Increased Expression of TSH Receptor by Fibrocytes in Thyroid-Associated Ophthalmopathy Leads to Chemokine Production

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Context: The molecular basis for anatomically dispersed clinical manifestations in Graves' disease (GD) eludes our understanding. Bone marrow-derived, pluripotent fibrocytes represent a subset of peripheral blood mononuclear cells and infiltrate the orbital and thyroid tissues in GD. These cells may be involved in the pathogenesis of thyroid-associated ophthalmopathy (TAO).

Objective: The objective of the study was to quantify fibrocyte display of functional cell surface TSH receptor (TSHR), identify the profile of chemokines they express after TSHR activation, and determine whether circulating $TSHR^+$ peripheral blood fibrocytes are more frequent *in situ* in patients with TAO.

Design/Setting/Participants: Using a newly developed technique, fibrocytes were directly identified in peripheral blood from 31 patients with TAO and 19 healthy subjects receiving care at a multidisciplinary academic center.

Main Outcome Measures: The frequency *in situ* of fibrocytes (collagen 1^+, $CD45^+$, $CD34^+$, $CD14^+$, $CD86^+$ peripheral blood mononuclear cells) was assessed by multiparameter flow cytometry and correlated to clinical disease activity and smoking status. Levels of TSHR-displaying fibrocytes and their response to TSH and TSHR-activating antibody, M22, were measured by flow cytometry, Luminex, and real-time PCR.

Results: The levels of TSHR expression by fibrocytes are substantially higher than those found in orbital fibroblasts. Moreover, the frequency of $TSHR^+$ fibrocytes in patients with TAO was greater than that in healthy subjects *in situ.* Their abundance is not influenced by disease activity or smoking history. These cells produce high levels of several cytokines and chemokines including IL-8, regulated upon activation, normal T cell expressed and secreted, and monocyte chemoattractant protein-1 when treated with TSH or M22. TSH induces IL-8 production at the pretranslational level. This induced cytokine can be detected in intact fibrocytes *ex vivo*.

Conclusions: Frequency of circulating $TSHR^+$ fibrocytes is markedly increased in patients with TAO, and they express proinflammatory chemokines in response to TSH. Because they infiltrate both orbit and thyroid in GD, they may represent the link between systemic immunoreactivity and organ-specific autoimmunity. **(*J Clin Endocrinol Metab* 97: E740–E746, 2012)**

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Abbreviations: ABC, Antibody molecules bound per cell; CAS, clinical activity score; Col1, collagen-1; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GD, Graves' disease; IGF-1R, IGF-I receptor; MCP-1, monocyte chemoattractant protein-1; PBMC, peripheral blood mononuclear cell; RANTES, regulated upon activation, normal T cell expressed and secreted; SB, staining buffer; PE, phosphatidylethanolamine; TAO, thyroid-associated ophthalmopathy; TSHR, TSH receptor; TSI, thyroid-stimulating immunoglobulin.

Despite substantial progress toward understanding the cellular and molecular underpinnings of autoimmune diseases, the pathogenesis of Graves' disease (GD) and its orbital manifestation, thyroid-associated ophthalmopathy (TAO), remains poorly defined (1). Activating antibodies generated against the TSH receptor (TSHR), known as thyroid-stimulating immunoglobulins (TSI), mediate the thyroid-centric processes associated with GD, but their role in TAO has not been established. Compelling, albeit circumstantial evidence, suggests that orbital fibroblasts represent the dominant autoimmune target in TAO (2). They exhibit distinct phenotypes, including exaggerated responses to proinflammatory cytokines, which could contribute to anatomic site-selective disease manifestations (3–5).

Fibrocytes are $CD45^+CD34^+$ bone marrow-derived, pluripoieitic cells that can migrate to sites of tissue inflammation and remodeling (6). They constitute approximately 0.5% of circulating leukocytes in healthy individuals, but their abundance increases in several diseases (7). These cells share phenotypic attributes with both fibroblasts [collagen-1 (Col1) and α-smooth muscle actin] and hematopoietic cells (CD34 and CD45). By virtue of their ability to promote antigen-specific immune responses (8) and to participate in tissue remodeling, fibrocytes appear to be outstanding candidate mediators of chronic inflammatory processes. Once they infiltrate tissues, they can differentiate into a variety of committed cells including adipocytes, capillary endothelium, and myofibroblasts, depending on the molecular cues they encounter (9).

The abundance of fibrocytes cultivated from peripheral blood mononuclear cells (PBMC) derived from patients with GD is greater than that from healthy subjects (10). Moreover, $CD34^+$ fibroblasts constitute a substantial fraction of orbital fibroblasts from patients with TAO but are absent in those from healthy donors (10). $CD34^+$ fibroblasts are present *in situ* in the orbit (10) and thyroid (11). We hypothesize that fibrocyte recruitment to the orbit represents a previously unrecognized bridge between tissues manifesting GD.

We have developed a novel method for directly identifying and quantifying $TSHR^+$ fibrocytes in peripheral blood. This technique has allowed us to determine that $TSHR^+$ fibrocytes are substantially more abundant in the circulation of patients with TAO than in healthy individuals. We also demonstrate that fibrocytes express high levels of TSHR and generate several inflammatory chemokines, including IL-8, regulated upon activation, normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein-1 (MCP-1) in response to TSH and to the monoclonal TSHR-activating antibody, M22. Our current findings connect the TSH/TSHR molecular bridge with the recruitment of immune competent cells to tissues in GD.

Materials and Methods

Patient samples

Individuals with TAO (n = 31) and healthy subjects (n = 19) were recruited from patients receiving care at the Kellogg Eye Center, University of Michigan. Informed consent was obtained in accordance with policies of the Institutional Research Board of the University of Michigan Health System. Immunosuppressed individuals and those with other autoimmune diseases, asthma, chronic inflammation, recent trauma, HIV, or active infection were excluded. Historical information and laboratory values for these patients as well as clinical activity score (CAS) are presented (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). A majority of subjects were Caucasian, including 25 of those with TAO (81%) and 12 healthy controls (86%). Most with TAO were female (n = 22; 71%) as were controls (n = 10; 71%) and were in the inactive phase (CAS $\leq$ 3, n = 22, 71%). All participants were euthyroid at the time of study participation as assessed by clinical examination and serum free T_4.

Flow cytometry

Staining for flow cytometry was performed within 24 h of blood collection. Staining buffer (SB) was prepared in PBS (Invitrogen Life Technologies, Frederick, MD) containing 2% fetal bovine serum (FBS) (Invitrogen) with 0.1% sodium azide (Sigma Aldrich, St. Louis, MO). One hundred microliters whole blood were placed in 12- × 75-mm polypropylene tubes, and 2 ml Pharm Lyse solution (BD Biosciences, San Jose, CA) was added for 10 min at room temperature. Cells were centrifuged at 500 × *g* for 5 min, washed, and resuspended in 100 μl SB. The following antihuman fluorochrome-conjugated monoclonal antibodies were used: CD14-fluorescein isothiocyanate (FITC; BD Biosciences, catalog no. 555397), CD45-peridinin chlorophyll protein (BD Biosciences; catalog no. 347464), CD11b-phosphatidylethanolamine (PE; BD Biosciences; catalog no. 555388), CD34-PE (BD Biosciences; catalog no. 550761), CD86-FITC (BD Biosciences; catalog no. 555657), CD90-FITC (BD Biosciences; catalog no. 555595), IGF-I receptor (IGF-1R)-PE (BD Biosciences; catalog no. 555999), CXCR4-PE (R&D Systems, Minneapolis, MN; catalog no. FAB173P), TSHR-PE (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. 53542), isotype control-FITC (BD Biosciences; catalog no. 555748), and isotype control-PE (BD Biosciences; catalog no. 554680). After 20 min incubation in the dark at 4 C, cells were resuspended and washed two times in SB. To quantify Col1 expression, cells were permeabilized with CytoFix/CytoPerm (BD Biosciences; catalog no. 554722) for 20 min at 4 C, washed twice, and resuspended in 100 μl Perm/Wash buffer (BD Biosciences; catalog no. 554723). After incubation with biotinylated goat antihuman Col1 polyclonal Ab (Millipore, Billerica, MA; catalog no. AB758B), cells were washed twice, incubated with streptavidin-conjugated FITC (BD Biosciences; catalog no. 554060), and fixed with 1% paraformaldehyde. Analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). At least 5×10^4 events were collected. Mean fluorescent intensity was calculated as a

ratio of mean fluorescence sample to isotype fluorescence. Percent positive expression was defined as the fraction of cells with increased fluorescent intensity compared with isotype control. Fibrocyte quantification was determined as the percentage of monocytes coexpressing CD45, Col1, and TSHR or CD34, CD45, Col1, and TSHR.

Detection of intracellular IL-8 in PBMC was conducted after 20 h stimulation, which directly followed isolation over Histopaque-1077 (Sigma Aldrich; catalog no. 10771), following the manufacturer's protocol. PBMC were washed and counted using a hemocytometer. Twenty-four-well plates were inoculated with 5×10^6 cells/well in 1 ml DMEM with 1% FBS and were then treated with bovine TSH (5 mU/ml) (Calbiochem EMD Biosciences Inc., La Jolla, CA; catalog no. 609385) or IL-1β (10 ng/ml) (Invitrogen; catalog no. PHC0815) for 20 h. Brefeldin A was added to each well (10 μg/ml) (Sigma Aldrich; catalog no. B7651) for the final 12 h of incubation. Staining for flow cytometry was performed as outlined above, with the addition of anti-IL-8-PE monoclonal antibody (BD Biosciences; catalog no. 55720) after permeabilization.

TSHR display on cultivated fibrocytes was quantified according to the antibody molecules bound per cell (ABC) method using the QuantiBRITE PE quantitation kit (BD Biosciences; catalog no. 340495). Cells were stained and subjected to flow cytometry in parallel with ABC standard according to the manufacturer's instructions.

Fibrocyte cultivation

PBMC were subjected to culture conditions similar to those described by Bucala *et al.* (12). Briefly, they were isolated and used to inoculate 24-well plates (5×10^6 cells/well) in 1 ml DMEM with 10% FBS). After 7 d in culture, nonadherent cells were removed by gentle aspiration. Medium was replaced every 3 d. After 12–14 d, adherent cells (<5% of initial PBMC population) were washed and removed from the substratum by scraping. Culture purity was greater than 90% fibrocytes by fluorescence-activated cell sorter analysis of CD34 and Col1. Viability was greater than 90% by trypan blue exclusion.

Cytokine measurements: Luminex

Cytokine production in cultured fibrocytes was determined by subjecting culture medium of cells stimulated with TSH or M22 for 24 h to Luminex analysis with a cytokine human multiplex panel (Invitrogen; catalog no. LHC6003). M22 is a monoclonal TSHR-activating antibody (2 μg/ml) (Kronus, Star, ID; catalog no. 5600).

Quantitative analysis of IL-8 mRNA

Relative IL-8 mRNA levels in cultivated fibrocytes were measured by quantitative real-time PCR using SYBR Green technique (Bio-Rad, Hercules, CA). RNA was extracted using an RNeasy minikit (QIAGEN, Valencia, CA) and reverse transcribed with the QuantiTect reverse transcription kit (QIAGEN). PCR involved the primer sequences (forward) 5′-GGCAGCCTTCCTGATTTCTG-3′ and (reverse) 5′-GGGTGGAAAGGTTTGGAGTATG-3′ for IL-8 and (forward) 5′-AAAGGACCCCACGAAGTGTT-3′ and (reverse) 5-TCAAGGGCATATCCTACAACAA-3′ for hypoxanthine-guanine phosphoribosyl transferase. Reaction conditions were as follows: 95 C for 5 min and then 40 cycles at 95 C for 10 sec and 60 C for 30 sec and then 95 C for 10 sec, melt curve 65–95 C, and increment 0.5 C for 5 sec.

Statistics

Statistical analysis was performed using two-tailed *t* test, paired *t* test, or one-way ANOVA without or with Bonferroni's multiple comparison posttest (as needed). Data are reported as the mean ± SE.

Results

Fibrocytes express greater levels of TSHR than orbital fibroblasts from patients with TAO

TSHR might play a pathogenic role in TAO, either as an autoantigen or by mediating immune cellular activation within the orbit. We have suggested previously that this receptor displayed by fibrocytes could contribute to the loss of peripheral immune tolerance and provoke generation of activating anti-TSHR antibodies. Alternatively, TSHR display on fibrocytes might convey immune tolerance. Cultured human fibrocytes express higher levels of TSHR than do orbital fibroblasts (Fig. 1). Comparisons in Fig. 1 were made using flow cytometric estimation of antibody molecules bound per cell, as described in *Materials and Methods*.

Patients with TAO exhibit an increased fraction of TSHR$^+$ fibrocytes *in situ*

Fibrocytes were identified in peripheral blood using a multiparametric flow cytometry technique modified from that described by Moeller *et al.* (13). The sequential gating strategy identified these cells based on the coexpression of CD45 and Col1. Fibrocytes assessed directly in peripheral blood from patients with TAO were significantly more frequent than were those from healthy donors (data not shown). The ages, genders, and ethnic profiles of the healthy study participants were similar to those with TAO (Supplemental Table 1).

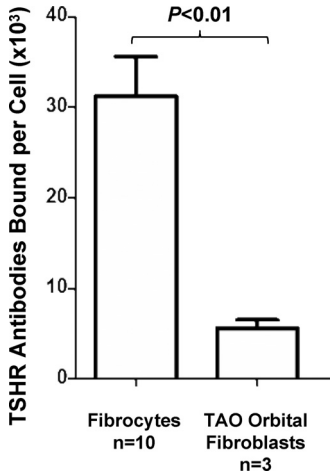

FIG. 1. Molecular density of TSHR is greater on fibrocytes than on TAO orbital fibroblasts ($P < 0.01$). Comparison utilized ABC determinations.

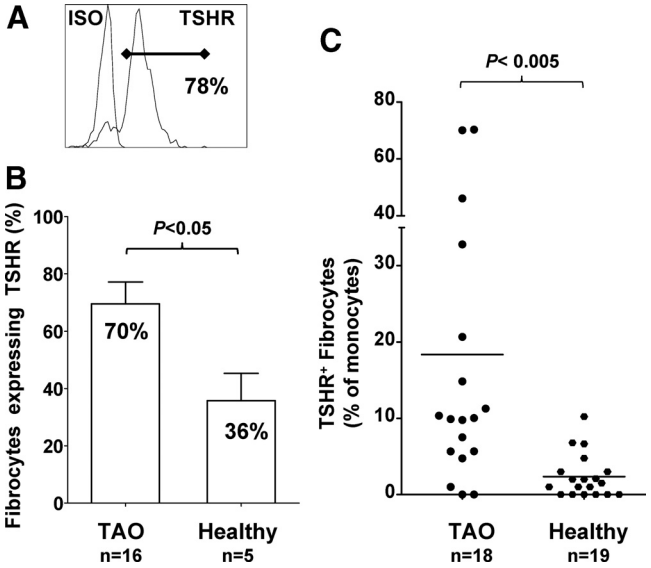

FIG. 2. TSHR$^+$ fibrocytes are more frequent in the circulation of TAO patients. A, A representative example of TSHR expression by fibrocytes from a TAO patient. B, Higher TSHR expression by fibrocytes from patients with TAO (70 ± 8%) than those from healthy controls (36 ± 10%, $P < 0.05$). The percentage of TSHR expression on CD45$^+$Col1$^+$ fibrocytes was assessed in donors with quantifiable fibrocyte levels (>5%) because of the limited number of fibrocytes for analysis in samples with fewer fibrocytes. C, Increased frequency of CD45$^+$Col1$^+$TSHR$^+$ fibrocytes in patients with TAO compared with healthy controls, as determined by percent of the monocyte population (TAO, 18 ± 5% *vs.* control, 2 ± 0.7%, $P < 0.005$). iso, Isotype control.

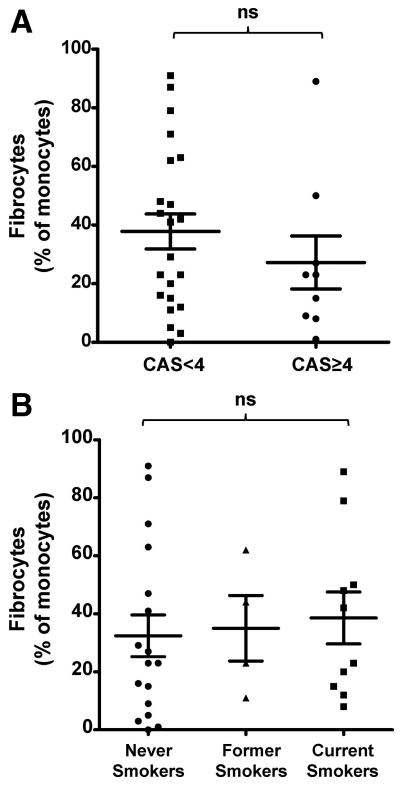

FIG. 3. Relationship between fibrocyte frequency and clinical disease activity or tobacco use. A, Correlation analysis between relative CD45$^+$Col1$^+$ fibrocyte abundance and CAS at single observational point. CAS of 4 or greater was defined as active and CAS less than 4 was inactive (27.2 ± 9.1 *vs.* 37.8 ± 6.0%, respectively; P = ns). B, Correlation between CD45$^+$Col1$^+$ fibrocyte abundance and cigarette smoking status [never-smokers 32.4 ± 7.2%, n = 17; former smokers (quit ≥ 6 months ago) 35.0 ± 11.3, n = 4; current smokers 38.6 ± 8.9%, n = 10; P = ns by one way ANOVA].

Additionally, a larger fraction of fibrocytes from patients with TAO were found to express TSHR than those from healthy donors (70 ± 8%, n = 16, *vs.* 36 ± 10%, n = 5, respectively; $P < 0.05$; Fig. 2, A and B). Circulating TSHR$^+$ fibrocytes are abundant in TAO patients but are vanishingly rare in most healthy donors. As shown in Fig. 2C, they comprise 18 ± 5% of the monocyte fraction in TAO patients but only 2 ± 0.7% in controls ($P < 0.005$). Moreover, 15 of 18 patients with TAO exhibit a detectable level of TSHR$^+$ fibrocytes (≥5%) compared with only 4 of 19 healthy controls. There was a trend toward females having a greater proportion of TSHR$^+$ fibrocytes, but this was not significant (females, n = 12, 24 ± 7% *vs.* males, n = 6, 7 ± 3%; $P = 0.10$).

Fibrocyte frequency *in situ* does not correlate with clinical activity score or smoking status

Disease activity and smoking history did not appear to affect fibrocyte frequency (Fig. 3). Their abundance was similar in nine patients with active TAO (CAS ≥ 4) and 22 with inactive (stable) disease (Fig. 3A) and was invariant with respect to cigarette smoking (Fig. 3B). In addition, no correlation between TSHR$^+$ fibrocyte frequency and disease activity or smoking was observed (data not shown).

TSH and M22 induce expression of multiple chemokines by fibrocytes, including IL-8

Given the high level of TSHR expression on fibrocytes, we determined the functional response of these cells to TSH and M22. TSH (5 mIU/ml) dramatically induced expression of several cytokines and powerful chemokines, including IL-8, RANTES, and MCP-1, in fibrocytes (Fig. 4). This effect was seen in cultured fibrocytes derived from donors with TAO and healthy controls. M22 also induced these chemokines after 24 h of treatment, albeit at lower levels. On the other hand, some cytokines such as IL-6 and IL-12 were induced to considerably higher levels by M22 than those achieved with TSH (Fig. 4). This lack of equivalence suggests that the impact of TSH and TSIs on inflammation may differ. We then determined that IL-8 induction by TSH is mediated at the pretranslational level. Fibrocytes were cultivated without or with TSH for 8 h. IL-8 mRNA as determined by real-time PCR was up-regulated in fibrocytes from both healthy donors and those with TAO (Fig. 5A). Baseline expression of IL-8 mRNA was nil and the response appears to peak at 12 h (Fig. 5B). We

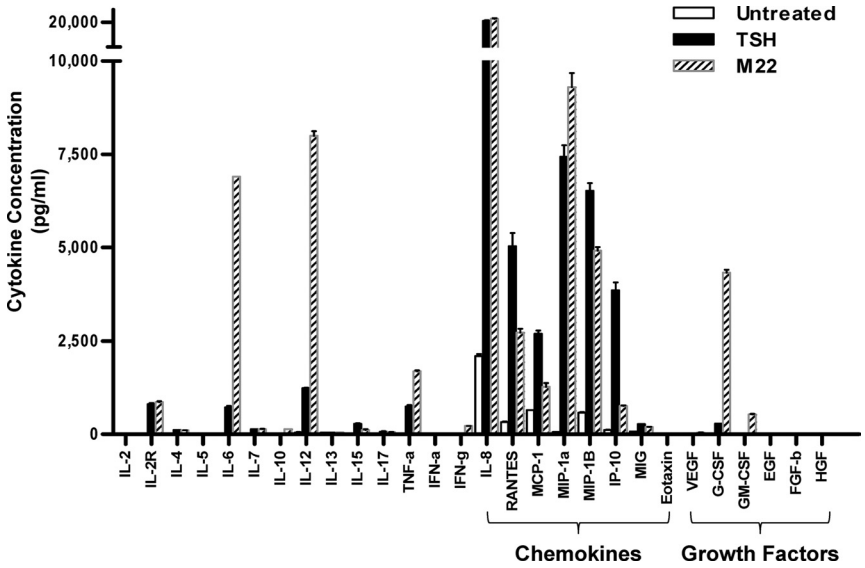

FIG. 4. TSH and M22 induce several cytokines and chemokines in TSHR$^+$ fibrocytes. Cells were cultured as described in *Materials and Methods* and treated with nothing, TSH (5 mU/ml), or M22 (2 μg/ml) for 24 h. Conditioned media were then subjected to Luminex assessment of the cytokines and growth factors indicated. Results are from one experiment, which are representative of three performed (IL-8: untreated *vs.* TSH and M22, $P < 0.001$; RANTES, MCP-1, MIP-1a, MIP-1b, IL-2R, IL-6, IL-12, and TNF-α: untreated *vs.* TSH and M22, $P < 0.01$). MIP, Macrophage inflammatory protein ; IL-2R, IL-2 receptor; IP, inositol phosphate; MIG, monokine induced by gamma interferon; VEGF, vascular endothelial growth factor; G-CSF, granulocyte colony-stimulating factor; EGF, epithelial growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor.

next determined whether treatment of fibrocytes with T_3 (1 nM) had an effect on steady-state IL-6 mRNA levels. The hormone increased the transcript abundance modestly after 24 h (control, 0.78 ± 0.16 fold *vs.* T_3, 1.00 ± 0.49 fold).

TSH induces intracellular IL-8 in TAO fibrocytes *ex vivo*

We next determined whether TSH could induce intracellular IL-8 accumulation in peripheral blood CD45$^+$Col1$^+$ fibrocytes from TAO patients. Intracellular IL-8 was measured after 20 h incubations without or with TSH or IL-1β (10 ng/ml). Vanishingly few untreated fibrocytes accumulated detectable intracellular IL-8 (Fig. 5, C and D). In contrast, TSH and IL-1β dramatically up-regulated IL-8. The chemokine could not be detected in identically treated lymphocytes from the same preparations, attesting to the specificity of the effects (data not shown). These findings strongly suggest that circulating fibrocytes synthesize chemokine in response to TSH and IL-1β.

Discussion

Explanation for anatomically separated manifestations of GD remains incomplete. Fibroblasts appear to be the principal immune target underlying orbital involvement (14), possibly as a consequence of autoantigen expression. Relatively high levels of both TSHR and IGF-1R have been detected on the surface of orbital fibroblasts from patients with TAO under specific culture conditions. Elevated IGF-1R levels can be detected under basal culture conditions relative to those displayed by orbital fibroblasts from healthy donors (15). In contrast, TSHR is essentially undetectable until the cells are subjected to conditions favoring differentiation into fat cells (16). Orbital fibroblasts from donors with TAO express other potentially important molecules, including CD34, CD40, and several cytokines (4, 14, 17). Fibrocytes share phenotypic and functional features with these fibroblasts (10); migrate to sites of injury; and can provide antigen-specific T cell stimulation, promote wound healing, and drive fibrosis (7, 12, 13, 18, 19). These fibrocytes are more frequent in the circulation of those with GD and appear to infiltrate orbital connective tissue in TAO and thyroid in GD (10, 11). They may provide a mechanistic link between involvement of the orbit, thyroid, and aberrant systemic immune responses associated with GD. We have reported previously that fibrocytes can be cultured from peripheral blood of patient with GD (10). We now show that fibrocytes express higher levels of TSHR than orbital fibroblasts. Not addressed in that earlier study was whether circulating fibrocytes are more abundant *in vivo* or whether their increased numbers in culture reflect a proliferative advantage *in vitro*. The current studies appear to answer that question. Moreover, the abundance of circulating TSHR$^+$ fibrocytes is significantly greater in patients compared with healthy individuals. We also show here that TSHR responds to TSH in circulating fibrocytes by up-regulating chemokines. We hypothesize that fibrocytes infiltrate orbital tissues in TAO and in so doing may participate in the inflammatory response and tissue remodeling through the activation of TSHR and the production of various chemokines.

IL-8 targets neutrophils and T lymphocytes as well as promotes cellular adhesion of immunocompetent cells to the endothelial surface (20). It has been implicated in multiple autoimmune diseases (21–23), and serum IL-8 levels can be elevated in hyperthyroid patients with GD

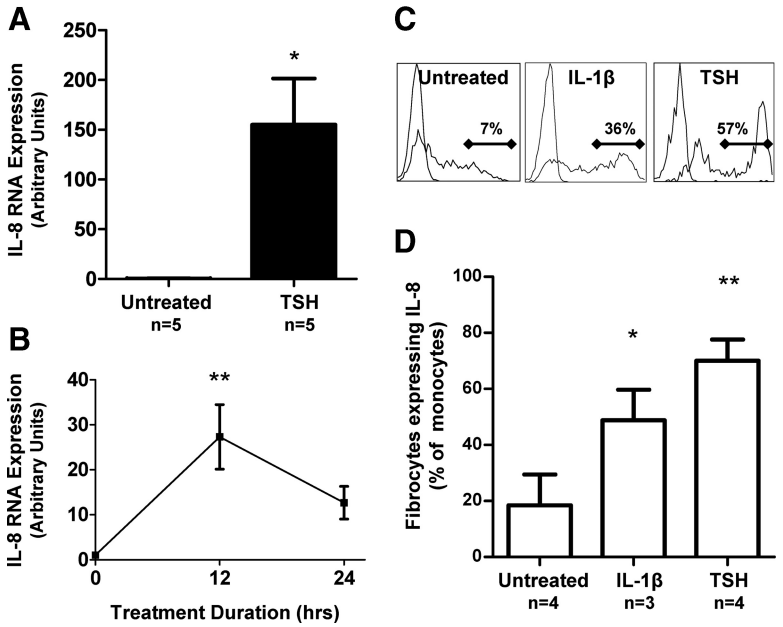

FIG. 5. A, IL-8 mRNA expression in cultivated fibrocytes as determined by real-time PCR before and after TSH treatment for 12 h. The n *under the abscissa* indicates the number of different fibrocyte strains analyzed. B, Time course of IL-8 mRNA up-regulation by TSH in cultivated fibrocytes. Cells were cultivated and treated as indicated in *Materials and Methods*. The RNA was harvested, reverse transcribed, and subjected to real-time PCR using the primers indicated. Data were normalized. Significance was determined by one-way ANOVA with Bonferroni's multiple comparison posttest showing a significant difference between 0 and 12 h only. C, Circulating $TSHR^+$ fibrocytes express intracellular IL-8 in response to TSH. $CD45^+Col1^+$ fibrocytes were incubated as PBMC for 20 h in the absence or presence of TSH (5 mU/ml) or IL-1β (10 ng/ml) and assessed for intracellular IL-8 by flow cytometry. Representative histograms from one experiment are shown. D, Aggregate data from the number of donors indicated below the abscissa. Significance of the differences was determined by paired *t* test (*, $P < 0.05$; **, $P < 0.01$).

(24, 25). The findings here suggest fibrocytes as a potential source of IL-8 production, in the specific context of their interactions with TSH and TSI. The magnitude of response to TSH was similar to that of IL-1β, an established inducer of IL-8 in monocytes (26). MCP-1 and RANTES, CC chemokines, also target specific immune cells (27, 28). Blocking either IL-8 or MCP-1 can attenuate B cell migration in an *in vitro* model of multiple sclerosis (29) and might reduce monocyte/lymphocyte infiltration in autoimmune anterior uveitis (30). Because fibrocytes appear to infiltrate the orbit in TAO, blocking their recruitment might represent a therapeutic strategy in GD.

Immunoglobulins specific to GD, of which TSI are a subset, up-regulate the production of both RANTES and IL-16 in fibroblasts from these patients. These effects are mediated through the IGF-1R (17). Immunoglobulins specific to GD and IGF-I also induce these chemoattractants in thyrocytes. IGF-1R and TSHR are autoantigens implicated in GD and TAO (31), and recent colocalization of these proteins strengthened the functional link (15). However, unlike IGF-1R, TSHR has not been shown previously to up-regulate chemokine production. Here we demonstrate a potentially important link between TSI activity and chemokine expression in fibrocytes. We also found that M22 induced higher levels of some cytokines, including IL-6 and IL-12, than did TSH. M22 was recently shown to provoke greater cAMP/protein kinase A/cAMP response element-binding protein activity and Akt and PKC phosphorylation than did TSH in thyroid cells (32). It is possible that these differences in signaling pathways are related to the divergent cytokine production elicited by the two agents. Thus, our findings potentially link TSI and TSH to the inflammatory activity of fibrocytes and may relate to the pathogenesis of TAO. The recent findings of Neumann *et al.* suggest a small molecule antagonist approach to inhibiting the activation of TSHR by TSI (33). That strategy could prove useful in treating TAO by attenuating signaling from TSHR displayed on fibrocytes.

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Disclosure Summary: All authors have nothing to declare.

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