig. 7B, panel c) than do TGF-β1-treated fibroblasts pretreated with Pio or Rosi (Fig. 7B, panels f or i, respectively).

**CD44^+ /CD3^+ Cells Infiltrate Graves Orbital Adipose Tissue**—The accumulation of GAGs and infiltration of inflammatory cells into orbital tissues are prominent histological markers of...
TED. The infiltration of T cells in Graves orbital adipose tissue was further investigated using immunofluorescence on orbital tissue sections. Orbital adipose tissues were obtained from TED patients undergoing orbital decompression surgery as described under “Experimental Procedures.” The tissue sections were stained with CD44 and CD3 antibodies, biotinylated HABP for HA staining, and DAPI for nuclear staining. Fig. 8 shows that CD44 (green) and CD3 (purple) are colocalized in small round cells (T cells). Concentrated infiltration of CD44+/CD3+ cells is visible in the adipose sections (Fig. 8, b, c, f, and g). HA staining is shown by narrow red bands outlining large vacuoles that indicate fat droplet deposits (Fig. 8a). The addition of free soluble HA to the HABP probe completely eliminated HA staining (Fig. 8e), suggesting that the HA staining is specific. CD44+/CD3+ cells are clustered around the vessel area and are attached to the vessel wall or just outside the vessel, suggesting that CD44+/CD3+ cells traversed through the vessel wall to the orbital tissue (Fig. 8, f and g, see arrows). Fat droplets were stained with Bodipy 493/503 dye, and CD3+ cells could be found among the green fat droplets (data not shown).

**DISCUSSION**

Orbital fibroblasts are believed to be the primary autoimmune target in TED (2, 6). Once orbital fibroblasts become activated, they undergo proliferation, differentiation, and/or
produce GAGs. The accumulation of GAGs, especially the hydrophilic GAG HA, is the most evident feature of tissue remodeling in TED. TGF-β levels are increased in human orbital tissue, and TGF-β increases HA secretion into the culture medium of orbital fibroblasts in vitro (29, 34). These studies indicate that TGF-β may be involved in the pathogenesis of TED. Our data presented here demonstrate for the first time that TGF-β-induced accumulation of HA is not only secreted HA but also pericellular HA (HA remaining on the cell surface). The increased HA levels mediated by TGF-β are most likely due to increased levels of HAS2 mRNA in orbital fibroblasts. Furthermore, TGF-β also increases the adhesion of activated T cells to orbital fibroblasts mediated by newly synthesized pericellular HA on orbital fibroblasts interacting with its cognate receptor CD44 on T cells.

The infiltration of orbital tissue by inflammatory cells (such as T cells, B cells, mast cells, and macrophages) and the accumulation of HA are two histological characteristics of TED (5). Surprisingly, the correlation between the two features is not clear. HA is a multifunctional ECM molecule that participates in regulation of inflammation and tissue remodeling. It is well documented that the HA-CD44 interaction can play a proinflammatory role in facilitating leukocyte recruitment and adhesion to inflammatory sites. Previous studies show that CD44 is expressed at elevated levels in Graves orbital connective tissue in situ (11), suggesting a role for HA/CD44 in regulating inflammatory responses in TED. Our results demonstrate that CD44+ cells are present in Graves orbital tissue sections and that these cells express the T cell marker CD3. Our experiments also show that TGF-β induced HA-rich pericellular matrix facilitates orbital fibroblast adhesion to activated T cells. The adhesion of fibroblasts and T cells depends on the HA-CD44 interaction as 1) activated human T cells highly express CD44 (Fig. 5A), 2) preincubation of T cells with CD44 antibody significantly reduced T cell adhesion to fibroblasts (Fig. 5B), 3) pretreatment of the fibroblasts with HAase to digest HA diminished cell-cell adhesion (Fig. 5B), 4) HAS2 siRNA blocked HA synthesis and significantly inhibited cell-cell adhesion (Fig. 6B), and 5) CD44+ T cells appear to attach to orbital fibroblasts with increases pericellular HA (Fig. 7B). Our findings suggest that the accumulation of HA in orbital tissue not only contributes to periocular edema but also participates in the inflammatory response by enhancing or facilitating inflammatory cell infiltration into orbital tissue. These studies also suggest that CD44 is a crucial cellular adhesion receptor required for the recruitment of leukocytes (not only T cells, but monocytes, macrophages, and neutrophils as well) to inflamed orbital tissue. Furthermore, CD44 may activate TGF-β and promote fibroblast migration to injured tissue, suggesting CD44 plays a role in tissue remodeling and fibrosis (51). Future studies are needed to explore the role of CD44 in activation of orbital fibroblasts.

Recent work reveals that PPARγ and its ligands have anti-inflammatory and anti-TGF-β activities. Overexpression of PPARγ suppresses TGF-β-induced activation of monocytes/macrophages and fibrosis in human subconjunctival fibroblasts (52, 53). TZDs and other PPARγ ligands such as 15-deoxy-Δ 12,14-prostaglandin J2 and 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid also show strong anti-TGF-β functions through PPARγ-dependent or independent pathways (38, 50, 54). Circulating levels of the chemokine CXCL10 and the cytokine interferon γ (IFNγ) are elevated in patients with Graves disease, particularly in those with active TED. Rosi and Pio exert a dose-dependent inhibition of IFNγ and TNFα-induced chemokines CXCL9, CXCL10, and CXCL11 secretion in orbital fibroblasts, preadipocytes, and thyrocytes (55, 56). These studies indicate that PPARγ activity is involved in the regulation of IFNγ-induced chemokine expression in thyroid autoimmunity and TED, and PPARγ activators might attenuate the recruitment of activated T cells at sites of T helper type 1 (Th1)-mediated inflammation, which predominates in early stage of TED (whereas Th2 cells are more abundant later in TED) (57). Our results demonstrate that the PPARγ ligands Pio and Rosi inhibit TGF-β mediated functions including elevating HAS1 and HAS2 mRNA levels, HA production, and T cell-fibroblast adhesion. Supporting evidence indicates that PPARγ ligands could interrupt the communication between activated T cells and orbital fibroblasts at sites of inflammation in early TED. However, in our experimental system, neither a PPARγ antagonist GW9662 nor PPARγ knockdown relieve the inhibition of HA induction by Pio and Rosi. Therefore, these studies imply a PPARγ-independent mechanism for the Pio- and Rosi-mediated reduction of TGF-β activity.

We tested several signaling pathways to identify a potential mechanism whereby TGF-β-driven HA synthesis is inhibited by Pio and Rosi in our experimental system. However, there was no clear evidence showing that Pio or Rosi are general inhibitors of TGF-β-induced responses in orbital fibroblasts. For example, the phosphorylation and nuclear translocation of Smad2 and -3 induced by TGF-β was unaffected by co-treatment with Pio or Rosi. Furthermore, Pio and Rosi did not inhibit the TGF-β-activated mitogen-activated protein kinase (MAPK) signaling pathway or the phosphorylation of p38 and p42/44. Other alternative pathways of TGF-β such as phosphorylation of AKT, c-Jun N-terminal kinase (JNK), or c-Abl were not detectable after TGF-β treatment in orbital fibroblast.

Although our study and other in vitro studies show the anti-inflammatory functions of PPARγ ligands and their potential therapeutic importance in early immunological processes
associated with TED, these anti-diabetic TZD drugs might activate PPARγ, an adipogenic transcription factor, and lead to increases in orbit volume through adipogenesis. Several case reports have described development of exophthalmos in patients receiving TZD treatment for type 2 diabetes (58–61). Because the results of these clinical studies are from TED patients with type 2 diabetes, the increase in exophthalmos might be the result of adipocyte accumulation due to a pre-existing hyperinsulinemic state (60). Furthermore, the success of TZD drugs as a therapy for type 2 diabetes is also paradoxical as they target PPARγ, which induces adipose tissue formation, a major risk factor for type 2 diabetes (36). Further studies are needed to explore whether the anti-inflammatory effects of PPARγ ligands can be exploited for TED patients without type 2 diabetes.

Taken together, our results demonstrated that TGF-β plays an important role in human orbital fibroblast HA synthesis, and the accumulation of HA in orbital tissue not only contributes to periorbital edema but also participates in inflammatory responses by enhancing or facilitating inflammatory cell infiltration into orbital tissue. Furthermore, we have demonstrated that the PPARγ ligands Pio and Rosi have strong inhibitory effects on TGF-β-mediated inflammatory processes and that their mode of action is PPARγ-independent.

REFERENCES