Inhibition of TGFβ1 by Anti-TGFβ1 Antibody or Lisinopril Reduces Thyroid Fibrosis in Granulomatous Experimental Autoimmune Thyroiditis

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Inhibition of TGFβ1 by Anti-TGFβ1 Antibody or Lisinopril Reduces Thyroid Fibrosis in Granulomatous Experimental Autoimmune Thyroiditis

Kemin Chen,* Yongzhong Wei,* Gordon C. Sharp,* and Helen Braley-Mullen2‡‡

In this study, a murine model of granulomatous experimental autoimmune thyroiditis (G-EAT) was used to determine the role of TGFβ1 in fibrosis initiated by an autoimmune inflammatory response. The fibrotic process was evaluated by staining thyroid tissue for collagen, α-smooth muscle actin, TGFβ1, and angiotensin-converting enzyme (ACE), and measuring serum thyroxine in mice given anti-TGFβ1 or the ACE inhibitor lisinopril. The role of particular inflammatory cells in fibrosis was tested by depletion experiments, and the cytokine profile in thyroids was examined by RT-PCR. Neutralization of TGFβ1 by anti-TGFβ1 or lisinopril resulted in less collagen deposition and less accumulation of myofibroblasts, and levels of active TGFβ1 and ACE were reduced in thyroids of treated mice compared with those of untreated controls. Other profibrotic molecules, such as platelet-derived growth factor, monocyte chemotactic protein-1, and IL-13, were also reduced in thyroids of anti-TGFβ1- and lisinopril-treated mice compared with those of controls. Confocal microscopy showed that CD4+ T cells and macrophages expressed TGFβ1. Fibrosis was reduced by injection of anti-CD4 mAb on day 12, when G-EAT was very severe (4–5+). Together, these results suggest a critical role for TGFβ1 in fibrosis initiated by autoimmune-induced inflammation. Autoreactive CD4+ T cells may contribute to thyroid fibrosis through production of TGFβ1. This G-EAT model provides a new model to study how fibrosis associated with autoimmune damage can be inhibited. The Journal of Immunology, 2002, 169: 6530–6538.

M uose thyroglobulin (MtG)3-sensitized spleen cells activated in vitro with MtG and IL-12 induce a very severe, destructive granulomatous form of experimental autoimmune thyroiditis (G-EAT) in recipient mice with proliferation of thyocytes, infiltration of the thyroid by lymphocytes and histiocytes, extensive neutrophils, fibrin deposition, and necrosis (1). The most extensively destroyed thyroids ultimately become fibrotic and atrophic (1–3).

TGFβs are a family of a group of polypeptides, which mediate a broad spectrum of biological activities important in embryogenesis, tissue repair, cell growth, and immune regulation (4–7). TGFβ is implicated in various models of fibrosis (2, 4), and it also regulates autoimmune diseases (5–7). Fibrosis can be a severe consequence of some autoimmune diseases (2, 8–14). Pulmonary fibrosis can be associated with rheumatoid arthritis in some patients (8–10), and fibrosis can occur in patients with scleroderma, Wegener’s granulomatosis, giant cell arteritis, and Riedel’s thyroiditis (11–13). However, the mechanisms involved in development of fibrosis, when the initiating event is an autoimmune inflammatory response, remain poorly understood.

The regulation of inflammatory cytokines is central to the pathogenesis of autoimmune diseases as well as fibrosis (4, 6–8). Immunocompetent cells, especially lymphocytes and macrophages, are probably the main source of these cytokines, and production of various cytokines and chemokines by CD4+ T cells plays a crucial role in regulation of autoimmune diseases. However, the role of autoreactive CD4+ T cells and other inflammatory cells in the development of fibrosis is not well understood. CD4+ T cells may contribute to fibrosis by producing or regulating profibrotic or antifibrotic molecules. Some cytokines and chemokines, such as IL-4, IL-13, and monocyte chemotactic protein (MCP)-1, are important profibrotic molecules (15–19) and may mediate fibrosis by promoting production of TGFβ1 (16). In G-EAT, various inflammatory cells infiltrate the thyroid, and CD4+ T cells are the primary effector cells, producing both Th1 and Th2 cytokines (16, 20, 21). The role of particular subsets of thyroid-infiltrating inflammatory cells in the development of fibrosis is unknown.

Our previous results showed that TGFβ1 protein expression is colocalized with myofibroblasts (myofib) and macrophages in areas of collagen deposition in G-EAT thyroids (2), suggesting TGFβ1 may contribute to thyroid fibrosis in G-EAT. Targeting TGFβ1 has been an effective strategy for treatment of fibrosis in other models (8). However, because TGFβ1 negatively regulates some autoimmune diseases (6, 7, 22), it is important to determine the effect of blocking TGFβ1 on fibrosis that occurs as a result of autoimmune inflammation. Angiotensin (ANG)-converting enzyme (ACE) inhibitors (ACEI), designed primarily to limit vasoconstriction, can have potent antifibrotic effects due to their ability to block the link between ANGII and TGFβ1 (14, 23–25). Blocking ANGII by ACEI inhibited fibrosis of the kidney and heart (14, 24–26), but it is not known whether this will also reduce fibrosis resulting from an autoimmune inflammatory response. The results of this study...
show that inhibition of TGFβ1 using anti-TGFβ1 or the ACEI lisinopril reduces thyroid fibrosis but has no effect on G-EAT severity scores at the peak of disease, demonstrating a direct role for TGFβ1 in thyroid fibrosis. An important role for CD4+ T cells in thyroid fibrosis was demonstrated by showing that anti-CD4 mAb, given under conditions that did not affect G-EAT severity scores at the peak of disease, inhibited fibrosis. A role for other inflammatory cells was inferred by assessing the localization of specific cells in relation to areas of collagen deposition in fibrotic thyroids. Moreover, comparison of cytokine profiles in thyroids of control and anti-TGFβ-treated mice suggests that interactions between TGFβ1 and other cytokines may be important in modifying the effects of TGFβ1 on fibrosis.

Materials and Methods

Mice

DBA/1 mice were bred in our animal facilities at the University of Missouri. Both male and female mice (6–10 wk old) were used for these experiments.

Induction of G-EAT

Donor DBA/1 mice were immunized twice with 150 μg MTg and 15 μg LPS (E. coli 0111:B4; Sigma-Aldrich, St. Louis, MO) i.v. at 10-day intervals (1, 20). Seven days after the second injection of MTg and LPS, donor spleen cells were cultured at 10^5 cells/ml in RPMI 1640 containing 25 mM HepES buffer (Cell and Immunobiology Core Facility, University of Missouri), 5% FCS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, MEM vitamin solution, nonessential amino acids, 1 mM sodium pyruvate, and 5 x 10^{-3} M 2-ME. Cells were cultured with 25 μg/ml MTg and 5 mg/ml IL-12 (PeproTech, Rocky Hill, NJ) as previously described (1). After 72 h, cells were harvested and washed, and 3.5 x 10^6 cells were injected i.v. into irradiated (500-rad) recipient mice. Recipient thyroids were evaluated for experimental autoimmune thyroiditis severity 17–21 days later, the time of maximal severity of G-EAT in this adoptive transfer model (1, 2, 20), or 35–60 days following cell transfer when fibrosis is maximal (2).

Anti-TGFβ1 and lisinopril treatment

Mice were given 250 μg anti-TGFβ1 mAb 2D11.16.8 (mouse IgG1) (ATCC HB 9849; American Type Culture Collection, Manassas, VA) every 4 days beginning 4 days after cell transfer and continuing throughout the experiment. Preliminary results indicated that thyroid lesions in recipient mice given normal mouse IgG according to the same injection schedule were indistinguishable from those of control recipient mice not given IgG. Therefore, in the experiments shown here, the control recipient mice received in vitro activated donor splenocytes but were not given mouse Ig. The ACEI lisinopril (Sigma-Aldrich) was used at a concentration of 60 mg/L administered in the drinking water beginning on the day of cell transfer and continuing throughout the experiment. The water was changed two to three times per wk. Preliminary experiments indicated that each mouse consumed ~5 ml water/day; therefore, the amount of lisinopril ingested per day was ~0.3 mg/mouse.

Evaluation of G-EAT histopathology

Thyroids were removed at various times after cell transfer, and one lobe of each thyroid was fixed in formalin. For histologic analysis, tissues were embedded in paraffin, sectioned (7 μm), and stained with H&E. Thyroids were scored quantitatively for G-EAT severity, using a scale of 0–5+ according to previously established criteria (1, 20). Measurements of thyroiditis were as follows: 1+, an infiltrate of at least 125 cells in one or several foci; 2+, 10–20 foci of cellular infiltration involving up to 25% of the gland; 3+, infiltration of 25–50% of the gland; 4+, >50% of the gland is destroyed; and 5+, virtually complete destruction of the gland, with few or no remaining follicles. Thyroid lesions were also evaluated qualitatively.

DBA/1 mice typically develop very severe (4–5+) G-EAT (2). Fourteen to 21 days after cell transfer, thyroids had extensive granulomatous changes with follicular cell proliferation, multinucleated giant cells, large numbers of histiocytes, and numerous lymphocytes and neutrophils. There were also microabscess formation, necrosis, and fibrin deposition. The inflammation in thyroids with 4–5+ severity scores characterized extended beyond the thyroid to involve adjacent connective tissue and muscle. By days 35–60, thyroids of most untreated mice were very small and atrophic, with fewer inflammatory cells, extensive collagen deposition, and virtually no remaining follicles. For qualitative evaluation of collagen deposition and fibrosis, thyroid sections were stained using Masson’s Trichrome (Histo-scientific Research Labs, Manassas, VA). All slides were evaluated separately by at least two of the investigators, one of whom had no knowledge of the experimental treatments. Differences in interpretation were very rare.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated through sequential ethanol, and rinsed in PBS. The immunohistochemical methods used immunoperoxidase staining as previously described (2), and the intensity of immunostaining was graded semiquantitatively. Staining of TGFβ1 and α-smooth muscle actin (α-SMA) was performed as previously described (2). ACE staining was performed on paraffin sections, and microwave irradiation was used for Ag retrieval as previously described (2). Sections were incubated overnight with anti-ACE mAb (ATCC HB 8191; American Type Culture Collection) followed by incubation with biotin-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min; color was visualized by VIP (very intense purple) chromogen (Vector Laboratories, Burlingame, CA). Infiltration of neutrophils and macrophages in G-EAT thyroids was detected on crossections using rat mAb against neutrophils (RB6-8C5; provided by Dr. R. Coffman, DNAX, Palo Alto, CA) or macrophages (F4/80; both American Type Culture Collection). Sections were blocked in 1% BSA for 30 min, washed with PBS, and endogeneous peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS for 30 min. This and all subsequent washes were in PBS (0.1 M, pH 7.6). TGFβ1, neutrophils, and macrophages were visualized by diaminobenzidine, and α-SMA by VIP. Sections were counterstained with hematoxylin. Negative controls were performed using non-immune mouse, rat, or chicken Ig at a protein concentration equivalent to the respective secondary Abs. These controls were always negative. All immunostaining was performed with tissue sections from three to four individual animals per group from at least three separate experiments. Results shown are representative of all animals tested, and representative areas of each slide are shown.

Confocal laser-scanning double-immunofluorescence microscopy

To detect the differential expression of TGFβ1 by CD4+ T cells during development of thyroid fibrosis, dual-color immunofluorescence and confocal laser-scanning microscopy were done using established markers for CD4 and macrophages. Thyroid frozen sections were fixed with methanol (4°C for 5 min) and then in acetone (4°C for 5 min) and 25% parafomaldehyde for 15 min. Sections were then washed with PBS and blocked with 0.5% casein diluted in PBS. Sections were incubated with chicken anti-TGFβ1 Ab (diluted 1/200) for 1 h at room temperature, and visualized with Alexa 488 conjugated anti-chicken secondary Ab (1/500; Molecular Probes, Eugene, OR). For CD4+ T cell and macrophage staining, slides were incubated with monoclonal rat IgG anti-CD4 mAb (25 μg/ml) (Becton Dickinson, San Jose, CA) as a primary Ab, and then with monoclonal rat IgG anti-CD68 (Serotec, Oxford, UK) as the secondary Ab. Sections were then incubated with anti-rat secondary Ab (1/500) for 1 h, and visualized by streptavidin-conjugated Alexa 568 (Molecular Probes) for 30 min. Slides were stored at 4°C in the dark until observation. Preparations were observed with a Bio-Rad (Hercules, CA) Radiance 2000 confocal system coupled to an Olympus (Melville, NY) IX70 inverted microscope.

Serum thyroxine (T4) assay

Serum T4 levels were determined using a T4 enzyme immunoassay kit (Biotect Labs, Houston, TX) according to the manufacturer’s instructions. Results are expressed as micromgrams of T4 per deciliter of serum. These kits were recently reoptimized for detection of T4 in human serum, so the values reported here tend to be lower than those reported in our previous study (2). Using the current kits, values for normal mouse serum range from 4 to 6 μg T4/dl of serum, and any values >3 are considered normal.

RT-PCR

Thyroid lobes from individual mice were removed at different times after adoptive transfer, and one lobe was stored at −80°C before processing. Frozen thyroids were homogenized in TRIzol; RNA was extracted and reverse transcribed, and RT-PCR was done as previously described (21). To determine the relative initial amounts of target cDNA, each cDNA sample was serially diluted 1/5, 1/25, and 1/125, and amplified with cytokine-specific primers (21). Hypoxanthine phosphoribosyltransferase (HPRT) was used as a housekeeping gene to verify that the same amount of RNA was amplified. The PCR products were electrophoresed in 2% agarose gel, visualized by UV light after staining with ethidium bromide, and normalized between samples relative to levels of HPRT using an IS-1000 Digital Imaging System (Life Sciences, St. Louis, MO). Most cytokine gene primers used in this study have been described previously (21).
Table I. Administration of anti-TGFβ1 or lisinopril reduces thyroid fibrosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of Assessment</th>
<th>Severity of G-EAT</th>
<th>Fibrosis</th>
<th>Atrophy</th>
<th>T4 (μg/dl)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0 1+ 2+ 3+ 4+ 5+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>0 0 0 0 0 5</td>
<td>+</td>
<td>0/5</td>
<td>2.8, 5.0, 2.3, 4.7, 4.8</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>14</td>
<td>0 0 0 0 0 5</td>
<td>-/+</td>
<td>0/5</td>
<td>3.8, 6.2, 5.1, 4.5</td>
</tr>
<tr>
<td>Anti-TGFβ1</td>
<td>14</td>
<td>0 0 0 0 0 5</td>
<td>-/+</td>
<td>0/5</td>
<td>8.2, 5.9, 4.5</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>0 0 0 0 1 5</td>
<td>+</td>
<td>6/6</td>
<td>0.9, 0.5, 0.9, 1.1</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>45</td>
<td>0 0 0 0 0 1</td>
<td>+</td>
<td>+/</td>
<td>4.2, 1.1, 1.4, 1.3</td>
</tr>
<tr>
<td>Anti-TGFβ1</td>
<td>45</td>
<td>0 0 0 0 1 2</td>
<td>3</td>
<td>+</td>
<td>2/6</td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>0 0 0 0 0 3</td>
<td>-/+</td>
<td>0/3</td>
<td>2.3, 2.3, 2.9</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>55</td>
<td>0 0 0 0 0 3</td>
<td>-/+</td>
<td>0/3</td>
<td>2.7, 2.7, 1.9</td>
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<tr>
<td>Lisinopril</td>
<td>55</td>
<td>0 0 0 0 0 3</td>
<td>-/+</td>
<td>5/7</td>
<td>1.0, 1.0, 1.0, 1.0</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>55</td>
<td>0 0 0 0 0 3</td>
<td>-/+</td>
<td>2/6</td>
<td>4.1, 0.5, 1.2, 5.1</td>
</tr>
</tbody>
</table>

*Spleen cells from MTg-immunized DBA/1 donors were activated in vitro with MTg and IL-12, and 3 × 10⁶ cells were transferred to 500-rad DBA1 recipients. Mice in lines 3 and 6 received 250 μg anti-TGFβ1 mAb every 4 days beginning 4 days after cell transfer and continuing to day 41. Mice in lines 5, 8, and 10 received lisinopril (60 mg/kg) in their drinking water beginning at the time of cell transfer. Thyroids were removed 14, 19, 45, or 55 days after cell transfer as indicated.

1Number of recipient mice with various degrees of severity of G-EAT as described in Materials and Methods.

2Intensity of fibrosis was graded as follows: +, negative; +, weak; +, moderate; and +++, strong. The range of fibrosis intensity for each group is indicated (e.g., +/+/+).

3Number of recipients with atrophic thyroid of total. Thyroids designated atrophic were one-quarter to one-half the size of the other thyroids in the group that were not atrophic (see Fig. 1B vs C and D).

4T4 values for selected individual mice in each group. T4 levels were not determined on the animal with the 2+ severity score in line 2 or the animals with 1+ and 3+ scores in line 9. In line 10, the animals with 4+ and 5+ scores had T4 values of 1.0 and 1.2, and in line 6, two of the mice with 5+ scores had T4 values of 1.6 and 1.9.
in thyroids of anti-TGFβ1- or lisinopril-treated mice compared with those of controls, probably due to the reduced myofbs and macrophages.

_**Inflammatory cells involved in development of thyroid fibrosis in G-EAT**_

The inflammatory cells infiltrating the thyroid at days 14–21 included CD4⁺ and CD8⁺ T cells, macrophages, plasma cells, myofbs, histiocytes, giant cells, and many neutrophils. Several types of inflammatory cells in G-EAT thyroids have the potential to produce TGFβ1, and all can express the high affinity TGFβ1R. To begin to address the potential role of various inflammatory cells in thyroid fibrosis, the infiltration of inflammatory cells in the thyroid was monitored over time by immunohistochemical staining. CD4⁺ T cells were the first cells detected in the thyroid, as early as day 3 after cell transfer. This was followed several days later, usually by days 7–8, by infiltration of CD8⁺ T cells, neutrophils, and macrophages (data not shown). Neutrophil infiltration was
mice is comparable to that in controls at days 35 and our previous studies have shown that Ig/\text{H}9252 Ig/\text{H}9252 are also predominant in only partially reduced in the thyroids (data not shown). When thy-

rs were not reduced in either spleens or thyroids (data not shown). Many CD4+ T cells may produce some of the TGFβ1 in G-EAT thyroids.

Cytokine mRNA expression in thyroids of control and anti-TGFβ1-treated mice

The action of TGFβ1 is dependent not only on the cell type and its state of differentiation but also on the cytokine milieu in an inflammatory site (5, 6, 8). Expression of cytokine mRNA in thy-

roids of mice with or without anti-TGFβ1 or ACEI treatment was examined by RT-PCR. Administration of anti-TGFβ1 prevented the overexpression of TGFβ1 mRNA, and inhibited ACE gene expression at day 19 (Fig. 3A). Because PDGF and MCP-1 are important in many models of fibrosis (34–36), their gene expression was also examined. Both PDGF and MCP-1 were highly expressed in thyroids of control mice at day 19, and PDGF remained relatively high at day 35 (Fig. 1B). Their expression was significantly decreased at day 19 in thyroids of anti-TGFβ1-treated mice, and PDGF was also decreased at day 35. Expression of IFN-γ was increased in the thyroids of anti-TGFβ1-treated mice (Fig. 3C), which may suggest antagonism between TGFβ1 and IFN-γ (6, 32). The effect of TGFβ1 on the synthesis and deposition of extracellular matrix are mediated by the type I receptor (TGFβR1) (4). However, expression of TGFβR1 was not decreased in thyroid of anti-TGFβ1-treated mice (Fig. 3C), suggesting the reduction in fibrosis in anti-TGFβ1-treated mice was mainly due to neutralization of TGFβ1. Recently, IL-4 and IL-13 have been identified as key mediators of tissue fibrosis (8, 15–17), and expression of both cytokines was significantly higher in thyroids of control mice compared with those of anti-TGFβ1-treated mice at day 19, but not at day 35 (Fig. 3D). Similar results were obtained with RT-PCR analysis of thyroids of mice given lisinopril (data not shown). Taken together, reduction of fibrosis by anti-TGFβ1

Table II. Summary of expression of TGFβ1, collagen, α-SMA, and ACE in thyroids of control mice or anti-TGFβ1- or lisinopril-treated mice

<table>
<thead>
<tr>
<th></th>
<th>Days 14-21</th>
<th>Days 45-60</th>
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<tbody>
<tr>
<td>Immunostaininga</td>
<td>Control</td>
<td>Anti-TGFβ1</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>+ + +</td>
<td>+/+ +</td>
</tr>
<tr>
<td>Trichrome</td>
<td>+/+ +</td>
<td>-/+ +</td>
</tr>
<tr>
<td>α-SMA</td>
<td>+/+ + +</td>
<td>+/+ + +</td>
</tr>
<tr>
<td>ACE</td>
<td>+ + +</td>
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*Expression of TGFβ1 and ACE was visualized by immunostaining; α-SMA was used to stain myofbs, and Trichrome staining was used to evaluate collagen deposition in thyroids. Staining intensity was graded as follows: −, not detectable; +, weak; + +, moderate; and + + +, strong. The range of staining intensity for each group is indicated (e.g., +/+ + +). Three to four thyroids were examined from each group from at least three separate experiments. This includes animals from each group in Table I as well as animals from other similar experiments not shown in Table I. The results are a summary of results of all thyroids examined by immunohistochemical or Trichrome staining. Thyroids from normal DBA/1 mice were also stained and were negative in all cases.

maximal at days 14–21. At day 14, neutrophils were predomin-
antly located at the periphery of the thyroid (Fig. 2A), in the same location as myobfs and collagen (Fig. 1, A and M). At days 19–21, large groups of neutrophils accumulated inside the thyroid (Fig. 2B), and the aggregated neutrophils were surrounded by macro-

phages (Fig. 2E). The general pattern and extent of neutrophil infiltration was similar in thyroids of both control and treated mice, and few neutrophils persisted in thyroids examined at day 35 or later in any groups (data not shown). (Higher power views of the cells in Fig. 2, A and B, are shown in Fig. 2, C and D.)

Macrophages were also present in thyroids at day 19 (Fig. 2E), and many macrophages persisted, particularly in thyroids of control mice, through day 60 (Fig. 2F). Macrophages could promote fibrosis by producing ACE and TGFβ1 (26), and macrophages were shown to produce TGFβ1 in thyroids that progress to fibrosis (2).

Because CD4+ T cells are the primary effector cells for G-EAT (20), it was of interest to determine whether CD4+ T cells were necessary for the development of fibrosis. Injection of anti-CD4 mAb 2 days after cell transfer almost completely prevented develop-

ment of G-EAT (data not shown). However, if a single injection of anti-CD4 mAb was given 12 days after cell transfer, when G-

EAT severity scores were 4–5+, G-EAT severity at days 19–20 was only minimally reduced (Table III). However, fibrosis was markedly reduced at days 40–60 (Table III), most thyroids were not atrophic, and thyroid follicles were beginning to regenerate (not shown). In addition, serum T₄ levels were higher at days 40–60 in most anti-CD4-treated mice compared with those of controls (Table III). These results indicate that CD4+ T cells, the pri-

mary effector cells for G-EAT (20), were also important for de-
velopment of thyroid fibrosis. A single injection of anti-CD4 given 12 days after cell transfer resulted in nearly complete depletion of CD4+ T cells in the spleen for 10–14 days, but CD4+ T cells were only partially reduced in the thyroids (data not shown). When thy-

roids were removed 40–60 days after cell transfer, CD4+ T cells were not reduced in either spleens or thyroids (data not shown).

CD8+ T cells play an important role in G-EAT resolution and are also predominant infiltrating cells in G-EAT thyroids (27). Se-

vere G-EAT develops in mice in which CD8+ T cells are depleted, and our previous studies have shown that fibrosis in CD8-depleted mice is comparable to that in controls at days 35–50 (28). Thus, CD8+ T cells are not required for development of fibrosis.

TGFβ1 is produced by CD4+ T cells and macrophages in the thyroid during development of fibrosis

The mechanism by which CD4+ T cells might promote thyroid fibrosis could be due to their ability to produce TGFβ1. Confocal microscopy analysis of thyroids that ultimately developed fibrosis showed that some CD4+ T cells expressed TGFβ1 as early as day 7, and many CD4+ T cells were strongly TGFβ1 positive at day 10 (data not shown). At day 19, TGFβ1 expression in thyroids further increased, and TGFβ1+ cells included CD4+ T cells, as well as non-CD4+ T cells (Fig. 2G). A few CD4+ T cells still expressed TGFβ1 at day 35 (Fig. 2H). Many macrophages were also TGFβ1 positive at both day 19 and day 35 (Fig. 2, I and J). Taken together, these results indicate that CD4+ T cells produce TGFβ1 primarily from days 10 to 19, while macrophages were predominant pro-
ducers of TGFβ1 at day 19 and also at day 35, when fibrosis was extensive. As a potent chemotactic factor for many inflammatory cells (5, 29–31), TGFβ1 secreted by early infiltrating CD4+ T cells may attract other inflammatory cells to the thyroid, thus pro-
moting further production of TGFβ1 (32, 33). TGFβ1 expression in G-EAT thyroids at day 19 was reduced after anti-CD4 treatment (data not shown), consistent with the idea that CD4+ T cells may produce some of the TGFβ1 in G-EAT thyroids.

Summary of expression of TGFβ1, collagen, α-SMA, and ACE in thyroids of control mice or anti-TGFβ1- or lisinopril-treated mice

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or ACEI was accompanied by reduction of several profibrotic molecules in the thyroid, suggesting that TGFβ1 is an important cytokine in development of fibrosis as demonstrated by others (4, 37).

**Discussion**

Fibrosis can occur in many tissues and organs as a result of various types of damage, and is a major cause of tissue damage and organ failure (4, 8). These studies were designed to address the mechanisms involved in fibrosis that can develop as a consequence of an autoimmune inflammatory response. This adoptive transfer murine model of G-EAT is, to our knowledge, the only well-characterized animal model of autoimmune disease in which a granulomatous inflammatory response that can result in organ fibrosis can be experimentally induced. Granulomatous inflammatory lesions, some of which have fibrotic manifestations, can be a pathologic feature of several human diseases such as Wegener granulomatosis, allergic granulomatosis, giant cell arteritis, rheumatoid arthritis, Riedel thyroiditis, and sarcoidosis (12), and fibrosis is a major pathologic feature of scleroderma (11). Therefore, G-EAT provides a unique model.
for understanding mechanisms of fibrosis and for experimentally determining how fibrosis initiated by tissue or organ destruction resulting from an autoimmune inflammatory response might be inhibited or controlled.

The current results demonstrate that inhibition of TGFβ1 by anti-TGFβ1 Ab or ACEI can reduce thyroid fibrosis after induction of G-EAT, indicating that TGFβ1 has a critical role in fibrosis that develops as a consequence of an autoimmune inflammatory response. Blocking the activity of TGFβ1 by anti-TGFβ1 or by adenovirus-mediated local expression of a dominant-negative TGFβ1R prevented or reduced fibrosis resulting from nonautoimmune damage to the liver, lung, and kidneys (4, 37). Our data expand the concept that TGFβ1 expression in a particular organ is central to the development of fibrosis. TGFβ1 has been reported to have both pro- and anti-inflammatory effects on autoimmune diseases. TGFβ1 suppressed experimental autoimmune encephalomyelitis in vivo (7, 22) but enhanced activation of experimental autoimmune encephalomyelitis (38), experimental autoimmune thyroiditis (39), and experimental autoimmune uveitis effector cells in vitro (40). Arthritis was promoted by intra-articular injection of TGFβ1 (41), and neutralization of TGFβ1 inhibited development of spontaneous autoimmune thyroiditis in NOD-H-2h4 mice (42). Despite the potent immunosuppressive effects of TGFβ (5–7), inhibition of TGFβ1 by anti-TGFβ1 or ACEI did not increase inflammation in G-EAT thyroids. Thyroids of mice given anti-TGFβ1 or ACEI had G-EAT severity scores at days 14–19 comparable to those of the controls (Table I), but at days 35–60, thyroids of anti-TGFβ1- and ACEI-treated mice were less atrophic and had many regenerating thyroid follicles and less deposition of collagen.

**Table III. Inhibition of fibrosis by administration of anti-CD4 to recipient mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of Assessment</th>
<th>Severity of G-EAT</th>
<th>Fibrosis</th>
<th>Atrophy</th>
<th>T4 (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0 0 0 3 2</td>
<td>–</td>
<td>0/5</td>
<td>2.0, 5.8, 2.0, 2.4</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>0 0 0 0 2</td>
<td>+</td>
<td>0/3</td>
<td>2.8, 2.8, 4.3</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>20</td>
<td>0 0 1 0 2</td>
<td>++</td>
<td>0/3</td>
<td>3.7, 3.7, 4.0</td>
</tr>
<tr>
<td>Control</td>
<td>41</td>
<td>0 0 0 1 0</td>
<td>–/+</td>
<td>5/6</td>
<td>1.9, 2.0, 2.3, 1.3, 3.5</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>41</td>
<td>0 0 0 2 2</td>
<td>–/+</td>
<td>1/6</td>
<td>5.3, 3.7, 2.3, 5.1, 5.4</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>0 0 0 0 3</td>
<td>+</td>
<td>0/3</td>
<td>1.8, 2.1, 3.3</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>19</td>
<td>0 0 0 0 1</td>
<td>+/++</td>
<td>0/3</td>
<td>4.0, 4.0, 2.3</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>0 0 0 1 5</td>
<td>–/++</td>
<td>6/6</td>
<td>0.6, 0.4, 0.4, 1.3, 1.3, 1.5</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>60</td>
<td>0 0 0 1 4</td>
<td>–/+</td>
<td>2/6</td>
<td>1.8, 1.5, 1.7, 5.1, 5.1</td>
</tr>
</tbody>
</table>

* Spleen cells from MTg-sensitized DBA/1 donors were activated in vitro with MTg and IL-12, and $3 \times 10^7$ cells were transferred to 500-rad DBA/1 recipients. Twelve days after cell transfer, mice were given 300 µg rat Ig (control) or 300 µg anti-CD4 mAb (YTS191), and thyroids were removed at the indicated times after cell transfer.

* Number of recipient mice with various degree of severity of G-EAT as described in Materials and Methods.

* Intensity of fibrosis was graded as follows: –, negative; +, weak; ++, moderate; and ++++, strong. The range of fibrosis intensity for each group is indicated (e.g., +/++).

* Number of recipients with atrophic thyroids of total (see footnote d, Table I).

* T4 values of selected individual mice in each group. In line 4, the animal with 3 + G-EAT had the highest T4 (3.5); in line 5, the animals with 4 + severity scores had T4 values of 3.7 and 2.3; and in line 9, the animals with the 5.1 T4 values were those with the 2 + and 3 + severity scores.

**FIGURE 3.** Levels of TGFβ1 and ACE (A), PDGF and MCP-1 (B), IFN-γ and TGFβRI (C), and IL-4 and IL-13 (D) mRNA transcripts in thyroids of mice with or without anti-TGFβ1 treatment 19 or 35 days after cell transfer. At day 19, both control and anti-TGFβ1-treated mice had 4–5 + G-EAT severity scores; at day 35, the average G-EAT score was 5 + for controls and 4 + for anti-TGFβ1 mice. cDNA was prepared from thyroids of individual mice and amplified as described in Materials and Methods. Bars represent the means of data for thyroids of five individual mice ± SD. Results are expressed as the mean ratio of cytokine densitometric units/HPRT ± SD ($\times 100$) and are representative of three independent experiments. A significant difference between anti-TGFβ1 treatment or control thyroids is indicated by an asterisk ($p < 0.05$).
Effective inhibition of the fibrotic process by anti-TGFβ1 and ACEI treatment was demonstrated both by histologic (H&E as well as collagen and myoﬁb staining) and functional (serum T₄) criteria. Inhibition of ﬁbrosis was associated with reduced levels of TGFβ1 mRNA and protein in G-EAT thyroids. There was only a transient appearance of myoﬁb in thyroids of treated mice at day 19, followed by a decline of inﬂammatory cells in thyroids at days 35–60. At days 50–60, thyroids of most anti-TGFβ1- and ACEI-treated mice had regenerating follicles with no myoﬁb and little collagen deposition, whereas thyroids of control mice generally had extensive ﬁbrosis and were very small and atrophic through day 60 (Fig. 1). Serum T₄ levels were low in most control mice at this time, whereas T₄ levels were usually higher and often had returned to normal in treated mice (Tables I and III). The reason serum T₄ levels were still below normal in some treated mice even when thyroid ﬁbrosis and atrophy were clearly reduced may be that some time is needed for serum T₄ levels to return to normal after thyroid follicles begin to regenerate. Thus, T₄ levels might have been normal if serum had been obtained several days later.

G-EAT thyroids become ﬁbrotic only when most thyroid foci are destroyed (1–3). The fact that ﬁbrosis develops in the presence or absence of CD8⁺ T cells (28) suggests CD8⁺ T cells are not essential for the development of thyroid ﬁbrosis. However, CD4⁺ T cells apparently contribute to the development of thyroid ﬁbrosis because administration of anti-CD4 mAb 12 days after cell transfer, when G-EAT severity was nearly maximal, effectively inhibited ﬁbrosis (Table III). CD4⁺ T cells might promote ﬁbrosis through regulation and production of TGFβ1. Production of TGFβ1 by CD4⁺ T cells in the thyroid was noted as early as day 7, before the inﬁltration of most other inﬂammatory cells. Previous studies have shown that TGFβ1 can facilitate the migration of neutrophils and monocytes into tissues (5, 29–31). CD4⁺ T cells were the ﬁrst cells to accumulate in the thyroid, and thyroids of anti-CD4-treated mice expressed less TGFβ1 (our unpublished observations), even though CD4⁺ T cells were not completely depleted from thyroids. Thus, as primary effector cells, MTh-speciﬁc CD4⁺ T cells may initially promote inﬁltration of neutrophils and monocytes by releasing TGFβ1 and other chemokines. Following inﬁltration of various inﬂammatory cells, activated macrophages and myoﬁb produce high levels of TGFβ1. The high expression of TGFβ1 by CD4⁺ T cells, macrophages, and myoﬁb results in excessive TGFβ1 production in the thyroid and in the development of ﬁbrosis. CD4⁺ T cells may also contribute to ﬁbrosis by producing other proﬁbrotic cytokines or chemokines such as IL-13 and MCP-1 (15, 16, 43). TGFβ1 can also activate neutrophils at inﬂammatory sites and enhance their survival (30, 31). In G-EAT that progresses to ﬁbrosis, neutrophil inﬁltration is initially prominent at the periphery of the thyroids, where ﬁbrosis typically begins. Extensive accumulation of neutrophils is observed only when thyroid destruction is very severe and ﬁbrosis ultimately develops, and increased neutrophils have been observed in other models of ﬁbrosis (44, 45). Although neutrophil accumulation in thyroids was not apparently affected by anti-CD4, anti-TGFβ, or lisinopril treatment, we cannot yet rule out a role for neutrophils in the development of ﬁbrosis. Studies are in progress to determine whether depletion of neutrophils will reduce thyroid ﬁbrosis in this model.

The interaction between TGFβ1 and other cytokines (2, 16, 17, 32, 46, 47) as well as their direct action on ﬁbroblasts suggests that other cytokines may be able to modify the proﬁbrotic effects of TGFβ1 (16, 17). For example, IL-4, IFN-γ, TNF-α, MCP-1, PDGF, and ACE are all involved in regulation of inﬂammation, tissue remodeling, and ﬁbrosis (15–18, 43, 47). In this study, expression of PDGF mRNA, known to be induced by TGFβ1 (46, 47), decreased following anti-TGFβ1 and ACEI treatment. MCP-1 and IL-13, which have frequently been implicated in ﬁbrosis (15–19), were also decreased after neutralization of TGFβ1. Reduction of MCP-1 may result from decreased inﬁltration of macrophages and T cells (11) and/or decreased IL-13 production (16). IL-13 is a potent inducer of MCP-1 in vivo (16), and it promotes ﬁbrosis through TGFβ1 signaling (17). Although IL-4 has also been implicated in ﬁbrosis (8), IL-4 is probably not critical for thyroid ﬁbrosis because ﬁbrosis develops in IL-4⁻/⁻ mice with G-EAT (our unpublished observations). Because reduced expression of proﬁbrotic cytokines was accompanied by reduced numbers of myoﬁb, the activated form of ﬁbroblasts, TGFβ1 may interact with other cytokines to modulate ﬁbroblast behavior (15, 33, 48). This may in turn regulate production of cytokines and/or chemokines, resulting in a persistent positive feedback loop between certain cytokines and ﬁbroblasts.

ACE was highly expressed in G-EAT thyroids at the time of maximal inﬂammation, and was localized in areas of collagen deposition. Myoﬁb and macrophages may be the major producers of ACE (26). The ACE lisinopril decreased TGFβ1 production in the thyroid and reduced thyroid ﬁbrosis. ACE may also reduce MCP-1 induced by ANGH (49), further interfering with MCP-1-mediated CD4⁺ T cell proliferation and cytokine production (43). These multiple effects may account for the potent effects of ACE in inhibiting ﬁbrosis and reducing tissue damage (14). These data support the notion that inhibition of TGFβ1 by ACEI can be a useful treatment for thyroid ﬁbrosis, consistent with results of other studies demonstrating its effectiveness for treatment of ﬁbrosis in other organs (14, 24, 25).

The complex interaction between TGFβ1 and various cells and mediators reﬂects the well-regulated process that should follow termination of an inﬂammatory response. This control can be interrupted by an excessive inﬂux of inﬂammatory cells and dysregulation of TGFβ1 and other cytokines. G-EAT provides a new model to study pathogenic mechanisms and therapeutic interventions in ﬁbrosis. The knowledge gained from these studies could have important implications for understanding speciﬁc mechanisms of ﬁbrosis involved in other autoimmune diseases, in particular those associated with granulomatous immunopathology, arthritis, vasculitis, and ﬁbrotic sequelae (8–10, 12). The ability of ACEI to block TGFβ1 suggests they may be clinically useful antifibrotic agents for such diseases.

References


