Increased expression of tumor necrosis factor-α and decreased expression of thyroglobulin and thyroid peroxidase mRNA levels in the thyroids of iodide-treated BB/Wor rats

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Abstract
Several lines of evidence suggest that tumor necrosis factor-α (TNFα) may contribute to the pathogenesis of autoimmune thyroid disease. It is not known, however, whether increased thyroidal TNFα levels are associated with changes in thyroid function. The purpose of the present study was to utilize in situ hybridization histochemistry and immunohistochemistry to determine if the expression of TNF-α in the thyroid is associated with a decrease in thyroglobulin (Tg) and thyroid peroxidase (TPO) mRNA levels. Lymphocytic thyroiditis was induced in BB/Wor rats by iodide administration, and thyroidal Tg and TPO mRNA levels were assessed by Northern blot analysis and in situ hybridization, and TNFα expression by Northern blot analysis and immunohistochemistry. Thyroids were obtained before and 1 and 2 months after iodide administration. Hematoxylin and eosin staining revealed that there was a progressive increase in mononuclear cells in the thyroids of BB/Wor rats ingesting iodide for 1 and 2 months. Northern blot analysis revealed that during the same time course there was a progressive increase in TNFα mRNA levels and a progressive decrease in Tg and TPO mRNA levels in the thyroids. In situ hybridization histochemistry was performed to determine if the decrease in Tg and TPO mRNA levels was associated with thyroid follicular cells in contact with infiltrating mononuclear cells. In rats treated with iodide for 1 month, there was a modest decrease in Tg and TPO mRNA levels in follicular cells in contact with infiltrating mononuclear cells. After 2 months of iodide treatment there was clearly a localized decrease in Tg and TPO mRNA levels in follicular cells in contact with infiltrating mononuclear cells. After 2 months of iodide treatment TNFα was easily detected in infiltrating mononuclear cells and in some thyroid follicular cells. Together, these results suggest that the suppression of Tg and TPO mRNA levels was associated with the expression of TNFα and thus are in agreement with in vitro studies demonstrating that TNFα inhibits thyroid cell function.

European Journal of Endocrinology 139 539–545

Introduction
Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine implicated in the regulation of thyroid growth and differentiated functions. Several lines of evidence suggest that TNFα may play a role in the development of autoimmune thyroid disease (ATD). Studies suggest that increases in endogenous TNFα production may be involved in the regulation of thyroid function in ATD. That is, TNFα and TNFα mRNA are found in human thyroid tissues from patients with Graves’ disease and Hashimoto’s thyroiditis (1–4). In FRTL-5 cells, we (5, 6) and others (7–10) have observed that TNFα inhibits type I 5’-deiodinase activity and thyroglobulin (Tg), thyroid peroxidase (TPO) and type I 5’-deiodinase gene expression. In vivo and in vitro, TNFα induces the expression of class II major histocompatibility complex antigens (11–13) and intercellular adhesion molecule-1 in thyroid cells (14). Further, TNFα triggers the expression of many proinflammatory mediators, such as interleukin-1, in several cell lines including the thyroid (15). Whereas these studies suggest that TNFα may contribute to the pathogenesis of ATD, it is not known whether increased thyroidal TNFα levels are associated with local changes in thyroid function in ATD.

Diabetes-prone Biobreeding Worcester (BB/Wor) rats have a high incidence of spontaneous lymphocytic thyroiditis (LT) which resembles Hashimoto’s disease (16). They develop circulating anti-thyroid colloid and anti-thyroglobulin antibodies, and in young rats, iodine intake markedly increases the incidence and earlier occurrence of LT (17). In the present study, we used this
model of iodide-augmented LT in BB/Wor rats to

determine if the development of LT is associated with
decreases in Tg and TPO mRNA levels in thyroid
epithelial cells and with infiltrating lymphocytes and
increased expression of TNFα.

Materials and methods

Animals

Forty-day-old BB/Wor rats were fed Purina chow
available ad libitum and 0.05% iodide water (0.64 g
NaI/l) for up to 2 months as previously described (17).
Thyroids were obtained before and 1 and 2 months after
iodide administration. One of the thyroid lobes was
quickly frozen in liquid N2 and stored at −80°C until used
for RNA isolation. The other lobe was fixed in 4%
paraformaldehyde solution (PFA) and embedded in
paraffin. Animals were maintained in accordance with
the NIH guidelines for the care and use of animals
approved by the Institutional Animal Care and Use
Committee at the University of Massachusetts Medical
Center.

Preparation of cRNA probes

Rat Tg and TPO cDNA (kindly provided by Dr Gilbert
Vassart and Dr Shioko Kimura respectively) in pGEM3Z. 

Figure 1 Hematoxylin and eosin staining of BB/Wor rat thyroids
obtained before (a) and after 1 (b) or 2 (c) months of iodide
administration. Magnification × 400.

Figure 2 Effect of iodide treatment on Tg, TPO and TNFα mRNA
levels in the thyroids of BB/Wor rats. The relative amounts of mRNA
were normalized to GAPDH.

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plasmid (Promega, Madison, WI, USA) and a rat TNFα cDNA in Bluescript (a gift from Dr Thomas Gill) served as templates for in vitro cRNA synthesis. Sense and antisense cRNA probes were transcribed from the templates by Sp6, T3 or T7 RNA polymerase (Promega) in the presence of [35S]UTP or [32P]UTP (New England Nuclear Corp., Boston, MA, USA). The specificity of antisense and sense probes was verified by Northern blot analysis.

Isolation of total cellular RNA and Northern blot analysis

Total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method (18). Equal amounts of total RNA were fractionated by electrophoresis and blotted on Duralose-UV filters (Stratagene, La Jolla, CA, USA) as previously described (5). The filters were hybridized to 32P-labeled rat Tg cDNA, rat TPO cDNA or rat TNFα cRNA probes overnight at 42 or 55°C. After several washes, filters were exposed to Fuji RX film (Fuji Photo Film Co., Tokyo, Japan). The autoradiogram was quantified by densitometry. The relative amounts of mRNA were normalized to glyceraldehyde-3-phosphate (GAPDH) RNA content.

In situ hybridization

Paraffin-embedded 4 μm thick thyroid sections were attached to slides coated with 1× Denhardt’s. The morphology of the thyroid sections was evaluated by hematoxylin and eosin staining. After deparaffinization in xylene and rehydration in graded ethanol, the sections were treated with 10 μg/ml proteinase K (Sigma Chemical Co., St Louis, MO, USA) followed by post-fixation in 4% PFA and acetylation in 0.25% acetic anhydride in 0.1 mol/l triethanolamine. The sections were incubated overnight at 55°C with hybridization solution containing 50% deionized formamide, 10 mmol/l Tris–HCl, pH 8.0, 300 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l dithiothreitol, 10% dextran sulfate, 500 μg/ml tRNA, 1× Denhardt’s solution and 35S-labeled antisense or sense cRNA probes. Subsequently, the sections were washed in buffer containing 10 mmol/l Tris–HCl, pH 8.0, 10 mmol/l sodium phosphate, 50% deionized formamide, 5 mmol/l EDTA, 1 mmol/l dithiothreitol and 1× Denhardt’s solution. They were incubated with

Figure 3 In situ expression of Tg mRNA, detected with Tg antisense cRNA probe, in BB/Wor rat thyroids obtained before and after 1 or 2 months of iodide administration. The arrow on the photograph at × 200 magnification indicates the area shown in the photograph at × 400 magnification.
50 µg/ml RNase A (Sigma Chemical) and 10 U/ml RNase T1 (Boehringer-Mannheim Co., Indianapolis, IN, USA) followed by extensive washes. Autoradiography was performed using NTB-2 photoemulsion (Eastman Kodak, Rochester, NY, USA) for 2–3 weeks.

**Immunohistochemistry**

After deparaffinization and rehydration, thyroid sections were incubated with 5% normal goat serum (Vector Labs, Burlingham, CA, USA) for 30 min. They were incubated with rabbit anti-mouse TNFα (Genzyme, Cambridge, MA, USA) overnight at 4 °C. After endogenous peroxidase activity had been blocked with 0.3% hydrogen peroxide, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega) for 30 min. The immunoreaction was visualized by the addition of diaminobenzidine (Dojin, Kumamoto, Japan), and then the sections were counterstained with methyl green. The specificity of immunostaining was verified by incubation of the sections with preimmune rabbit serum instead of anti-mouse TNFα and by the omission of the primary and/or secondary antibodies from the reaction buffer.

**Results**

**Expression of Tg and TPO mRNAs levels in the thyroids of BB/Wor rats ingesting iodide**

In BB/Wor rats fed iodide for 1 or 2 months there was a progressive increase in infiltrating mononuclear cells in the thyroids (Fig. 1). To determine if the progression of ATD was associated with a change in thyroid function, Tg and TPO mRNA levels were evaluated by Northern blot analysis. Northern blot analysis revealed a slight but progressive decrease in Tg and TPO mRNA levels in BB/Wor rats fed iodine for up to 2 months (Fig. 2). Northern blot analysis, however, could not determine if there was a general decrease in thyroidal gene expression or if the decreases in thyroidal Tg and TPO mRNA levels were associated with infiltrating mononuclear cells. To address this question, in situ hybridization was performed to determine if the decrease in Tg and TPO mRNA levels was associated with infiltrating mononuclear cells. Tg and TPO mRNAs were easily detected by in situ hybridization in thyroid epithelial cells of BB/Wor rats (Figs 3 and 4). Induction of ATD following iodide ingestion for 1 month was associated with a

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**Figure 4** In situ expression of TPO mRNA, detected with TPO antisense cRNA probe, in BB/Wor rat thyroids obtained before and after 1 or 2 months of iodide administration. The arrow on the photograph at ×200 magnification indicates the area shown in the photograph at ×400 magnification.
Figure 5 Expression of TNFα, examined immunohistochemically, in BB/Wor rat thyroids obtained before (top) and after 1 (middle) or 2 (bottom) months of iodide administration. Sections were counterstained with methyl green. The arrow on the photograph at ×200 magnification indicates the area shown in the photograph at ×400 magnification. Arrowheads in the ×400 magnification photograph, after 2 months of iodine administration, indicate TNFα positive cells.
decrease in the Tg mRNA levels in some follicles in contact with infiltrating mononuclear cells (Fig. 3). In rats ingesting iodine for 2 months, however, there was a marked localized decrease in Tg mRNA levels in follicles in contact with infiltrating mononuclear cells. Similarly, TPO mRNA levels were decreased in some follicles in contact with infiltrating cells in rats ingesting iodide for 1 month (Fig. 4). Further, a localized decrease in TPO mRNA levels in follicular cells was clearly associated with infiltrating mononuclear cells in rats ingesting iodide for 2 months.

**TNFα expression in BB/Wor rat thyroids**

As shown in Fig. 2, there were detectable levels of TNFα mRNA in the thyroids of BB/Wor rats without iodide treatment. Iodide administration for 1 or 2 months, however, resulted in a progressive increase in TNFα mRNA levels in the thyroids of BB/Wor rats. To determine if an increase in expression of TNFα is associated with infiltrating mononuclear cells and/or thyroid follicles, we examined TNFα protein expression in the thyroids of BB/Wor rats by immunohistochemistry. As shown in Fig. 5, TNFα was not detected in BB/Wor rat thyroids obtained before and 1 month after iodide administration. In rats treated for 2 months, however, TNFα protein was easily detected in infiltrating mononuclear cells and thyroid follicular cells.

**Discussion**

Using a model of iodide-augmented spontaneous LT in BB/Wor rats, we found a progressive increase in infiltrating mononuclear cells and intrathyroidal TNFα mRNA levels in BB/Wor rat thyroids after 1 and 2 months of iodide ingestion. TNFα is synthesized in a number of cell types, including lymphocytes and thyroid epithelial cells (3, 4, 19, 20). To determine the distribution and identify the cell types expressing TNFα, immunohistochemical studies were performed using a TNFα-specific antibody. Before initiation of iodine ingestion, TNFα levels were not detectable by immunocytochemistry. While TNFα mRNA levels had increased by approximately 150% after 1 month of iodine ingestion, TNFα protein levels remained below the level of detection by immunocytochemistry. In contrast, we found that after 2 months of iodine ingestion, TNFα protein was easily detected in infiltrating mononuclear cells and in thyroid follicular cells associated with the infiltrating mononuclear cells. These findings in the BB/Wor rat are consistent with previous studies demonstrating an increase in TNFα protein and mRNA levels in thyroids obtained from patients with Graves’ disease and Hashimoto’s thyroiditis (3, 4). Further, reports show an increase in TNFα levels in other autoimmune diseases such as synovial tissues in rheumatoid arthritis (21) and in the pancreas in type I diabetes mellitus (22), which is associated with the infiltration of mononuclear cells.

In addition to the localization of TNFα in infiltrating mononuclear cells, we found that TNFα protein is expressed in thyroid follicular cells, suggesting that thyroid follicular cells, through TNFα production and secretion, may control thyroid function in a paracrine and autocrine manner. Thus, our results suggest that an increase in TNFα levels in the areas with mononuclear cell infiltration may be a critical step in the pathogenesis of ATD.

We have shown that TNFα blocks thyrotropin-induced increases in Tg and TPO mRNA levels in FRTL5 cells (5). However, it is not known whether an increase in intrathyroidal TNFα levels is associated with a decrease in Tg and TPO mRNA levels in ATD. In the present study, we clearly demonstrate that after 2 months of iodide ingestion there is a decrease in Tg and TPO mRNA levels, which is localized in thyroid follicular cells in contact with infiltrating mononuclear cells expressing TNFα. This is consistent with a recent report showing that apoptosis of thyroid follicular cells is associated with mononuclear cell infiltration in thyroids obtained from patients with Hashimoto’s thyroiditis (23). Thus our results suggest that, in ATD, infiltrating mononuclear cells may suppress Tg and TPO gene expression, at least in part, through TNFα production. Further, in ATD, thyroid cells themselves may suppress their function through TNFα production in an autocrine or paracrine manner.

**Acknowledgements**

This paper was presented in part at the 69th Annual Meeting of the American Thyroid Association, San Diego, CA, USA, November 1996, and was supported in part by DK18931, NIDDK and AA11277, NIAAA, Bethesda, MD, USA.

**References**


Received 17 July 1998
Accepted 6 August 1998