Evidence for Enhanced Thy-1 (CD90) Expression in Orbital Fibroblasts of Patients with Graves' Ophthalmopathy

Teck Kim Khoo,¹ Michael J. Coenen,¹ A. Reagan Schiefer,¹ Seema Kumar,² and Rebecca S. Bahn¹

Background: Thy-1 is a surface protein that defines functionally distinct subpopulations of fibroblasts, with those lacking the antigen being capable of adipogenesis. Because increased fat cell development is a hallmark of the orbit in Graves' ophthalmopathy (GO), we wished to compare baseline Thy-1 expression in orbital fibroblasts from GO patients and normal individuals, and determine whether levels of the protein might be impacted by adipogenesis following peroxisome proliferator activator- γ ligation.

Methods: Orbital adipose/connective tissue specimens were obtained from euthyroid patients undergoing orbital decompression surgery for severe GO ($n=9$) and from normal individuals ($n=9$). Thy-1 mRNA and protein levels were assessed in tissue specimens and in orbital fibroblast cultures at baseline using RT-PCR, quantitative immunofluorescent staining, and flow cytometry using a specific Thy-1 mouse anti-human CD90/Thy-1 monoclonal antibody. In addition, some orbital fibroblast cultures were treated with rosiglitazone ($1\ \mu\text{L}/\text{mL}$; $2\ \text{nM}$) or control for 10 days in culture.

Results: We found that Thy-1 mRNA and protein expression was higher in uncultured GO connective/adipose tissue specimens (3.8-fold; 0.835 ± 0.116 relative expression) compared with normal (0.22 ± 0.062 ; $p=0.002$) and in cultured orbital fibroblasts from GO patients (3.3-fold; 9.28 ± 1.82 relative expression) compared with normal cultures (2.80 ± 0.42 ; $p=0.013$). Adipocyte differentiation had no effect on Thy-1 expression. Flow cytometry and immunofluorescent staining showed increased numbers of Thy-1–positive cells in the GO (mean $77.9 \pm 4.09\%$; range $66.5\text{--}84.8\%$) compared with the normal fibroblast cultures ($66.8 \pm 1.6\%$; range $63.3\text{--}71.0\%$ positive; $p=0.046$), as well as higher levels of expression on the positive cells.

Conclusions: Increased Thy-1 expression in GO orbital tissues and cultures is likely a consequence of the orbital disease process, reflecting both the presence of increased numbers of Thy-1–positive cells and higher expression on those cells. Adipogenesis itself does not appear to impact Thy-1 expression. Increased expression of this protein in GO could represent an adaptive response to cell injury, in effect limiting disease progression within the orbital adipose/connective tissues.

Introduction

MANY OF THE CLINICAL SYMPTOMS and signs of Graves' ophthalmopathy (GO) can be explained on a mechanical basis by an increase in the volume of intraorbital tissues (1). While most individuals with GO have evidence of both extraocular muscle and orbital adipose tissue enlargement, some exhibit a predominance of either muscle or fat expansion. Fibroblasts contained both within the adipose/connective tissue of the posterior orbit and surrounding the extraocular muscles are involved in the pathogenic process (2). These cells exhibit remarkable phenotypic heterogeneity

as regards surface receptor expression, glycosaminoglycan (GAG) synthesis, and adipogenic potential (3).

Thy-1 is a classical T lymphocyte marker that is expressed in both humans and mice (4–5). Recent studies have shown it to be expressed on fibroblasts where it defines functionally distinct subpopulations of cells; those displaying this antigen (Thy-1+) produce hyaluronan and prostaglandin E₂, interleukin (IL)-6, and are capable of myofibroblast differentiation (6–8). Fibroblasts lacking this antigen (Thy-1–) have the ability to differentiate into mature adipocytes, express Human Leukocyte Antigen-DR (HLA-DR), and produce high levels of IL-8 following activation. Since both Thy-1+ and

¹Division of Endocrinology, Metabolism and Nutrition; ²Division of Pediatric Endocrinology, Mayo Clinic, Rochester, Minnesota. This work was presented in part at the 78th Annual meeting of the American Thyroid Association (New York, 2007).

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH GO AT TIME OF ORBITAL DECOMPRESSION SURGERY

Patient	Age/ sex	Smoker	Duration of Graves' disease (years)	Duration of GO (years)	Optic neuropathy	Previous antithyroid medication	Glucocorticoid use in previous 3 months	Previous orbital radiotherapy	Previous radioiodine therapy	Time since radioiodide treatment (years)	TSI (≤ 1.3 TSI index)
#1	58/F	No	1	1	Yes	No	No	No	Yes	1	ND
#2	47/F	No	1	1	Yes	No	Yes	No	No	NA	ND
#3	53/F	Yes	2	2	Yes	No	Yes	Yes	Yes	2	ND
#4	56/M	No	20	20	No	Yes	No	No	Yes	18	ND
#5	36/F	Yes	2	1	No	Yes	No	No	No	NA	13
#6	49/F	No	6	6	No	No	Yes	No	Yes	6	7.4
#7	55/M	Yes	3	1	Yes	No	Yes	No	Yes	3	11
#8	53/F	No	2	1	No	No	Yes	No	Yes	2	8.5
#9	29/F	Yes	6	1	No	Yes	No	No	Yes	4	7.6

GO, Graves' ophthalmopathy; TSI, thyroid stimulating immunoglobulin.

Thy-1 – fibroblasts express the adipogenic transcription factor peroxisome proliferator activator- γ (PPAR- γ), adipogenic potential in Thy-1 – cells likely stems from phenotypic characteristics downstream from this receptor. Thy-1+ cells comprise the majority of the fibroblast population within the fatty connective tissues of the posterior orbit, while the remaining third are Thy-1 – and thus capable of adipocyte differentiation (9). Fibroblasts investing the extraocular muscles uniformly display this antigen and therefore do not differentiate into adipocytes when cultured under identical conditions.

Previous studies of Thy-1 expression in GO orbital fibroblasts have compared those derived from the posterior orbit with fibroblasts investing the extraocular muscles (7,9). We undertook these studies to determine whether the level of Thy-1 displayed in orbital adipose/connective tissues from GO patients differs from that found in tissues from normal individuals. In addition, we wished to determine whether Thy-1 expression is impacted by adipogenesis induced through ligation of the PPAR- γ receptor.

Materials and Methods

Tissue collection and cell cultures

Orbital adipose/connective tissue specimens were obtained from euthyroid patients undergoing orbital decompression surgery for severe GO ($n = 9$) and retrieved at very early autopsy from individuals with no history of Graves' disease whose corneas were being procured for transplantation ($n = 9$). Clinical characteristics of the GO patients are shown in Table 1. Tissues were placed in a container on saline-soaked gauze and transported at room temperature to the laboratory where it was either frozen immediately at -70°C or minced and placed directly in plastic culture dishes as previously described (10). Cells were propagated in medium 199 containing 20% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 U/mL), and gentamicin (20 $\mu\text{g/mL}$) in a humidified 5% CO_2 incubator at 37°C and maintained in 80-mm² flasks until confluent.

Cultures were studied either at confluence (baseline) or following 10 days in serum-free DMEM/Ham's F-12 (1:1; Sigma Chemical, St. Louis, MO) supplemented with biotin (33 μM), panthothenic acid (17 μM), transferrin (10 $\mu\text{g/mL}$), T3 (0.2 nM), insulin (1 μM), carbaprostacylin (0.2 μM ; Calbiochem, La Jolla, CA), and for the first 4 days only, dexameth-

asone (1 μM) and isobutylmethylxanthine (0.1 nM). Some cultures were treated with rosiglitazone (1 $\mu\text{L/mL}$; 2 nM) for the entire 10-day differentiation period. Media were replaced in all cultures every 3–4 days.

Quantitative RT-PCR

Orbital tissue (100–150 mg) was homogenized in 4.0 mL lysis buffer (QIAGEN, Valencia, CA) for 45–60 seconds. RNA was extracted and subsequently reverse transcribed to cDNA using methods previously described (10). Briefly, total RNA was isolated from tissue samples using the RNeasy kit (QIAGEN) according to the manufacturer's protocol. This was then purified using an affinity resin column (RNeasy; QIAGEN, Chatsworth, CA). The cDNA was subsequently synthesized using the Superscript cDNA synthesis kit (Life Technologies–BRL, Gaithersburgs, MD) using 200 ng of total RNA incubated with random hexamers, followed by a 100- μL RT reaction with 6.25 U of Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). Conditions used were 25°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes. Real-time quantitative RT-PCR for Thy-1, adiponectin, PPAR- γ , and GAPDH was performed using Taqman gene expression probes (Applied Biosystems). Expression of mRNA species was assessed using standard curve method and normalized to GAPDH. Data were expressed as relative expression units, and statistical analyses were performed using the statistical software SigmaStat (Version 3.0.1; Systat Software, Chicago, IL).

Quantitation of Thy-1 protein expression using digital imaging

Confluent cell cultures were trypsinized, and approximately 50,000 cells per chamber were plated on two-chamber slides in medium 199. After 2 days in culture, mouse anti-human CD90/Thy-1 monoclonal antibody (2.0 $\mu\text{g/mL}$; # MAB2067; R & D Systems, Minneapolis, MN) was added to the cultures. Following overnight incubation at 4°C , slides were counterstained with a goat anti-mouse fluorescein-conjugated secondary antibody (R & D Systems). Control slides were counterstained with the secondary antibody only, without prior exposure to the Thy-1 antibody. Twelve photographs (850-millisecond exposure; 10 $\times$ magnification) per chamber were obtained at random locations using a Zeiss

FIG. 1. Relative expression of Thy-1 mRNA in whole orbital adipose/connective tissue specimens obtained from patients with GO ($n=5$) and normal individuals ($n=5$). Expression was 3.8-fold greater in GO than in normal specimens ($*p=0.002$).

AxioPlan 2 compound microscope with an FITc filter attached to a Zeiss AxioCam HRc camera. Fields containing obvious artefacts (contaminants and bubbles) were excluded. Thy-1 protein expression was quantitated using digital imaging software (Adobe Photoshop 7.0; Adobe Systems Incorporated, San Jose, CA). Areas with appropriate color ratios (red/green/blue 49:84:35; fuzziness 80) were selected and the number of positive pixels counted. Data were expressed as means of positive pixels per set of 12 images, and statistical analyses were performed using *t*-tests.

Flow cytometry

Cells plated in medium 199 were trypsinized, and approximately 5×10^5 cells were incubated for 1 hour at room temperature with the specific Thy-1 antibody ($2.0 \mu\text{g/mL}$), then counterstained with the goat anti-mouse fluorescein-conjugated secondary antibody. Control slides were counterstained with the secondary antibody only, without prior exposure to the Thy-1 antibody. Cells were washed immediately and studied using standard flow cytometric techniques. Data were analyzed using unpaired *t*-test.

Results

Orbital adipose/connective tissue specimens

Quantitative RT-PCR analysis of Thy-1 mRNA expression in whole tissue specimens derived from the orbital adipose connective tissues of patients with GO ($n=5$; patients 1–5 in Table 1) and normal individuals ($n=5$) revealed a 3.8-fold higher levels of Thy-1 mRNA in the GO samples (0.835 ± 0.116 relative expression) compared with normal (0.22 ± 0.062 ; $p=0.002$; Fig. 1).

The GO patients in our study were somewhat younger than the normal individuals studied (mean age 48.4 ± 3.3 years vs. 64.9 ± 6.3 years, $p=0.03$; Table 1). However, we found no correlation between age and Thy-1 expression either overall, or within the GO or normal groups. We further found no relationship between history of previous antithyroid medi-

FIG. 2. Relative expression of Thy-1 mRNA in cultures of orbital fibroblasts derived from patients with GO ($n=4$; dark circles) and normal individuals ($n=4$; open circles) at confluence (Baseline) and following 10-day treatment with the thiazolidinedione rosiglitazone (TZD; 2 nM). Expression was 3.3-fold greater in GO than in normal cultures at baseline ($*p=0.01$) and was 2.5-fold higher in GO than in normal cultures following TZD treatment ($**p=0.03$). Neither GO nor normal cultures significantly increased levels of Thy-1 mRNA expression following TZD treatment.

cation, radioiodine ablation (including time since the treatment), orbital radiotherapy, or glucocorticoid therapy and Thy-1 expression in the GO patients. Neither did thyroid stimulating immunoglobulin (TSI) level, smoking status, duration of GO or Graves' disease, or presence of optic neuropathy correlate with Thy-1 levels.

Orbital fibroblast cultures

Quantitative RT-PCR analysis of Thy-1 mRNA expression in baseline cultures of orbital fibroblasts derived from patients with GO ($n=4$; patients 6–9 in Table 1) and normal individuals ($n=4$) revealed 3.3-fold higher levels of Thy-1 mRNA in the GO cultures (9.28 ± 1.82 relative expression) compared with normal cultures (2.80 ± 0.42 ; $p=0.013$; Fig. 2). Following 10 days of treatment with the thiazolidinedione rosiglitazone (TZD; 2 nM), neither the GO (7.38 ± 1.47) nor the normal (2.94 ± 0.68) cultures increased expression of Thy-1 mRNA, with the GO cultures maintaining 2.5-fold higher levels of Thy-1 expression compared with the normal cultures (Fig. 2; $p=0.034$).

Quantitation of Thy-1 protein expression using digital imaging revealed significantly greater levels of Thy-1 protein in GO cultures ($n=4$; patients 6–9 in Table 1; mean 2147 ± 371 positive pixels; range 1318–3035) compared with normal cultures ($n=4$; 599 ± 112 ; range 378–889; $p=0.007$; Fig. 3). This difference in fluorescence was generally visible under the microscope at $10\times$ magnification even without formal quantitation (Fig. 4).

Flow cytometric measurement of Thy-1 protein expression in GO ($n=4$; patients 6–9 in Table 1) and normal ($n=4$) orbital fibroblast cultures at baseline revealed increased Thy-1 expression in a greater proportion of the GO fibroblasts (mean

FIG. 3. Quantitation of Thy-1 protein expression in GO ($n=4$) and normal ($n=4$) orbital fibroblast cultures at baseline using digital imaging. Higher levels of Thy-1 protein were measured in GO cultures (mean 2147 ± 371 positive pixels; range 1318–3035) compared with normal cultures (599 ± 112 ; range 378–889; $*p=0.007$).

$77.9 \pm 4.09\%$; range 66.5–84.8%) compared with the normal fibroblasts ($66.8 \pm 1.6\%$; range 63.3–71.0% positive; $p=0.046$; Fig. 5).

Discussion

The hyperthyroidism of Graves' disease results from the liganding of thyrotropin receptor (TSHR) on thyrocytes by stimulatory autoantibodies. However, while the ocular manifestations of Graves' disease are thought to result from autoimmunity targeting orbital fibroblasts, the pathogenic mechanisms underlying this process are less clear. The characteristic clinical features of GO are proximally caused by inflammation and expanded orbital tissue volume resulting from increased adipogenesis and accumulation of hydrated GAGs within the fixed bony cavity of the orbit (1,11). The expanded tissues include both the adipose/connective tissues of the orbit and the extraocular muscles, the latter reflecting primarily the accumulation of GAG.

Thy-1 is a 25–37 kDa glycoposphatidylinositol (GPI)-anchored surface glycoprotein expressed on human fibroblasts, neurons, blood stem cells, endothelial cells, and murine T cells (4). This protein plays both immunological roles in T cell activation (5) and widespread nonimmunological roles in cell–cell and cell–matrix interactions, including nerve regeneration, apoptotic signaling, metastasis, inflammation, and fibrosis (4). Because GO is an autoimmune disease characterized by profound remodeling of the intercellular matrix within the orbit, and previous studies by Smith *et al.* have implicated differential expression of this protein in the clinical manifestations of GO (9), we undertook these studies to further define its role in development of the disease.

Smith *et al.* reported that only about 50% of cultured fibroblasts from the connective/adipose tissue depot of GO orbits stained positively for Thy-1, while extraocular muscle-derived fibroblasts and pretibial fibroblasts uniformly displayed this antigen (9). Thy-1+ extraocular muscle-derived fibroblasts were incapable of adipocyte differentiation, while approximately 50% of fibroblasts from the connective/adipose tissue depot (presumably the Thy-1 – subset) differentiated after 18 days in culture. They postulated that the relative numbers of Thy-1+ and Thy-1 – cells within the orbital connective/adipose tissue in patients with GO may explain why some have predominant eye muscle disease while others exhibit increased orbital adipose tissue volume as the major disease feature. Patients over 60 years are prone to severe eye muscle involvement and inflammation without much expansion of the connective/adipose tissues, while patients younger than 40 years tend to exhibit expanded connective/adipose tissues and little muscle involvement or inflammation. This clinical observation led Smith *et al.* to postulate that the adipogenic potential of orbital fibroblasts might diminish with age. This could be in part mediated by Thy-1 in that fibroblasts from younger individuals might exhibit intrinsically lower levels of Thy-1 expression (favoring adipogenesis) than cells derived from older individuals. However, while the GO patients in our study were somewhat younger than the normal individuals studied (Table 1), their cells and tissues in fact exhibited uniformly higher Thy-1 expression than those derived from the normal individuals. As there were no correlations between age and Thy-1 expression either overall or within the GO or normal groups, it is unlikely

FIG. 4. Representative photomicrograph (10× magnification) of normal (A) and GO (B) orbital fibroblasts plated in chamber slides, stained with a specific Thy-1 antibody ($2.0 \mu\text{g/mL}$), and visualized using a Zeiss Axioplan 2 compound microscope.

FIG. 5. Representative flow cytometric analysis of orbital fibroblasts from a GO patient compared with orbital cells from a normal individual, showing a rightward shift in the GO cultures indicating Thy-1 protein expression in a greater proportion of the GO orbital fibroblasts. Overall, GO cultures ($n = 4$) showed Thy-1 protein expression in a $77.9 \pm 4.09\%$ of cells (range 66.5–84.8%) compared with $66.8 \pm 1.6\%$ in normal cultures ($n = 4$; range 63.3–71.0% positive; $p = 0.046$).

that Thy-1 expression is a function of age or that the age difference between the GO and normal individuals accounts for the higher Thy-1 expression found in the GO tissues.

We found Thy-1 mRNA and protein to be higher in uncultured connective/adipose tissue specimens and in cultured orbital fibroblasts (including baseline and differentiated cultures) from patients with GO compared with the same preparations derived from normal individuals. We showed using flow cytometry and immunohistochemical staining that this enhanced expression in GO likely reflects both increased numbers of Thy-1-positive cells and higher levels of expression on positive cells. Adipocyte differentiation itself (stimulated using the PPAR- γ agonist rosiglitazone) had no effect on Thy-1 expression. These findings suggest that increased Thy-1 expression in GO is likely a consequence of the orbital disease process itself and that it is not a direct consequence of the increased adipogenesis known to be present within the GO orbit (10,11).

It is possible that orbital Thy-1 expression is stimulated in GO owing to the presence of particular cytokines shown to be highly expressed in the GO orbit (2,12). However, Smith *et al.* noted that treatment of fibroblasts with glucocorticoids and “a wide array of inflammatory cytokines” did not influence Thy-1 expression (9). While we did not study the effect of cytokines on Thy-1 expression, we found no association between previous glucocorticoid therapy and levels of Thy-1 expression in the GO cells. Another group found tumor necrosis factor- α to induce loss of Thy-1 surface expression in cultured pulmonary fibroblasts through shedding of the antigen (13), opening up the possibility that Thy-1 expression may in fact be impacted by the cytokine milieu. Alternately, TSHR or other circulating autoantibodies in patients with GO

that target orbital fibroblasts may be the stimulus for enhanced Thy-1 expression in the disease.

In conclusion, the significantly enhanced Thy-1 mRNA and protein expression in GO orbital fibroblasts appears to be a consequence of the disease process reflecting a greater proportion of the cells expressing the protein. Adipocyte differentiation, which is enhanced in the GO orbit, does not itself appear to stimulate Thy-1 expression. Increased expression of this protein in the GO orbit could represent an adaptive response to cell injury, in effect limiting disease progression within the orbital adipose/connective tissues. Thy-1+ cells that become Thy-1+ would no longer be capable of adipogenesis, HLA-DR expression (and thus antigen presentation), or production of high levels of inflammatory IL-8. Further study is warranted to better understand the distribution and regulation of Thy-1 expression within the orbit, the impact of disease activity on its expression, and whether enhancing expression of this protein in GO might represent a novel approach to treatment.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Rebecca S. Bahn, M.D.
200 First St. SW,
Rochester, MN 55905

E-mail: bahn.rebecca@mayo.edu