Orbital Fibroblasts From Thyroid Eye Disease Patients Differ in Proliferative and Adipogenic Responses Depending on Disease Subtype

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PURPOSE. Thyroid eye disease (TED) patients are classified as type I (predominantly fat compartment enlargement) or type II (predominantly extraocular muscle enlargement) based on orbital imaging. Orbital fibroblasts (OFs) can be driven to proliferate or differentiate into adipocytes in vitro. We tested the hypothesis that type I OFs undergo more adipogenesis than type II OFs, whereas type II OFs proliferate more than type I OFs. We also examined the effect of cyclooxygenase (COX) inhibitors on OF adipogenesis and proliferation.

METHODS. Type I, type II, and non-TED OFs were treated with transforming growth factor-beta (TGFβ) to induce proliferation and with 15-deoxy-Delta-12,14-prostaglandin J2 (15d-PGJ2) to induce adipogenesis. Proliferation was measured using the [3H]thymidine assay, and adipogenesis was measured using the AdipoRed assay, Oil Red O staining, and flow cytometry. The effect of COX inhibition on adipogenesis and proliferation was also studied.

RESULTS. Type II OFs incorporated 1.7-fold more [3H]thymidine than type I OFs (P < 0.05). Type I OFs accumulated 4.8-fold more lipid than type II OFs (P < 0.05) and 12.6-fold more lipid than non-TED OFs (P < 0.05). Oil Red O staining and flow cytometry also demonstrated increased adipogenesis in type I OFs compared to type II and non-TED OFs. Cyclooxygenase inhibition significantly decreased proliferation and adipogenesis in type II OFs, but not type I OFs.

CONCLUSIONS. We have demonstrated that OFs from TED patients have heterogeneous responses to proproliferative and proadipogenic stimulators in vitro in a manner that corresponds to their different clinical manifestations. Furthermore, we demonstrated a differential effect of COX inhibitors on type I and type II OF proliferation and adipogenesis.

Keywords: orbital fibroblasts, thyroid eye disease, proliferation, adipogenesis
The adherent cells did not express CD45, factor VIII, or cytokeratin, but did express vimentin and type I and III collagen; and the cells were morphologically consistent with fibroblasts. The fibroblast strains (called stains because they were each derived from different human beings) were stored in liquid N$_2$ and used at the earliest possible passage, between passages 4 and 10.

The PPARY ligand 15d-PGJ$_2$ (Biomol, Plymouth Meeting, PA) was prepared as 10 mM stocks in dimethyl sulfoxide (DMSO) and added to cell cultures to the final concentration indicated, as previously described. Recombinant human TGFβ1 (No. 240B) was purchased from R&D Systems (Minneapolis, MN) and added to cell cultures as previously described. A dual COX I and 2 inhibitor (indomethacin; Sigma-Aldrich, St. Louis, MO), a selective COX-2 inhibitor (celecoxib; Sigma-Aldrich), and SC-650 (a selective COX-1 inhibitor; Cayman Chemical, Ann Arbor, MI) were prepared as previously described and added to cell culture at the final concentrations indicated.

Analysis of Computed Tomography Scans
Computed tomography scans were obtained from TED patients who were undergoing orbital decompression for optic neuropathy, cosmesis, and exposure at the Flaum Eye Institute. Written consent was obtained within the guidelines of the institutional review board, and the tenets of the Declaration of Helsinki were followed. The coronal CT image immediately posterior to the globe was measured in all patients to measure the area of the entire orbit and the area of the EOMs (superior rectus, inferior rectus, medial rectus, lateral rectus, levator palpebrae superioris, and superior oblique muscles) using IDX Imagecast software. The areas of all the EOMs were summed to create an EMA:OA ratio for each patient.

Classification of Type I and Type II Patients
The average control patient EMA:OA ± two standard deviations (SD) was used as the range within which TED patients were classified as type I patients. Patients with an EMA:OA larger than the average control EMA:OA ± 2 SD were classified as type II patients. Orbital fibroblasts for patients (all inactive) and patients (one active) were selected for in vitro experimentation. Disease activity was determined based on clinical presentation and use of steroid therapy.

Fibroblast Proliferation
Proliferation of OFs was studied by assessing [3H]thymidine incorporation as previously described. Orbital fibroblasts were plated in 96-well plates at 5000 cells/well in RPMI containing 10% FBS. The next day, the medium was changed to 0.5% FBS RPMI. Orbital fibroblasts were maintained in the 0.5% FBS RPMI or treated with 5 ng/mL TGFβ1. In some experiments, OFs were cotreated with 5 ng/mL TGFβ1 and different drugs as described below. After 24 hours, 10 μCi [methyl-3H]thymidine (DuPont NEN Products, Boston, MA) was added, and [3H] incorporation was determined after another 48 hours using a microplate scintillation counter (TopCount; PerkinElmer, Meriden, CT) as previously described.

All assays were performed on triplicate cultures, and the results are reported as means ± SE.

Materials and Methods
Cells and Reagents
Primary OFs were isolated and established by standard explant techniques from TED patients undergoing orbital decompression and non-TED patients undergoing orbital surgery at the Flaum Eye Institute (Rochester, NY) as previously described. None of the non-TED patients had inflammatory orbital diseases; the indications for orbital surgery included cavernous hemangioma, lacrimal gland tumor, and orbit fracture. The protocol for tissue procurement was approved by the Research Subjects Review Board, and informed, written consent was obtained from all patients. The OFs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2-mercaptoethanol (Eastman Kodak, Rochester, NY), L-glutamine (Life Technologies, Grand Island, NY), HEPES (United States Biochemical Corp., Cleveland, OH), nonessential amino acids, sodium pyruvate, and gentamicin (Life Technologies). The adherent cells did not express CD45, factor VIII, or cytokeratin, but did express vimentin and type I and III collagen; and the cells were morphologically consistent with fibroblasts.

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Classification of Type I and Type II Patients
The average control patient EMA:OA ± two standard deviations (SD) was used as the range within which TED patients were classified as type I patients. Patients with an EMA:OA larger than the average control EMA:OA ± 2 SD were classified as type II patients. Orbital fibroblasts for type I (all inactive) and type II (one active) patients were selected for in vitro experimentation. Disease activity was determined based on clinical presentation and use of steroid therapy.

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All assays were performed on triplicate cultures, and the results are reported as means ± SE.
Fibroblast Adipogenesis

Orbital fibroblasts were grown in RPMI +10% FBS medium to confluence. To induce adipogenesis, the OFs were treated with 5 μM 15d-PGJ2 every 2 days for a total of 8 days as previously described.4 In some experiments, OFs were cotreated with the PPARγ ligand, 15d-PGJ2, and different drugs as described below. Adipogenesis was assessed using one or more of the assays described below on the eighth day.

AdipoRed Assay

Adipogenesis of OFs was studied by analyzing triglyceride accumulation using the AdipoRed assay reagent (Cayman) as previously described.24 Orbital fibroblasts from three type I patients, three type II patients, and three non-TED patients were treated with 10% FBS culture media with or without 5 μM 15d-PGJ2. The difference in OF proliferation in 0.5% FBS culture media and 0.5% FBS culture media with 5 μg/mL TGFβ1 was calculated by measuring the uptake of [3H]thymidine. Orbital fibroblasts from type II patients incorporated 1.7 times as much [3H]thymidine as did OFs from type I patients (P < 0.05). Type II OFs incorporated 1.5 times more [3H]thymidine compared to non-TED OFs.

Oil Red O Staining

Orbital fibroblasts were plated on an eight-chamber glass slide at 2500 cells/well in RPMI medium containing 10% FBS. The following day, they were treated with 5 μM 15d-PGJ2 as described above to induce adipogenesis. After 8 days of treatment, the supernatants were discarded, and cells were washed in PBS and fixed with 10% formaldehyde (Sigma-Aldrich) for 10 minutes at room temperature. Orbital fibroblasts were then incubated for 10 minutes with Oil Red O stain, which stains lipid red. Treatment with 5 μM 15d-PGJ2 resulted in a large accumulation of Oil Red O staining in the OFs from a type I TED patient compared to minimal staining in the OFs from a type II and a non-TED patient.
Shandon, Astmoor, UK) and glass coverslips. The slides were viewed using a Zeiss Axioplan microscope (Zeiss, Thornwood, NY).

**Imaging Flow Cytometry**

Orbital fibroblasts were plated in 25-mL flasks (Greiner Bio-One, Frickenhausen, Germany) in 10% FBS media and treated as described above. They were harvested with trypsin-EDTA solution and then washed with PAB (phosphate-buffered saline [PBS; Gibco Invitrogen, Grand Island, NY] supplemented with 0.1% sodium azide and 1% bovine serum albumin [both from Sigma-Aldrich]) as previously described. The OFs were then washed with PAB twice and fixed with 2% paraformaldehyde (Sigma-Aldrich) at 4°C. After 24 hours, the cells were again washed with PBS twice and then stained with LipidTOX neutral lipid stain (1:100; Molecular Probes Invitrogen, Eugene, OR) and Draq5 nucleic acid stain (1:200; Axxora, San Diego, CA) in PBS. Signals from brightfield, darkfield, LipidTOX, and Draq5 were collected. Samples were analyzed on an Imagestream System 100 (Amnis, Seattle, WA). Statistical analysis was performed as previously described.

**Statistical Analysis**

For the experiments performed in triplicate, the error bars in graphical depiction of data represent the standard error of the mean (SEM) from the mean of triplicate samples. One-way ANOVA with post hoc Tukey test was performed on GraphPad Prism 5 (GraphPad Software, La Jolla, CA) software for statistical analysis of the effect of drugs on proliferation or adipogenesis. A P value less than 0.05 was considered statistically significant.

**RESULTS**

**TED Patients Have Varying Degrees of Extraocular Muscle Enlargement**

To determine the degree of EOM enlargement in TED patients, orbit CT scans were analyzed to ascertain the EMA:OA ratio for 30 TED patients and for five non-TED patients. The average TED patient EMA:OA ratio was 0.31 (range, 0.15–0.51). The average non-TED patient EMA:OA ratio was 0.22 (range, 0.17–0.26). Thyroid eye disease patients with an EMA:OA ratio greater than 2 SD from the normal mean were classified as type II TED patients (n = 17), while those with an EMA:OA ratio within 2 SD from the normal mean were classified as type I TED patients (n = 13).

**Orbital Fibroblasts From Type II TED Patients Proliferate to a Greater Degree Than Orbital Fibroblasts From Type I TED and Non-TED Patients**

Differences in proliferation of OFs from type I and type II TED patients and non-TED patients were studied by assessing tritiated (H)thymidine incorporation. Orbital fibroblasts from three type I patients, three type II patients, and three non-TED patients were treated with 5 ng/mL TGFβ, a proproliferative and proinflammatory cytokine, for 3 days. Orbital fibroblasts from type II patients incorporated 1.7-fold more (H)thymidine than OFs from type I patients (P < 0.05). Type II OFs
incorporated 1.5-fold more \[ ^3 \text{H} \] thymidine compared to non-TED OFs (Fig. 2).

**Orbital Fibroblasts From Type I TED Patients Undergo Adipogenesis to a Greater Degree Than Orbital Fibroblasts From Type II TED and Non-TED Patients**

Differences in adipogenesis of OFs from type I and type II TED patients and non-TED patients were studied using the AdipoRed assay (Cayman). Orbital fibroblasts from three type I patients, three type II patients, and three non-TED patients were treated with the endogenous PPAR\(\gamma\) ligand 15d-PGJ\(_2\) (5 \( \mu \)M) every other day for 8 days. Orbital fibroblasts from type I patients accumulated 4.8-fold more lipid than type II OFs (\( P < 0.05 \)) and 12.6-fold more lipid than non-TED OFs (\( P < 0.05 \)) (Fig. 3).

Oil Red O staining provided further evidence of increased adipogenesis in type I OFs. Orbital fibroblasts from one type I TED patient, one type II TED patient, and one non-TED patient were either untreated or treated with 5 \( \mu \)M 15d-PGJ\(_2\) every other day for 8 days. A greater amount of Oil Red O staining was evident in the type I TED OFs compared to the type II TED and non-TED OFs (Fig. 4).

Additionally, imaging flow cytometry was used to demonstrate increased adipogenesis in type I TED OFs. Orbital fibroblasts from one type I TED patient and one type II TED patient were treated with 15d-PGJ\(_2\) (5 \( \mu \)M) every other day for 8 days. Cells were stained for lipid using LipidTOX to identify OFs that underwent adipogenesis into adipocytes (Fig. 5A). Treatment with 15d-PGJ\(_2\) resulted in 54% of type I OFs undergoing adipogenesis compared to only 5% of type II OFs (Fig. 5B).

**Indomethacin Is an Antiadipogenic Agent in OFs From Type II Patients**

The ability of indomethacin (a nonspecific COX-1/2 inhibitor), celecoxib (a COX-2-specific inhibitor), and SC-560 (a COX-1-specific inhibitor) to inhibit adipogenesis was studied in OFs from type I, type II, and non-TED patients by using the AdipoRed assay (Cayman). Orbital fibroblasts were either untreated or treated for 10 days with 5 \( \mu \)M 15d-PGJ\(_2\) alone or with cotreatment of 20 \( \mu \)M indomethacin, 10 \( \mu \)M celecoxib, or 1 \( \mu \)M SC-560 added every other day (Fig. 6). 15d-PGJ\(_2\) treatment of OFs compared to untreated OFs resulted in an increase in the amount of OF lipid accumulation by 2.1-fold in type I OFs (\( P < 0.001 \)), 1.4-fold in type II OFs, and 1.8-fold in non-TED OFs (\( P < 0.01 \)). The amount of 15d-PGJ\(_2\)-induced lipid accumulation by type I OFs was 2.8-fold higher than by type II OFs (\( P < 0.001 \)) and 2.8-fold higher than by non-TED OFs (\( P < 0.001 \)). 15d-PGJ\(_2\)-induced lipid accumulation in type II OFs was inhibited by 47% by indomethacin (\( P < 0.01 \)), 1.3% by celecoxib, and 36% by SC-560. The COX inhibitors did not significantly alter 15d-PGJ\(_2\)-induced lipid accumulation in type I or non-TED patients.

**Figure 6.** Indomethacin inhibits 15d-PGJ\(_2\)-induced adipogenesis in type II OFs. Orbital fibroblasts from one type I patient, one type II patient, and one non-TED patient were either untreated or treated for 10 days with 5 \( \mu \)M 15d-PGJ\(_2\) alone or with cotreatment of 20 \( \mu \)M indomethacin, 10 \( \mu \)M celecoxib, or 1 \( \mu \)M SC-560 added every other day. 15d-PGJ\(_2\) treatment of OFs compared to untreated OFs resulted in an increase in the amount of OF lipid accumulation by 2.1-fold in type I OFs (\( P < 0.001 \)), 1.4-fold in type II OFs, and 1.8-fold in non-TED OFs (\( P < 0.01 \)). The amount of 15d-PGJ\(_2\)-induced lipid accumulation by type I OFs was 2.8-fold higher than by type II OFs (\( P < 0.001 \)) and 2.8-fold higher than by non-TED OFs (\( P < 0.001 \)). 15d-PGJ\(_2\)-induced lipid accumulation in type II OFs was inhibited by 47% by indomethacin (\( P < 0.01 \)), 1.3% by celecoxib, and 36% by SC-560. The COX inhibitors did not significantly alter 15d-PGJ\(_2\)-induced lipid accumulation in type I or non-TED patients.
COX Inhibitors Are Potent Antiproliferative Agents in OFs From Type II Patients

The ability of the nonspecific COX-1/2 inhibitor indomethacin, the COX-2-specific inhibitor celecoxib, and the COX-1-specific inhibitor SC-560 to inhibit proliferation was studied in OFs from type I, type II, and non-TED patients by measuring \[^{3}\text{H}]\text{thymidine incorporation. Orbital fibroblasts were either untreated or treated for 3 days with 5 ng/mL TGF\(_{\beta}\) alone or with cotreatment of 20 \(\mu\)M indomethacin, 10 \(\mu\)M celecoxib, or 1 \(\mu\)M SC-560 on day 1. TGF\(_{\beta}\) treatment of OFs compared to untreated OFs resulted in an increase in the amount of \[^{3}\text{H}]\text{thymidine incorporated by 2.7-fold in type II OFs (}\(P < 0.001\), 1.6-fold in type I OFs, and 1.2-fold in non-TED OFs. The amount of TGF\(_{\beta}\)-induced \[^{3}\text{H}]\text{thymidine incorporated by type II OFs was 1.7-fold higher than that by type I OFs (}\(P < 0.01\)) and 2.9-fold higher than that by non-TED OFs (}\(P < 0.001\)). TGF\(_{\beta}\)-induced \[^{3}\text{H}]\text{thymidine incorporation was inhibited by 57\% by indomethacin (}\(P < 0.05\), 59\% by celecoxib (}\(P < 0.001\), and 31\% by SC-560 (}\(P < 0.05\)) in type II OFs. The COX inhibitors did not significantly alter TGF\(_{\beta}\)-induced \[^{3}\text{H}]\text{thymidine incorporation in type I or non-TED patients.}

**DISCUSSION**

Thyroid eye disease is a clinically heterogeneous disease process in which there are subsets of patients with more predominant fat compartment enlargement (type I) or EOM enlargement (type II).\(^{10-12}\) Orbital fibroblasts are thought to play a major role in the inflammatory process in TED.\(^{19,29}\) Orbital fibroblasts from TED patients have demonstrated the ability to be driven to adipogenesis or to proliferate under different in vitro conditions.\(^{17,18,21,22}\) We have demonstrated that OFs from TED patients have heterogeneous responses to proproliferative and proadipogenic stimulators in vitro in a manner that corresponds to their different clinical manifestations: Type II OFs are driven by TGF\(_{\beta}\) to proliferate to a greater extent than type I OFs (Fig. 2), while type I OFs are driven by 15d-PGJ\(_2\) to adipogenesis to a greater extent than type II OFs (Figs. 3–5). Our data suggest that patients’ OFs may play a significant role in determining their clinical subtype of TED.

Previous studies have demonstrated that activated T cells from TED patients express high levels of COX-2 and that COX-2 is expressed in the orbital fibroadipose tissue of TED patients at higher levels than in non-TED patients.\(^{22,30}\) Cyclooxygenase is part of the arachidonic acid metabolism pathway and the rate-limiting enzyme for the expression of eicosinoids, including the prostaglandins (PG).\(^{30}\) Cyclooxygenase-1 is constitutively expressed by cells, whereas COX-2 expression is induced by proinflammatory conditions.\(^{30}\) Cyclooxygenase has a potential role in mediating both fat enlargement via induction of adipogenesis and EOM enlargement via induction of hyaluronan.\(^{13,22,30}\) Activated T cells from TED patients drive OF adipogenesis in vitro via COX-2-dependent 15d-PGJ\(_2\) production (a downstream product of PGD\(_2\)) through transcellular communication.\(^{22}\) Furthermore, mast cells also produce PGD\(_2\) that is taken up by OFs and serves as a signal to produce hyaluronan.\(^{13,30}\)
We demonstrated that inhibition of COX-1 and/or COX-2 significantly inhibited TGFβ-induced proliferation in OFs from type II patients, and that inhibition of both COX-1 and COX-2 by indomethacin inhibited 15d-PGJ2–induced adipogenesis in type II patients (Figs. 6, 7). A similar effect by COX inhibition was not seen in the type I or non-TED OFs. The difference in the effect of COX inhibition on proliferation in type II TED OFs compared to type I and non-TED OFs can be explained by the lack of significant TGFβ-induced proliferation among the latter two groups of OFs. One potential explanation for the lack of effect of COX inhibition on 15d-PGJ2–induced adipogenesis in type I and non-TED OFs is that COX does not play a significant role in adipogenesis in type I or non-TED OFs. This is supported by a study by Konuk and colleagues, which demonstrated that orbital fibroadipose tissue from type II patients had higher levels of COX-2 expression than that from type I patients and non-TED patients. Another potential explanation is that the exogenous proadipogenic 15d-PGJ2 overwhelms any endogenous proadipogenic PGs produced by the OFs, which are potentially inhibited by COX inhibition. This explanation is more likely, as a study from our laboratory found that COX inhibition significantly inhibited activated T cell–induced adipogenesis. However, this does not exclude a potential larger role for COX inhibition in type II TED patients compared to type I TED patients. Furthermore, celecoxib, a selective COX-2 inhibitor, has been used to effectively treat a type II TED patient who was refractory to corticosteroid treatment.

One potential explanation for decreased adipogenesis and proliferation with COX inhibition is a toxic effect of the COX inhibitors. However, this is an unlikely explanation, as no toxicity was noted by phase contrast microscopy or by inspection of cell attachment to culture dishes. Furthermore, our lab has shown that low-dose treatments with COX inhibitors are not toxic to human fibroblasts.

One limitation of our study is that we did not assess other inflammatory cells (e.g., lymphocytes, macrophages, mast cells), which have been shown to play a role in the pathophysiology of TED. We chose to specifically examine the role of OFs as the main contributor to the clinical heterogeneity of TED due to previous in vitro data demonstrating that OF populations from TED are heterogeneous with respect to their expression of Thy1, a cell surface glycoprotein, and that Thy1-expressing OFs are less likely to undergo adipogenesis due to production of a paracrine antiadipogenic factor. Another limitation of the study is the patients' disease activity (one of the three type II OF strains had active disease, and the patient was treated with oral steroids), which may have an effect on the in vitro findings. Furthermore, in our study we focused on the use of specific proproliferative (TGFβ) and proadipogenic (15d-PGJ2) stimulators. Other cytokines, such as interleukin-1 beta (IL-1β), have pleiotropic effects on OFs, stimulating both hyaluronic acid production and adipogenesis. Future studies could be performed to examine the effect of such pleiotropic cytokines on OFs from type I, type II, and non-TED patients. A final limitation of the study is the small number of OF strains used to study the effect of COX inhibitors on TGFβ-induced proliferation and 15d-PGJ2–induced adipogenesis. These data were for hypothesis generation, and future studies will examine differences in response to COX inhibition in a larger number of OF strains.

In summary, we have shown that type II TED OFs are driven by TGFβ to proliferate more than type I TED OFs, whereas type I OFs are driven by 15d-PGJ2 to adipogenesis more than type II OFs. Furthermore, we have demonstrated that COX inhibition decreased the amount of TGFβ–induced proliferation and 15d-PGJ2–induced adipogenesis in OFs from type II TED patients. Further studies are needed to elucidate the mechanism for the difference in response to proadipogenic and proliferative agents by OFs from type I and type II TED patients. Potential mechanisms include different levels of TGFβ or 15d-PGJ2 receptor expression in type I and type II OFs and differences in intracellular signaling in response to TGFβ or 15d-PGJ2. Clinical trials are necessary to establish the role of COX inhibition in TED patients and whether there is a difference in response among type I and type II patients.

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References


