Activated Human T Lymphocytes Express Cyclooxygenase-2 and Produce Proadipogenic Prostaglandins that Drive Human Orbital Fibroblast Differentiation to Adipocytes

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The differentiation of preadipocyte fibroblasts to adipocytes is a crucial process to many disease states including obesity, cardiovascular, and autoimmune diseases. In Graves' disease, the orbit of the eye can become severely inflamed and infiltrated with T lymphocytes as part of the autoimmune process. The orbital fibroblasts convert to fat-like cells causing the eye to protrude, which is disfiguring and can lead to blindness. Recently, the transcription factor peroxisome proliferator activated receptor (PPAR)- γ and its natural (15d-PGJ₂) and synthetic (thiazolidinedione-type) PPAR- γ agonists have been shown to be crucial to the *in vitro* differentiation of preadipocyte fibroblasts to adipocytes. We show herein several novel findings. First, that activated T lymphocytes from Graves' patients drive the differentiation of PPAR- γ -expressing orbital fibroblasts to adipocytes. Second, this adipogenic differentiation is blocked by nonselective small molecule cyclooxygenase (Cox)-1/Cox-2 inhibitors and by Cox-2 selective inhibitors. Third, activated, but not naïve, human T cells highly express Cox-2 and synthesize prostaglandin D₂ and related prostaglandins that are PPAR- γ ligands. These provocative new findings provide evidence for how activated T lymphocytes, through production of PPAR- γ ligands, profoundly influence human fibroblast differentiation to adipocytes. They also suggest the possibility that, in addition to the orbit, T lymphocytes influence the deposition of fat in other tissues. (*Am J Pathol* 2006, 169:1183–1193; DOI: 10.2353/ajpath.2006.060434)

Fibroblasts form the structure of most tissues.¹ Aside from their well-known roles as structural cells that synthesize collagen, fibronectin, and other extracellular matrix proteins, they are also capable of participating in the initiation and propagation of acute and chronic inflammatory responses.¹ On occasion, the process of inflammation drives some types of fibroblasts to differentiate to cells called myofibroblasts, important in wound healing and in scar formation.^{2,3} Interestingly, in some tissues such as those of the orbit of the eye, the bone marrow, blood vessels, and the liver, inflammatory processes drive preadipocyte fibroblasts to adipocytes.^{4–6} Thus, fibroblasts can differentiate to adipocytes, a process that can severely compromise tissue integrity and lead to loss of tissue function.

Recently, much interest has focused on a transcription factor called peroxisome proliferator activated receptor gamma (PPAR- γ). This transcription factor is the target of insulin-sensitizing drugs belonging to the thiazolidinedione family (eg, Rosiglitazone, Pioglitazone, and so forth).⁷ Putative natural ligands for PPAR- γ include prostaglandin (PG) products of the cyclooxygenase (Cox) pathway such as the PGD₂ metabolite 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂).^{8,9} PPAR- γ is crucial for the differentiation of preadipocyte fibroblasts to adipocytes.^{10–12} This process can be driven *in vitro* both by natural and synthetic PPAR- γ ligands (ie, 15d-PGJ₂, Rosiglitazone, and so forth).¹³ Indeed, type II diabetics using insulin-sensitizing thiazolidinedione-type drugs gain, on average, 3.5 kg in 26 weeks, in part because of fat accumulation.¹⁴

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The nature of the preadipocyte fibroblasts and the driving forces that induce their differentiation to adipocytes has yet to be elucidated. Nothing is known about this interaction between bona fide primary human fibroblasts and inflammation-associated infiltrating white blood cells that may drive fat accumulation as a type of tissue remodeling.

Graves' eye disease (also called thyroid eye disease) is a nonthyroidal consequence of an autoimmune process. The orbit becomes inflamed and infiltrated primarily with T lymphocytes and monocytes.^{15–17} We speculate that this drives the resident fibroblasts either to proliferate or to differentiate to adipocytes. The abundance of fat and inflammatory tissue then pushes the eye out of the orbit (exophthalmos) causing not only disfigurement but also double vision and sometimes blindness.¹⁸ We chose to study the process of adipogenesis using Graves' orbital preadipocyte fibroblasts because of the clinical relevance of this disease and to the juxtaposition of T cells with fibroblasts in the inflamed orbit.¹⁹ Herein, we demonstrate that activated human T cells express Cox-2, produce PPAR- γ ligands, and drive preadipocyte human orbital fibroblasts to adipocytes. These findings are interpreted within the context of inflammation, T-cell activation, and pathogenic tissue remodeling.

Materials and Methods

Materials

15d-PGJ₂ and Ciglitazone were purchased from Biomol (Plymouth Meeting, PA). PGD₂, 15-deoxy- $\Delta^{12,14}$ -PGD₂ (15d-PGD₂), GW9662, NS398, a mouse IgG₁ anti-Cox-2 unlabeled or fluorescein isothiocyanate antibody, a mouse IgG₁ anti-Cox-1 unlabeled or phycoerythrin-conjugated antibody, and an anti-aP2 [also known as fatty acid-binding protein 4 (FABP4)] antibody were purchased from Cayman Chemical (Ann Arbor, MI). Indomethacin, Oil Red O, recombinant human interleukin (IL)-2, and phytohemagglutinin (PHA) were from Sigma (St. Louis, MO). A polyclonal rabbit anti-PPAR- γ antibody was purchased from Calbiochem (San Diego, CA), the mouse monoclonal anti-PPAR- γ 2 antibody was from Chemicon International (Temecula, CA), and the anti-C/EBP- α antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All reverse transcriptase-polymerase chain reaction (RT-PCR) reagents were from Invitrogen (Carlsbad, CA).

Tissue Collection and Orbital Fibroblast Cell Culture

Primary orbital fibroblasts were isolated from individual Graves' patients undergoing orbital decompression surgery as previously described.²⁰ The primary fibroblasts were established by standard explant techniques, as previously described,²¹ and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2-mercaptoethanol (Eastman Kodak, Rochester, NY), L-glutamine (Life Technologies, Grand Island, NY),

HEPES (US Biochemical Corp., Cleveland, OH), nonessential amino acids, sodium pyruvate, and gentamicin (Life Technologies). These pure strains of adherent fibroblasts do not express CD45, factor VIII, or cytokeratin but do express vimentin and types I and III collagen, consistent with a fibroblast phenotype. Strains were stored in liquid N₂ until needed and were used between passages 4 to 9. The fibroblast strains were used at the earliest passage possible to obtain enough cells to complete the experiments.

T Cell Enrichment

Lymphocytes were isolated from 60 ml of peripheral blood obtained during the orbital decompression surgery. Whole blood was separated over a Ficoll-Paque Plus gradient (Amersham Biosciences, Piscataway, NJ) to obtain peripheral blood mononuclear cells. Lymphocytes were later frozen in RPMI supplemented with 2-mercaptoethanol, L-glutamine, HEPES, nonessential amino acids, sodium pyruvate, gentamicin, 20% fetal bovine serum, and 10% dimethyl sulfoxide and stored in liquid N₂ until needed. T-cell enrichment and culturing was done in RPMI 1640 media as described for the fibroblasts except the media were supplemented with 10% heat-inactivated human AB sera (Gemini Bio Products, Calabasas, CA). T cells were enriched using nylon wool fiber columns (Polysciences, Inc., Warrington, PA). Specifically, the nylon column was preincubated with prewarmed media at 37°C in 5% CO₂ for 1 hour. After that, a 2-ml lymphocyte cell suspension ($<7.5 \times 10^7$ cells/ml) was added to the column and incubated for 1 hour at 37°C in 5% CO₂. Nonadherent T cells were collected. Enrichment for T cells ranged from 70 to 90%. The column was refilled with media and incubated at 4°C for 30 minutes. Adherent B cells (referred to as autologous non-T-cell population) were collected via plunging. T-cell expansion was later accomplished by co-culture of the T-cell-enriched population with irradiated (1500 Rad) autologous non-T cells, in media supplemented with rIL-2 (25 U/ml) and PHA (5 μ g/ml) for 8 days. Additional rIL-2 was supplemented on the 4th day of co-culture. Several other T-cell activation methods were tested and IL-2 and PHA were found to be the best for induction of PG production (data not shown) and subsequent fibroblast activation.²² After this the enriched T-cell population was isolated using Ficoll-Paque Plus (Amersham Biosciences). All cells were examined for purity by staining with an anti-CD3 phycoerythrin-labeled antibody (BD Biosciences, San Jose, CA) and analyzed on a FACSCalibur flow cytometer (BD Biosciences). After expansion, the T-cell purity was $>95\%$.

Co-Culture of T Cells with Orbital Fibroblasts

Orbital fibroblasts from Graves' patients were plated at 5000 cells per treatment group. The following day, 1×10^5 autologous, enriched peripheral T cells were added. For all experiments the ratio of orbital fibroblasts to T cells was maintained at $5 \times 10^3:1 \times 10^5$ cells. The addition of

different drug treatments was done simultaneously with the addition of the enriched peripheral T cells. The drugs (eg, Cox inhibitors) were also added on subsequent days of the experiment as described below. The co-culture was maintained for 8 days in RPMI supplemented with 10% fetal bovine serum. In some experiments, fibroblasts and T cells were co-cultured in a transwell system with the fibroblasts on the bottom and the T cells in the top chamber separated by a polyester membrane with a pore size of 0.4 μm (Corning Inc., Corning, NY). In the co-culture system, the T cells loosely adhere and are juxtaposed to the fibroblasts. For experiments looking at only the orbital fibroblasts after T cell co-culture, the T cells were washed off with several phosphate-buffered saline (PBS) washes, and the removal of the T cells was monitored by microscopy.

Oil Red O Staining

Orbital fibroblasts were plated at 5000 cells/well in an eight-chamber glass slide. The following day, 1×10^5 autologous enriched peripheral T cells were added to the wells. The cells were co-cultured up to 8 days. Adipogenesis in orbital fibroblasts was assessed via staining of internal triglyceride vesicles. At the endpoint of the autologous mixed cell reaction, supernatants were removed, and cells were washed in PBS and fixed with 10% formaldehyde for 10 minutes at room temperature. Cells were briefly rinsed with distilled water and then 60% isopropanol. After this they were stained with filtered 0.3% Oil Red O in isopropanol/aqueous dextrin for 35 minutes at room temperature and washed with 60% isopropanol followed by an additional rinsing with distilled water. Occasionally, slides were also counterstained with hematoxylin for 10 seconds (Sigma). Washed glass slides were covered with Immu-Mount (Shandon, Pittsburgh, PA) and glass coverslips and visualized using the Olympus BX51 microscope (Melville, NY) at either $\times 400$ or $\times 600$ magnification. Quantification of the observed lipid vesicles was done using the Image-Pro Plus software version 4.5 (Media Cybernetics, Silver Spring, MD). Previous studies demonstrated that orbital fibroblasts contain lipid droplets and differentiate to adipocytes after 6 to 8 days of PPAR- γ ligand exposure.⁵ This timing for adipocyte differentiation is consistent with the 3T3-L1 preadipocyte model²³ but is slightly shorter than that reported for other preadipocyte models.^{24,25}

Western Blotting

The fibroblasts or T cells were washed in PBS, lysed in Nonidet P-40 lysis buffer containing a protease inhibitor cocktail [4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transepoxysuccinyl-L-leucylamido (4-guanidino) butane, bestatin, leupeptin, and aprotinin] (Sigma), and total protein was quantified using the bicinchoninic acid protein assay (BCA assay kit) (Pierce, Rockford, IL). Graves' decompression tissue was homogenized in Nonidet P-40 buffer with protease inhibitors. Five μg of total protein (for PPAR- γ /PPAR- $\gamma 2$) or 10 μg of total protein (for

Cox-1/2) was electrophoresed on 10% denaturing polyacrylamide stacking gels and transferred to nitrocellulose membrane. The membranes were blocked for 2 hours at room temperature in 10% Blotto (PBS/0.1% Tween 20, and 10% milk). Rabbit anti-PPAR- γ , mouse anti-PPAR- $\gamma 2$, mouse anti-Cox-1, or mouse anti-Cox-2 primary antibodies were added in 2.5% Blotto for 1 hour at room temperature, washed with PBS/Tween 20, and the secondary antibodies, goat anti-rabbit IgG-horseradish peroxidase or goat anti-mouse IgG-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), were added for 1 hour in 2.5% Blotto. Membranes were washed in PBS/Tween 20 and developed by chemiluminescence using a Western Lightning kit (Perkin-Elmer Life Sciences, Boston, MA). For loading control, membranes were reprobed with an antibody against actin (monoclonal mouse anti-actin; Oncogene, Boston, MA) and a goat anti-mouse IgM-horseradish peroxidase secondary antibody (Oncogene). Densitometry was performed using Kodak 1D Image Analysis Software (Eastman Kodak). The band intensities were normalized to the actin control and plotted as relative intensity. Western blots for the adipocyte proteins C/EBP α and aP2 were also performed on orbital fibroblast lysates and demonstrated expression of these proteins (data not shown).

RNA Isolation, RT-PCR for PPAR- $\gamma 1$ and PPAR- $\gamma 2$, and Quantitative RT-PCR for H-PGDS

RNA was isolated from homogenized Graves' orbital tissue using QIAzol reagent (Qiagen, Valencia, CA) and from orbital fibroblast strains and T cells using an RNeasy kit according to the manufacturer's protocol (Qiagen). RNA (0.5 μg) was incubated with 5 $\times$ reaction buffer, 3 μg of random primers, 40 U of recombinant RNasin ribonuclease inhibitor, 0.1 mmol/L dithiothreitol, 10 mmol/L of each dNTP, and 200 U of reverse transcriptase superscript III (RT) for 15 minutes at 25°C, 1 hour at 50°C, and 5 minutes at 95°C. A negative control without RT was performed, and there was no product formation. The PCR reaction for PPAR- $\gamma 1$ and PPAR- $\gamma 2$ included 10 $\times$ PCR buffer, 2 μl of cDNA, 10 mmol/L of each dNTP, 50 mmol/L MgCl_2 , 0.8 $\mu\text{mol/L}$ oligonucleotide primers specific for PPAR- $\gamma 1$ or PPAR- $\gamma 2$, 1.25 U of Platinum Hotstart TAQ DNA polymerase, and water to a final volume of 25 μl . Primer sequences (as previously published²⁶) were as follows: common PPAR- γ primer: 5'-CTTCCATTACGAGAGATCC-3'; PPAR- $\gamma 1$ -specific: 5'-AAAGAAGCCGACACTAAACC-3'; and PPAR- $\gamma 2$ -specific: 5'-GCGATTCCTTCACTGATAC-3'. PCR was performed for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds) in a PTC-200 DNA thermal cycler (MJ Research, Watertown, MA). Gel electrophoresis was performed on a 3% agarose gel, and PCR products were visualized with ethidium bromide. Quantitative real-time RT-PCR was performed on T cells for the hematopoietic prostaglandin D synthase (H-PGDS) and for 18S ribosomal RNA as a control. RNA isolation and the RT reaction were performed as described for PPAR- γ . The PCR reaction contained 50 mmol/L MgCl_2 , 0.8 $\mu\text{mol/L}$ primers,

12.5 μ l of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and 2 μ l of cDNA. The primers were as follows: H-PGDS sense oligo: 5'-ACCATGCCAACTACAACTC-3'; and the anti-sense oligo: 5'-AGCTTGTTCTATTCTGTGGTC-3'; 18S ribosomal RNA sense oligo: 5'-TGAGAAACGGCTACCACATC-3'; and anti-sense: 5'-ACTACGAGCTTTTAACTGC-3'. PCR was performed for 50 cycles (95°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute) in a Bio-Rad iCycler.

Immunohistochemistry

T cells (1×10^4) were cytospun onto glass microscope slides and fixed with 2% paraformaldehyde. For co-culture experiments, fibroblasts and T cells were cultured in glass chamber slides. The cells were washed in PBS, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide. After washing in PBS-0.1% Tween, nonspecific binding was blocked with 5% normal horse serum (Vector Laboratories, Burlingame, CA) for 1 hour. Next, the cells were incubated overnight at 4°C with a mouse monoclonal IgG₁ anti-15d-PGJ₂ antibody (a kind gift from Dr. Koji Uchida, Nagoya University, Nagoya, Japan),²⁷ a mouse anti-Cox-2 antibody, or an isotype control mouse IgG₁ antibody (Caltag, Burlingame, CA) in PBS/0.5% bovine serum albumin. The slides were washed three times with PBS/0.1% Tween, incubated with a horse anti-mouse IgG-biotin secondary antibody (Vector) for 1 hour at room temperature, washed two times with PBS/0.1% Tween, and incubated with streptavidin-peroxidase conjugate (Zymed, South San Francisco, CA) for 1 hour at room temperature. The slides were developed with aminoethylcarbazole substrate (Zymed), and coverslipped with Immu-Mount. Slides were visualized with an Olympus BX51 microscope, and photographs were taken using a SPOT camera with SPOT RT software (New Hyde Park, NY).

Enzyme Immunoassay for PGD₂, 15d-PGJ₂, and Related Compounds

PGD₂ and 15d-PGJ₂ were measured in T-cell supernatants using a MOX EIA kit from Cayman Chemical (highly specific for PGD₂), and an enzyme immunoassay (EIA) kit from Assay Designs, Inc. (Ann Arbor, MI) (15d-PGJ₂) as per the manufacturers' protocol. The EIA kit for 15d-PGJ₂ recognizes 100% of the 15d-PGJ₂; however, according to the manufacturer, it does cross-react with related PGs of the PGJ series.

Triglyceride Assay

Orbital fibroblasts and T cells were co-cultured for 8 days with or without indomethacin (20 μ mol/L added every day), NS398 (10 μ mol/L added on days 1 and 4), or the irreversible PPAR- γ antagonist GW9662 (1 μ mol/L added every other day) for 8 days. As a positive control, orbital fibroblasts alone were treated with 5 μ mol/L 15d-PGJ₂ (added every other day) for 8 days. The cells were rinsed

three times with $1 \times$ PBS and incubated with isopropanol for 45 minutes on a shaker. Cellular debris was removed by centrifugation, and the triglyceride content of the isopropanol fraction was measured using the L-type TG-H enzyme color kit from Wako Diagnostics (Richmond, VA) as per the manufacturer's protocol. The concentration of triglycerides in the samples was calculated based on the lipid calibrator standard curve (Wako Diagnostics). The colorimetric reaction was measured at 610 nm on a Benchmark microtiter plate reader (Bio-Rad, Hercules, CA).

Transfection of PPAR- γ Reporter Construct

Orbital fibroblasts (2×10^6) were co-transfected using Nucleofector solution (Amaxa Biosystems, Gaithersburg, MD) with 4 μ g of a PPRE-LUC reporter construct containing three copies of the acyl CoA oxidase-PPRE (a kind gift from Dr. Brian Seed, Massachusetts General Hospital, Boston, MA)²⁸ and with 1 μ g of a GFP reporter construct as a control for transfection efficiency (Amaxa). Cells were plated at 1×10^5 per well of a 12-well plate. Twenty-four hours after transfection, the fibroblasts were cultured with 5 μ mol/L 15d-PGJ₂ as a positive control or with autologous T cells for 48 hours at which time a luciferase assay was performed on cell extracts using a luciferase assay kit (Promega) and a multiwell format luminometer (Perkin-Elmer). Some cells were kept from each transfection and assayed for GFP expression by flow cytometry. A transfection efficiency of ~50% was achieved.

Statistical Analysis

Error bars represent the SD from the mean of triplicate samples. A two-tailed Student's *t*-test was performed and a *P* value of less than 0.05 was considered significant. All experiments were performed at least three times.

Results

Human Orbital Fibroblasts and Graves' Orbital Tissue Express PPAR- γ Protein

Findings using mouse systems indicate that PPAR- γ expression is crucial for fibroblastic preadipocyte differentiation to adipocytes; such systems require both PPAR- γ expression, as well as exposure to natural or synthetic PPAR- γ ligands.^{12,29} Figure 1A shows that orbital tissue from two Graves' patients and three human orbital fibroblast strains (each from a separate Graves' disease patient) all highly expressed PPAR- γ protein. Importantly, these samples contained PPAR- γ 2 protein, as determined using a monoclonal antibody specific for only the PPAR- γ 2 isoform (Figure 1A). In addition, Graves' orbital tissue and orbital fibroblast strains expressed mRNA for both isoforms of PPAR- γ (PPAR- γ 1 and PPAR- γ 2) (Figure 1B), suggesting the potential to differentiate to an adipocyte phenotype.

Figure 1. Human Graves' orbital fibroblasts express PPAR- γ protein and mRNA. **A:** A Western blot for total PPAR- γ (both $\gamma 1$ and $\gamma 2$ isoforms) and for PPAR- $\gamma 2$ (using a monoclonal antibody specific for the unique N terminus of PPAR- $\gamma 2$) was performed on orbital tissue from decompression surgery of two patients and orbital fibroblasts from three different Graves' patients. Five μ g of protein were loaded for each lane and the membranes were re-probed for total actin as a loading control (for fibroblasts only). **B:** RT-PCR for PPAR- $\gamma 1$ and PPAR- $\gamma 2$ was performed on 0.5 μ g of RNA from Graves' orbital decompression tissue (two patients) and orbital fibroblasts (three patients).

Activated Human T Lymphocytes Drive Human Orbital Fibroblasts to Adipocytes

Experiments were next performed using natural (15d-PGJ₂) and synthetic PPAR- γ ligands (eg, Rosiglitazone, Ciglitazone), and these ligands induced differentiation of orbital fibroblasts to Oil Red O-staining adipocytes (Figure 2A). The inflammation in the human orbit in Graves' disease is characterized mainly by infiltrates of T lymphocytes.^{15–17} This observation suggested to us that in susceptible tissue sites T cells may be driving fat production. Thus, sets of Graves' patient orbital fibroblasts were incubated with autologous, activated peripheral blood T cells. The cells were co-cultured up to 8 days to permit fibroblast differentiation to adipocyte-like cells. Adipogenesis was monitored by the observance of morphological changes, accompanied by the presence of lipid-filled refractile bodies, which stained red with Oil Red O (Figure 2, A and B). Orbital fibroblasts cultured with the natural PPAR- γ ligand 15d-PGJ₂ or with the synthetic ligand ciglitazone had abundant Oil Red O-staining cells (Figure 2A). Most interesting was the observation that the addition of activated, but not naïve, T lymphocytes strongly drove fibroblasts to adipocytes (Figure 2, B and C). In these studies, the percentage of Oil Red O-positive cells varied between 20 to 50% depending on the strain of orbital fibroblast. The up-regulation of the adipocyte-specific proteins C/EBP α and FABP4 (aP2) was established by Western blotting, confirming that the fibroblasts differentiated to adipocytes (data

not shown). Orbital fibroblasts cultured with IL-2 and PHA (T cell activators) were negative for Oil Red O staining and did not differentiate (data not shown).

T-Lymphocyte-Induced Fibroblast Differentiation to Adipocytes Is a Cyclooxygenase-Dependent Process

Fibroblasts are well known to be capable of expressing both Cox-1 and Cox-2.³⁰ One of the known ligands for PPAR- γ is 15d-PGJ₂, a Cox product.^{8,9} We hypothesized that orbital fibroblasts and/or the T lymphocytes were expressing Cox-2 and that a PG(s) was responsible for the adipogenesis process. As a first step, co-cultures of orbital fibroblasts and T lymphocytes were treated with the Cox-1/Cox-2 inhibitor indomethacin or with the Cox-2 selective inhibitor NS-398. Interestingly, co-cultures treated with either indomethacin or NS-398 failed to differentiate to adipocyte-like cells (Figure 2B and Figure 3). To further show the Cox-2 dependence of this process, triglyceride levels were measured in cells after 8 days of culture (Figure 3). Activated, but not unactivated, T lymphocytes strongly increased triglyceride levels in the orbital fibroblasts. Co-culture of fibroblasts plus activated T cells exposed to the Cox-1/Cox-2 inhibitor indomethacin or the Cox-2 selective inhibitor NS-398 reduced triglyceride levels to near background. These findings strongly support a role for Cox-2 in the adipogenesis process.

Activated Human T Lymphocytes from Graves' Patient Peripheral Blood Strongly Express Cox-2

Recent reports reveal that activated human T lymphocytes can express Cox-2.^{31–33} Two Graves' patients' T cells were evaluated for Cox-1 and Cox-2 expression by Western blotting (Figure 4A). Interestingly, human peripheral blood T cells highly express Cox-2 when activated. In contrast, our studies with the orbital fibroblasts showed little or no Cox-2 expression (data not shown; Figure 5). The peripheral blood T cells also highly express Cox-1, which was shown by Western blot in Figure 4A to be down-regulated on activation. In addition, Graves' orbital tissue from two patients was positive for Cox-1 and Cox-2 by Western blot (Figure 4B). These findings suggested that it was the T lymphocytes in the co-culture system that were producing a Cox-2-derived product(s) that acted as a PPAR- γ ligand.

T Lymphocytes in Co-Culture with Fibroblasts Highly Express Cox-2

Co-cultures of human orbital fibroblasts and unactivated or activated T lymphocytes were stained *in situ* for Cox-2 (Figure 5). Unactivated T cells and their companion fibroblasts showed no Cox-2 expression in either cell type. In contrast, strong Cox-2 expression (Figure 5) occurred in the activated T cells cultured with orbital fibroblasts. The orbital fibroblasts express little or no Cox-2 (Figure 5).

Figure 2. Activated human T lymphocytes drive human Graves' orbital fibroblasts to adipocytes. **A** and **B**: Oil Red O staining was performed on 8-day cultures of orbital fibroblasts exposed to the PPAR- γ agonists 15d-PGJ₂ or ciglitazone (**A**) or co-cultured with activated (rIL-2 and PHA) or unactivated peripheral blood T cells from Graves' patients (**B**). **B**: The Cox inhibitor indomethacin blocked T-cell-induced adipocyte differentiation of orbital fibroblasts. **C**: The amount of lipid droplet formation for the T-cell orbital fibroblast co-culture was quantified using Image-Pro Software and was plotted as area of lipid droplet formation. * $P < 0.007$ for orbital fibroblast (OF) plus T cells versus OF alone.

Fibroblast-T Lymphocyte Contact Is Not Needed for Adipogenesis

If T lymphocytes were producing a Cox-2 derived PG product(s) that was driving adipogenesis, then activated

Figure 3. T-lymphocyte-induced fibroblast differentiation is a Cox-dependent process. Orbital fibroblasts were co-cultured with either activated or unactivated T cells in the presence or absence of vehicle, or the Cox-1/Cox-2 inhibitor indomethacin, or with the Cox-2-specific inhibitor NS-398. After 8 days of co-culture, the triglyceride content of the orbital fibroblasts was measured in a colorimetric triglyceride assay. * $P < 0.01$ for orbital fibroblasts cultured with activated T cells versus co-cultures treated with Cox inhibitors.

T cells should be capable of driving adipogenesis in a contact-independent manner. Orbital fibroblasts were separated from activated T cells using a transwell system. Fibroblasts were cultured in the lower chamber and T cells in the upper chamber. Interestingly, even when T cells were separated from the fibroblasts, differentiation to adipocytes proceeded (Figure 6). These findings supported that a T-cell-derived secreted product stimulated adipogenesis.

Activated Human T Lymphocytes Produce PGD₂-Derived PPAR- γ Ligands

Controversy remains about the synthesis of the PPAR- γ ligand 15d-PGJ₂.³⁴ Moreover, it is not known which human cells produce it. Our data reported, herein, show that a Cox-2-derived T-cell product can drive adipogenesis. The most likely candidate would be the PPAR- γ ligand 15d-PGJ₂ or a closely related PGJ or PGD family member. We tested activated human T cells (Cox-2 expressing) for the ability to produce both PGD₂ and 15d-PGJ₂ (and PGs of the PGJ series). PGD₂, which spontaneously undergoes a series of de-

Figure 4. Activated human T lymphocytes from Graves' patient peripheral blood strongly express Cox-2. **A:** A Western blot for Cox-1, Cox-2, and total actin was performed on activated and unactivated T cells from two Graves' patients. Densitometry was performed to calculate the relative intensities of Cox-1 and Cox-2 expression. **B:** A Western blot for Cox-1 and Cox-2 was performed on equal amounts of protein from Graves' orbital tissue (two patients) obtained from decompression surgery.

hydration reactions to form the PGJ family of prostaglandins, is the precursor for 15d-PGJ₂.³⁵ An enzyme immunoassay, specific and sensitive for PGD₂, first showed that activated but not unactivated T cells produce PGD₂ (Figure 7A). These findings suggest that the PGD₂ metabolites of the PGJ series, such as 15d-PGJ₂, would also be produced. A sensitive enzyme immunoassay revealed that activated human T cells do produce prostaglandins of the PGJ family, including 15d-PGJ₂ (Figure 7A). It is interesting to note that activated T cells from a normal donor produced reduced amounts of PGs compared with T cells from Graves' patients. This result may be because of the high expression of Cox-2 in the Graves' patient T cells, compared with a normal donor (data not shown). Further studies are clearly needed to define differences between Graves' and normal T cells. A final set of experiments were performed using a monoclonal antibody specific for 15d-PGJ₂.²⁷ Co-cultures of human

Figure 5. Activated, but not unactivated T lymphocytes from Graves' patients are positive for Cox-2 in co-culture with human orbital fibroblasts. Immunohistochemistry was performed for Cox-2 on 8-day co-cultures of activated (top) or unactivated (bottom) Graves' T cells with orbital fibroblasts. The activated T cells (arrows) are highly positive for Cox-2, whereas the fibroblasts are either weakly positive or negative for Cox-2.

orbital fibroblasts and activated human T lymphocytes were stained with an anti-15d-PGJ₂ antibody (Figure 7C). Interestingly, the majority of the activated human T cells strongly stained with this antibody (Figure 7, B and C). The 15d-PGJ₂ detected was strongly cytoplasmic and possibly surface displayed as well. The photograph also reveals the juxtaposition of 15d-PGJ₂ staining T cells with orbital fibroblasts. As further evidence for the production of PGD₂ and its metabolites, quantitative RT-PCR analysis demonstrated that activated Graves' T cells highly express H-PGDS, the en-

Figure 6. Fibroblast-T cell contact is not needed for adipogenesis. Fibroblasts and T cells were either co-cultured in a transwell system in which the fibroblasts and T cells were separated by a 0.4-μm membrane or cultured without separation. 15d-PGJ₂ (5 μmol/L) was added as a positive control. After 8 days of culture, the orbital fibroblasts in the transwell system were still able to differentiate to adipocytes as determined by Oil Red O staining.

Figure 7. Activated human T lymphocytes produce PGD₂ and its PGJ₂ metabolites, including the PPAR- γ ligand 15d-PGJ₂. **A:** Normal or Graves' T cells were either activated or not activated for 8 days. PGD₂ (left) and the PGJ₂ series of PGD₂ metabolites, including 15d-PGJ₂ (right) were measured in cell culture supernatants by enzyme immunoassay. **B:** Unactivated or activated Graves' T cells were stained with a monoclonal anti-15d-PGJ₂ antibody. Only activated T cells stained positive for 15d-PGJ₂. The isotype-stained cells are negative. **C:** Graves' T cells co-cultured with orbital fibroblasts for 3 days stain positive for 15d-PGJ₂. The orbital fibroblasts are negative for 15d-PGJ₂. The arrows indicate T cells. These experiments were repeated three times with T cells from different Graves' patients. **D:** Quantitative RT-PCR for H-PGDS was performed on RNA from unactivated Graves' T cells (one patient) and activated T cells from two Graves' patients. Cycle threshold values were normalized to 18S ribosomal RNA, and fold induction over unactivated T cells was calculated. Original magnifications: $\times 600$ (**B**, **C**); $\times 1000$ (insets).

zyme responsible for the conversion of PGH₂ to PGD₂ (Figure 7D).

T-Lymphocyte-Induced Fibroblast Differentiation to Adipocytes Is a PPAR- γ -Dependent Process

PGD₂ is the precursor for the PGJ family of PGs.^{35,36} PGD₂ also converts to 15d-PGD₂.^{37,38} These structurally similar molecules all can transactivate PPAR- γ .^{37,38} Therefore, we tested PGD₂, 15d-PGD₂, and 15d-PGJ₂ for their ability to induce adipogenesis of orbital fibroblasts. Indeed, all three PGs induced significant triglyceride accumulation (Figure 8A). The addition of the irreversible PPAR- γ antagonist GW9662 completely prevented triglyceride accumulation in the orbital fibroblasts exposed to PGs (Figure 8A), confirming that the adipogenesis process is PPAR- γ -dependent. To determine whether the T cell-derived PGs were indeed activating PPAR- γ to drive adipogenesis of the orbital fibroblasts, a PPAR- γ luciferase reporter construct (PPRE-Luc), using three copies of the PPAR-response element (PPRE), was transfected into human orbital fibroblasts.²⁸ The orbital fibroblasts were also co-transfected with a pGFP plasmid to determine transfection efficiency and an efficiency of 50% was achieved. As shown in Figure 8B, activated T cells induced PPAR- γ activity in the orbital fibroblasts as measured by luciferase activity after 48 hours of co-culture. The addition of 5 μ mol/L 15d-PGJ₂, which was shown to drive adipogenesis of orbital fibroblasts in Figure 2A, also induced PPAR- γ activation. Moreover, the irreversible PPAR- γ antagonist GW9662 prevented triglyceride accumulation in the orbital fibroblasts co-cultured with activated T lymphocytes (Figure 8C). These findings strongly support a PPAR- γ -mediated process.

Discussion

Our findings provide strong support for the novel concept that activated human T lymphocytes drive human fibroblasts to differentiate to adipocytes. Using Graves' disease as a model system of the link between inflammation and tissue remodeling to fat, we show that human peripheral blood T cells convert preadipocyte orbital fibroblasts to adipocytes *in vitro*. These findings appear to recapitulate the remodeling to fat that occurs in Graves' patients with ocular complications.³⁹ Our new findings strongly support that the Graves' T cell is a culprit in thyroid eye disease. Indeed, the orbit of afflicted Graves' patients is infiltrated with T lymphocytes.^{15–17} We suggest that these activated T cells express Cox-2 and produce PGD₂ and members of the PGJ family of PGs including 15d-PGJ₂. Determination of the actual PPAR- γ ligands produced in human orbital tissue will be the focus of future investigation. Sensitive PPAR- γ -expressing orbital fibroblasts would be expected throughout time to drive the orbital remodeling as evidenced by fat deposition.⁴⁰ Indeed, our *in vitro* data using human T-cell orbital fibroblast co-cultures and subsequent adipocyte differentiation supports this scenario. We believe that T cells are a key contributor

Figure 8. Prostaglandin and T-lymphocyte-induced fibroblast differentiation to adipocytes is a PPAR- γ -dependent process. **A:** Graves' orbital fibroblasts were exposed to PGD₂ (5 μ mol/L), 15d-PGD₂ (5 μ mol/L), and 15d-PGJ₂ (5 μ mol/L) with or without the irreversible PPAR- γ antagonist GW9662 (1 μ mol/L) for 8 days at which time an assay to measure triglyceride content was performed. The data are the mean of triplicates $\pm$ the SD. * P < 0.001 compared with PGs alone. **B:** Graves' orbital fibroblasts were co-transfected with the PPRE-LUC reporter construct and a GFP control plasmid, and then co-cultured with Graves' T cells. 15d-PGJ₂ was used as a positive control PPAR- γ ligand. A luciferase assay was performed after 2 days of co-culture. The data are the mean of triplicate samples ($\pm$ SD), and the luciferase results were plotted as fold increase over the untreated control. * P < 0.05 versus untreated OF cells. **C:** The irreversible PPAR- γ antagonist GW9662 inhibits the ability of activated T cells to induce adipogenesis in orbital fibroblasts. Fibroblasts were co-cultured with 1 μ mol/L GW9662 and activated T cells for 8 days. The results of a triglyceride assay are shown. The data are the mean of triplicates $\pm$ the SD. * P < 0.001 versus orbital fibroblasts (OF) plus T cells. There was no statistical difference between OF alone and OF plus T cells plus GW9662.

of proadipogenic PG production given their abundance in orbital tissue and high expression of Cox-2; however, it is possible that other infiltrating cells such as macrophages may express Cox-2 and generate PGs.^{16,27}

Our findings are the first to show that human T cells, when activated, strongly express Cox-2 and produce PGs, possibly 15d-PGJ₂, that are PPAR- γ ligands. Multiple approaches including enzyme immunoassay, immunostaining, mRNA analysis, and PPAR- γ reporter assays reveal that the T cells do produce PGD and PGJ family members. Moreover, T cells synthesize a molecule that drives orbital fibroblast differentiation to adipocytes, a

PPAR- γ -dependent process.^{10,11,41} Furthermore, the differentiation of orbital fibroblasts to adipocytes, in co-culture with activated T cells, is blocked by the Cox-2 inhibitor NS-398. The fact that the T cells can be physically separated from the fibroblasts and still induce adipogenesis supports that a secreted mediator (ie, a PG) was responsible for the fat accumulation. PGD₂ is a key PG mainly known to be produced by mast cells.⁴² Our data show that human T cells also produce PGD₂. Recent findings show that PGD₂ converts to the PGJ series of PGs with the final product being 15d-PGJ₂, a notable potent PPAR- γ ligand.^{8,9,35} It is likely that Cox-2-expressing T cells produce PGD₂, which is then converted by cell-independent mechanisms to the short-lived 15d-PGJ₂ or related family members, which then binds fibroblast PPAR- γ and initiates conversion to adipocytes. Indeed, we show (Figures 1 and 2) that human orbital fibroblasts express PPAR- γ and that 15d-PGJ₂, PGD₂, and 15d-PGD₂ strongly induce adipogenesis. Moreover, adipogenesis is a PPAR- γ -dependent process because T cells were unable to drive fibroblast differentiation in the presence of a small molecule PPAR- γ irreversible antagonist (Figure 8). It is likely a combination of multiple PGs of the PGD₂ family that are responsible for activating PPAR- γ to induce fibroblast adipogenesis. However, the determination of the most significant adipogenic PGs produced by activated T cells is a subject for future investigation. In support of our findings that PPAR- γ ligands induce orbital fibroblast adipogenesis, several case reports have described the development of proptosis in patients receiving thiazolidinedione treatment for type 2 diabetes.^{43,44} In particular, a patient with thyroid eye disease experienced exacerbated disease after starting pioglitazone therapy.⁴³

That activated peripheral blood T cells express Cox-2 and produce PGD₂ and possibly 15d-PGJ₂ is a provocative finding. Our previous work demonstrated that human T cells themselves express PPAR- γ and that natural (15d-PGJ₂) and synthetic (eg, Ciglitazone) PPAR- γ ligands promote T-cell synthesis of IL-8.^{45,46} Thus, T cells themselves possess an autocrine pathway where T-cell-synthesized PPAR- γ ligands may further activate T cells. Recent exciting findings also reveal that T cell Cox-2 expression is required for optimal T-cell survival.^{32,33} For example, in systemic lupus erythematosus, activated T cells express Cox-2 and inhibition of Cox-2 activity with a Cox-2 selective inhibitor enhanced the onset of apoptosis.³³

PPAR- γ and PPAR- γ ligands have proven to be a highly interesting and important field of research, not only for adipogenesis, but also for cancer and inflammation. PPAR- γ ligands are finding wide utility as anti-inflammatory agents. For example, both natural and synthetic PPAR- γ ligands dampen the synthesis of proinflammatory mediators including IL-6, tumor necrosis factor, and IL-1.²⁸ PPAR- γ ligands are also potent inducers of B lymphocyte and B lymphoma apoptosis.^{47,48} T cells are usually considered as proinflammatory cells. However, our new data on PPAR- γ ligand synthesis by T cells support that T cells could also play an important anti-inflammatory role. The synthesis of PGD₂ and related

PGJ molecules would be expected to dampen the activation of other cells, including fibroblasts. Fibroblasts are capable of synthesizing prodigious quantities of proinflammatory mediators including, IL-1, IL-6, IL-8, and so forth.^{49,50} T cells infiltrating a tissue may dampen traditional inflammation by attenuating regional synthesis of inflammatory cytokines and by remodeling the tissue to fat. Such a process would serve to render the tissue more quiescent.

Our studies suggest that T cells may drive the process of adipogenesis in other tissues. Fibroblasts can differentiate to myofibroblasts^{2,3} (scar-forming cells) or to adipocytes.⁵ Human tissues can form scars or fat as a consequence of chronic inflammation.¹ Our findings suggest that activated T cells may contribute to fat accumulation during atherosclerotic plaque formation. It is possible that tissues predisposed to become fatty (eg, orbit, bone marrow, liver) are driven to that pathway as a result of T lymphocyte synthesis of PPAR- γ ligands. These concepts will, of course, require further investigation. However, the implications for tissue derangement and for obesity are of obvious importance.

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