CD34⁻ Orbital Fibroblasts From Patients With Thyroid-Associated Ophthalmopathy Modulate TNF-α Expression in CD34⁺ Fibroblasts and Fibrocytes

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PURPOSE. Orbital fibroblasts from patients with Graves' disease (GD-OF) express many different cytokines when treated with bovine thyrotropin (bTSH). The present study aimed to determine why TNF-α cannot be induced by bTSH in GD-OF.

METHODS. Fibrocytes and GD-OFs were cultivated from donors who were patients in a busy academic medical center practice. Real-time PCR, Western blot analysis, reporter gene assays, cell transfections, mRNA stability assays, ELISA, and flow cytometry were performed.

RESULTS. We found that bTSH induces TNF-α dramatically in fibrocytes but is undetectable in GD-OF. The induction in fibrocytes is a consequence of increased TNF-α gene promoter activity and is independent of ongoing protein synthesis. It could be attenuated by dexamethasone and the IGF-1 receptor inhibiting antibody, teprotumumab. When separated into pure CD34⁺ OF and CD34⁻ OF subsets, TNF-α mRNA became highly inducible by bTSH in CD34⁻ OF but remained undetectable in CD34⁺ OF. Conditioned medium from CD34⁻ OF inhibited induction of TNF-α in fibrocytes.

CONCLUSIONS. Our data indicate that CD34⁻ OF appear to release a soluble(s) factor that downregulates expression and induction by bTSH of TNF-α in fibrocytes and their derivative CD34⁺ OF. We proffer that CD34⁻ OF produce an unidentified modulatory factor that attenuates TNF-α expression in GD-OF and may do so in the TAO orbit.

Keywords: fibrocytes, orbital fibroblasts, thyroid-associated ophthalmopathy, TNF-α

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Medium conditioned by CD34⁺ OF can attenuate TNF-α induction by bTSH when used to cover fibrocytes in culture. Thus, CD34⁺ OF appear to release a modulating factor(s) that reduces TNF-α expression induction by CD34⁺ OF and fibrocytes and thus might govern inflammation occurring in the TAO orbit.

**METHODS**

**Materials**

Ficoll-Paque PLUS was purchased from GE Healthcare Bio-Science (cat. no. 17-1440-03, Uppsala, Sweden). Dulbecco’s modified Eagle’s medium (DMEM; Cat. no. 11 965-092), fetal bovine serum (FBS; cat. no. 16 000-044), and penicillin-streptomycin mixture (cat. no. 15 140-122) were from Life Technology (Grand Island, NY, USA). Bovine TSH (bTSH; cat. no. 609385) came from EMD Millipore (Billerica, MA, USA). Teprotumumab (Tepro, RV001) was a gift from River Vision Development (New York, NY, USA). APC mouse anti-human CD34 (Cat. no. 560940) was from BD Biosciences (Gibbstown, NJ, USA). 5, 6-dichloro-2'-deoxyuridine (D4902) was from Sigma-Aldrich, LLC (St. Louis, MO, USA). Bovine TSH (bTSH; cat. no. 609385) came from EMD Millipore (Billerica, MA, USA). 5, 6-dichloro-β-D-ribofuranosylbenzimidazole (DRB; Cat. no. 10 010 302) was from Calbiochem/EMD Biosciences (Gibbstown, NJ, USA). 5, 6-dichloro-β-D-ribosyl-4-anosylbenzimidazole (DRB; Cat. no. 10 010 302) was from Cayman Chemical (Ann Arbor, MI, USA) while the pGL3 vector backbone was from GeneCopoeia (Rockville, MD, USA).

**Fibrocytes and Fibroblasts Culture**

Fibrocyte isolation from PBMCs was described previously. These cells are identified by the display of a CD34⁺ CXCR4⁺, Col 1⁺ phenotype. Fibrocytes from healthy donors (n = 20) and those with TAO (n = 5) were used in these studies. All study participants provided both verbal and written informed consent prior to study participation. These activities were approved by the Institutional Review Board of the University of Michigan Health System. They adhered to the tenets of the Declaration of Helsinki. Some fibrocytes were cultivated from PBMCs provided anonymously by the American Red Cross. Peripheral blood was centrifuged over density gradient media (GE Healthcare Bio-Science). Ten million PBMCs were inoculated in each well of 6-well plates. These were covered with DMEM supplemented with 2 mM glutamine, sodium pyruvate (110 mg/mL), penicillin (100 units/mL), streptomycin (100 units/mL). 4.5% glucose, and 10% fetal bovine serum (FBS). Cultures were maintained in a 37°C, humidified, 5% CO₂ environment. Unattached cells were discarded by gentle aspiration after 7 days and monolayers were incubated for an additional 3 to 5 days prior to experimental manipulation. Fibrocyte purity of cultures was verified to be >90% by fluorescence-activated cell sorter (FACS) analysis using the CD34 surface marker.

GD-OF derived from deep orbital connective tissue waste during surgical decompressions for severe TAO. These patients (n = 3) had not been exposed to high-dose glucocorticoids or external beam radiotherapy, were euthyroid at the time of their surgeries, when they exhibited stable TAO. Cells were cultured as described. Monolayers were covered with DMEM containing the additives listed above which was changed every 3 to 4 days. Culture strains were utilized between the 2nd and 11th passages from initial plating, an interval when their phenotype remains constant.

**RNA Isolation and Real-Time PCR**

RNA was extracted with the Aurum total RNA mini kit (Cat. no.732-6820; Bio-Rad; Hercules, CA, USA). cDNA was generated using a reverse transcription kit (QuantiTec, Cat. no. 205 310; QIAGEN; Germantown, MD, USA). RT-PCR was conducted with iQ SYBR Green supermix (Cat. no. 170-8882; Bio-Rad) in a thermocycler (model CFX96; Bio-Rad). PCR reactions were performed in triplicate and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene control. The primers used were as follows: TNF-α, forward 5'-ATGAGCAGCTAAAGATCATGC-3' and reverse 5'-GTGGTGCATAAATGGGCTA-3'; GAPDH, forward 5'-TGGCCATCAATGACCCTTCA-3' and reverse 5'-GCCGCCACTT GATT-TTG-3'. Amplification conditions: initial 3-minute activation at 95°C followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 61.7°C for 1 minute, and extension at 94.5°C for 30 seconds.

**Western Blot**

Cell lysates were prepared in lysis buffer (Invitrogen) containing PMSF (1 mM), Protein concentrations were normalized with a Dc protein assay kit (Bio-Rad) and loaded on SDS-PAGE, running at 80 V for 30 minutes and then 120 V for 45 minutes. Separated proteins were transferred to PVDF (Millipore Corp.). Membranes were incubated with primary antibodies against phospho-p38 MAPK (Thr180/Tyr182; Cat. no. 9216, Cell Signaling, Boston, MA, USA) and β-actin (Cat. no. 3700) overnight at 4°C. Washed membranes were incubated with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Cat. no. 7076, CST, Boston, MA, USA) for 2 hours at room temperature. ECL Plus (Thermo Fisher Scientific, Rockville, MD, USA) was used for signal generation. Bands were visualized using Fluor ChemE (Cat. no. 92-14800-00, ProteinSimple, Santa Clara, CA, USA) and subjected to densitometry, performed with commercial software (Image Pro Plus Version 6.0; Media Cybernetics, Warrendale, PA, USA).

**TNF-α mRNA Stability Assay**

Confluent fibrocyte cultures were pretreated with bTSH (5 μIU/mL) in DMEM with 1% FBS for 1 hour. Control wells were shifted to untreated conditions while the bTSH treated wells were continued in its presence. All cultures received DRB (50 μM) at time “0” and RNA was harvested at the intervals (see Fig. 4B). RT-PCR was performed with TNF-α mRNA as the target. Data were graphed as a best fit line.

**Cloning of the Human TNF-α Promoter, Transient Transfections and Luciferase Reporter Assays**

A 2456-bp fragment of the TNF-α gene promoter was amplified by PCR and subcloned into pG3 basic. The following primers were used: forward 5’-TAAACCCCTTTTTCTCTGAAATGCTG-3’ and reverse 5’-GGTCTCAGCCTTTTCTCTCCT-3’. Constructs were transfected into cultured fibrocytes using Human CD34 Cell Nucleofector Kit (Cat. no. VPA-1003, Lonza, Basel, Switzerland). Promoter-less pGL3 vector was used as a control. Following centrifugation at 2000g for 5 minutes, cell pellets were resuspended in buffer provided by the manufacturer and mixed with 2 μg DNA. After 24 hours, monolayers were treated with nothing or bTSH (5 μIU/mL) for 1 hour and luciferase activities were assessed in 2 μL cell extract mixed with luciferase assay reagent (Cat. no. E1980; Promega Corp., Madison, WI, USA). All transfection assays were performed in triplicate. Lumicount data were normalized to their Renilla transfection controls.
Cell Sorting

Pure CD34⁺ GD-OF and CD34⁻ GD-OF subsets were generated from parental (mixed) strains. They were stained with anti-CD34 antibodies for 30 minutes at 4°C. Washed cells were sorted under sterile conditions with a BD FACS Aria III instrument (BD Biosciences).

Flow Cytometry

Intracellular TNF-α in GD-OF was detected following treatment with Golgistop (Cat no. 554724; BD Biosciences, Franklin Lakes, NJ, USA) for 6 hours after bTSH stimulation. Cultures were then incubated for 12 hours, monolayers disrupted and centrifuged at 500g for 5 minutes, washed and resuspended in PBS containing 2% FBS with 0.1% sodium azide staining buffer. Cells were permeabilized and fixed with CytoFix/CytoPerm (Cat. no. 55472; BD Biosciences) for 20 minutes, and resuspended in 0.1 mL Perm/Wash buffer (Cat. no. 554723; BD Biosciences). Cells were incubated with anti-human TNF-α (Cat. no. 540511; BD Biosciences) Ab or isotype control Ab (Cat. no. 555748; BD Biosciences) for 1 hour and fixed with 1% paraformaldehyde. Flow cytometry of at least 10⁶ events was conducted. Mean fluorescent intensity was calculated as a ratio of sample geometric mean fluorescence and isotype geometric mean fluorescence.

ELISA

Extracellular TNF-α levels were assessed in triplicate by ELISA analysis using a human TNF-α ELISA kit (Cat. no. DATOOC; R&D Systems, Minneapolis, MN, USA).

Statistics

Statistical analysis was performed using 1-way ANOVA with a confidence level greater than 95%. Data are expressed as the mean ± SD. All experiments were conducted in triplicate and performed at least three times.

RESULTS

bTSH Induction of TNF-α in Fibrocytes and GD-OF

Fibrocytes and GD-OF were treated with or without bTSH (5 mIU/mL) for 12 hours and subjected to TNF-α ELISA (Fig. 1A). TNF-α protein increased significantly following exposure to
bTSH in fibrocytes ($P < 0.001$ versus control). In contrast, no TNF-α protein was detected in GD-OF without or following treatment with bTSH by ELISA or flow cytometry (data not shown).

Both cell types were treated with or without bTSH ($5 \text{ mIU/mL}$) for graded intervals and TNF-α mRNA levels determined by RT-PCR. The transcript in fibrocytes was detectable under basal conditions and dramatically induced with bTSH (Fig. 1B). The effects were near-maximal at 1 hour ($>100,000$-fold over basal) after which the induction decayed rapidly, returning to basal levels within 8 hours. Basal mRNA levels were considerably lower in GD-OF compared to fibrocytes ($P < 0.001$). The induction of TNF-α in these cells was substantially less robust (218-fold) than that in fibrocytes. The time-courses of effects of bTSH in the two cell-types were similar (both $P < 0.001$ compared to controls after 1 hour). The effects in fibrocytes were dose-dependent, detectable at 0.5 mIU/mL and maximal at 5.0 mIU/mL (Supplemental Fig. S1). Three additional strains of each cell-type were examined. In each, basal TNF-α mRNA expression and that following treatment with bTSH is uniformly greater in fibrocytes compared to GD-OF (Fig. 1C, $P < 0.001$).

Induction of TNF-α mRNA by M22 ($2 \mu g/mL$) was less robust than that seen with bTSH in fibrocytes (Fig. 1D).

**DEX and Teprotumumab Inhibit TSH-Induced TNF-α Expression in Fibrocytes**

Corticosteroids represent the current mainstay of anti-inflammatory therapies in active TAO. To determine whether they can block the induction of TNF-α mRNA by bTSH in fibrocytes, cultures were treated with nothing or bTSH ($5 \text{ mIU/mL}$) alone or with DEX ($10 \text{ nM}$). The steroid attenuated the upregulation of TNF-α mRNA ($50.4\%$ inhibition, $P < 0.05$ versus bTSH alone; Fig. 2A). Recent studies from our laboratory and those conducted elsewhere strongly suggest that TSHR-initiated actions are dependent on the activity of IGF-IR. Teprotumumab ($50 \mu g/mL$), an inhibitory anti-IGF-IR antibody, could also attenuate the induction of TNF-α mRNA by bTSH by $48.5\%$ ($P < 0.05$ versus bTSH alone; Fig. 2B). Thus, the actions of bTSH on TNF-α expression appear to require IGF-IR activity. However, IGF-I as a single agent failed
Thus, the p38 MAPK pathway mediates these actions of bTSH. bTSH provoked p38/MAP kinase phosphorylation (Fig. 3B). nt) fused to a luciferase reporter into fibrocytes. Cells were substantially attenuated by the inhibitor (Fig. 3A, l treated with nothing or SB203580 (20 M) for 1 hour. (B) Cultures were pretreated as in (A) and then treated with or without bTSH for 45 minutes. Monolayers were solubilized and subjected to Western blot analysis for phosphorylated p38 and b-actin. Bands were quantified by densitometric analysis ([B], bottom). Data are expressed as mean ± SD of triplicates. Representative of three independent experiments using cells from a different donor.

**Figure 3.** bTSH induced TNF-α mRNA expression is mediated through p38/MAPK. (A) Fibrocytes were pretreated with nothing or SB203580 (20 μM) for 1 hour and then treated with or without bTSH (5 mIU/mL) for 1 hour. (B) Cultures were pretreated as in (A) and then treated with or without bTSH for 45 minutes. Monolayers were solubilized and subjected to Western blot analysis for phosphorylated p38 and b-actin. Bands were quantified by densitometric analysis ([B], bottom). Data are expressed as mean ± SD of triplicates. Representative of three independent experiments using cells from a different donor.

to enhance bTSH-dependent TNF-α mRNA expression (Fig. 2C).

**bTSH-Induction of TNF-α Is Mediated Through p38/MAPK Signaling**

The signaling pathways downstream from TSHR utilized in the induction of TNF-α were explored next. Fibrocytes were pretreated with nothing or SB203580 (20 μM, a specific inhibitor of the p38 pathway). bTSH induction of TNF-α mRNA was substantially attenuated by the inhibitor (Fig. 3A, P < 0.05). bTSH provoked p38/MAP kinase phosphorylation (Fig. 3B). Thus, the p38 MAPK pathway mediates these actions of bTSH.

**bTSH Increases TNF-α Gene Promoter Activity in Fibrocytes**

We next began to determine the molecular mechanisms involved in the upregulation by bTSH on the expression of the cytokine. TNF-α gene promoter activity was assessed by transiently transfecting a 2456-bp fragment (~1440 nt to +1016 nt) fused to a luciferase reporter into fibrocytes. Cells were treated with nothing or bTSH for 1 hour. bTSH enhances the promoter/reporter construct activity by 1.60-fold (P < 0.001 versus control, Fig. 4A). TNF-α mRNA in fibrocytes decays rapidly under control conditions (Fig. 4B). Unlike its action on several cytokine-encoding mRNAs in fibrocytes, bTSH failed to retard TNF-α mRNA degradation significantly. Thus, it would appear that TSH exerts its principal actions on TNF-α expression through enhanced gene transcription.

A number of inflammatory cytokines are very rapidly induced as a consequence of their primary induction. To determine whether TNF-α might behave as an immediate early gene in fibrocytes, monolayers were incubated with or without cycloheximide (10 μg/mL) alone or with bTSH for 1 hour. TNF-α mRNA was modestly induced by cycloheximide as a single agent (2.6-fold; Fig. 4C). When added in combination with bTSH, the inhibitor of protein synthesis failed to attenuate the induction of TNF-α. Thus, in fibrocytes, TNF-α mRNA upregulation by bTSH represents a primary gene induction that does not require ongoing intermediate protein synthesis.

**TNF-α Induction by bTSH in Pure CD34+ OF and CD34− OF Subsets**

To directly determine whether CD34− OF subsets express a factor(s) that attenuates the induction of TNF-α in GD-OF mixed (parental) cultures comprising both CD34+ OF (typically approximately 60%) and CD34− OF (40%), were subjected to sorting into pure subsets. While TNF-α expression and induction by bTSH in parental GD-OF and CD34− OF cultures were barely detectable, both were considerably higher in pure CD34+ OF than in either the parental strain (P < 0.001) or pure CD34− OF (P < 0.001; Fig. 5A). Thus, it would appear that the presence of CD34+ OF downregulates TNF-α induction in CD34− OF. To determine whether the CD34− OF-derived inhibitory factor(s) was soluble and released from the cell layer, medium conditioned by those cells was added to fibrocytes cultured from PBMCs and incubate for 3 days. These fibrocytes were then left untreated or received bTSH for 1 hour. As the results in Fig. 5B indicate, TNF-α mRNA expression/induction was substantially attenuated by CD34− OF conditioned medium when compared to medium from CD34+ OF. Conditioned medium generated from CD34− OF used to cover autologous CD34+ OF depressed basal expression of TNF-α mRNA when compared to medium conditioned with CD34− OF (CD34+ OF, 356.25 ± 14.21 versus CD34− OF, 166.95 ± 9.73, P < 0.001). In addition, the induction of the transcript by bTSH was also reduced (2706.21 versus 899.04 ± 22.31, respectively; P < 0.01). Thus, CD34− OF release a soluble factor(s) that downregulates the generation of TNF-α in fibrocytes.

**Discussion**

The current studies reveal that CD34− OF functionally emerge from the inhibitory influence of their CD34+ OF counterparts following their separation into pure subsets. This is evidenced by the increased magnitude of TNF-α induction by bTSH (Fig. 5A). This change in cytokine expression could result from the release from a factor generated by CD34+ OF that interferes with/represses specific gene expression or it might reflect a more generalized transition of these fibroblasts to the fibrocyte phenotype. Further studies will be necessary to determine the underlying mechanisms involved.

Targeting TNF-α in several autoimmune processes has proven effective as a therapeutic strategy.12–14 This cytokine can be detected, albeit at low levels, in the TAO orbital connective tissue.16 GD-OF barely express the cytokine in
The current results indicate that the amplitude of TNF-α induction by bTSH is considerably greater in fibrocytes cultured from PBMCs than that in GD-OF despite the abundant induction by bTSH. TNF-α mRNA was quantified by RT-PCR. (C) Confluent fibrocyte cultures were pretreated with nothing or cycloheximide (CHX, 10 μg/ml) for 1 hour, then treated alone or in combination with bTSH for 1 hour. RNA was harvested and subjected to RTPCR for TNF-α mRNA. Data are expressed as the mean ± SD of triplicate, independent determinations. These studies were performed three times, each using cells from a different donor.

vitro, suggesting a potentially complex relationship may exist between discrete GD-OF cell populations. This concept has been substantiated by finding fibroblast heterogeneity in orbital tissues. The findings thus far suggest that multiple intercellular regulatory factors are at play. It is the balance between effector and regulatory pathways that maintains the immunologic steady-state achieved in the healthy orbit.

The current results indicate that the amplitude of TNF-α induction by bTSH is considerably greater in fibrocytes cultured from PBMCs than that in GD-OF despite the abundant presence of CD34+ OF. The magnitude of the induction varied substantially among cells derived from different donors, consistent with our previous experience with other cytokines. These cells exhibit a constellation of phenotypic attributes that unambiguously identify them as having been derived from fibrocytes. CD34+ OF apparently release a soluble factor(s) that downregulates the inflammatory phenotype of fibrocytes and their derivative CD34+ OF. This becomes apparent when GD-OF undergo sorting into pure populations of cells. Under that circumstance, TNF-α becomes inducible in pure CD34+ OF but remains essentially undetectable in CD34- OF. It should be noted that the magnitude of response in the pure CD34+ OF remains considerably lower than that in fibrocytes. We attribute this difference to the relatively short interval between cell sorting and the treatment with bTSH which may be inadequate to completely reverse the modulatory effects of the CD34+ OF. When medium conditioned by CD34+ OF was added to fibrocytes, induction of TNF-α was attenuated. This finding is consistent with CD34+ OF imposing a negative influence on CD34+ OF regarding thyroglobulin and TSHR expression. 23

bTSH induces TNF-α expression in fibrocytes as a consequence of increased TNF-α gene promoter activity and is independent of ongoing protein synthesis. Further it is mediated through activation of the p38/MAPK pathway. Thus, this signaling pathway might be utilized as another therapeutic target for attenuating those pathologic events occurring in TAO that involve fibrocytes. That interruption might involve small molecules targeting relevant kinases in the involved pathways, interfering small RNAs, or monoclonal Abs targeting the cytokine or its receptor. Pilot studies have suggested the potential for interrupting the TNF-α pathway as an effective therapy for TAO. Conduct of controlled, adequately powered, prospective studies will be necessary before safety and efficacy of this approach can be determined.

Crosstalk between IGF-1R and TSHR has been demonstrated in vitro in GD-OF. TSHR can form a physical and functional complex with IGF-1R. Teprotumumab, an IGF-IR inhibitory monoclonal antibody has recently demonstrated substantial therapeutic benefit in active, moderate to severe TAO. As demonstrated in the current studies, this agent attenuates the induction of TNF-α by TSH, suggesting a potential mechanism underlying its clinical effectiveness. Should that prove the case, our findings may have relevance to other autoimmune diseases where TNF-α appears to play an important role. On the other hand, IGF-I failed to enhance the actions of bTSH in these cells, a finding that differs from observations made in another laboratory.

Our current study identifies a putative regulatory loop through which TNF-α expression is controlled by a soluble factor released by CD34+ OF. It is possible that the balance between CD34+ OF and CD34- OF in the orbit represents an important determinant of the severity, duration, and clinical course of TAO. Thus, the relative abundance of the two cell subsets might change as a function of the disease phase and when disease activity ceases and the chronic, stable phase begins. Further, the ratio could change as a consequence of successful therapy. We proffer that in GD, the orbit in which CD34+ OF dominates may never develop clinically important
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TAO. We await those rare occasions when patients with GD but without TAO require orbital surgery to test this hypothesis.

Identification of the inhibitory factor(s) released by CD34+OF may allow development of novel therapeutic approaches for TAO. That molecule might be introduced locally into the orbit. Alternatively, a means for specifically enhancing its synthesis by CD34+OF might be developed. In any event, the current findings may help explain why TNF-2 expression in GD-OF is substantially lower than that found in fibrocytes. These findings might be relevant to other processes where fibrocytes infiltrate tissues and cohabitate a disease-manifesting niche.

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