Autoinduction of tumor necrosis factor-α in FRTL-5 rat thyroid cells

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Abstract

Tumor necrosis factor-α (TNFα) may play a role in the development of autoimmune thyroiditis such as Hashimoto’s thyroiditis. In the present study, we examined whether TNFα induced its own expression in FRTL-5 rat thyroid cells. Lipopolysaccharide (LPS) markedly increased TNFα mRNA levels in FRTL-5 cells as assessed by semiquantitative RT-PCR. In addition, LPS-stimulated cells released TNFα protein into the culture medium. Similarly, TNFα induced its own gene and protein expression in FRTL-5 cells as assessed by RT-PCR and metabolic labeling and immunoprecipitation of TNFα. The autoinduction of TNFα gene was also observed in TNFα-stimulated human thyroid epithelial cells. TNFα induction was specific to LPS and TNFα since interferon-γ or amiodarone failed to increase TNFα mRNA levels in FRTL-5 cells. Human TNFα induced rat TNFα gene expression, indicating that type 1 TNF receptor (TNF-R) is involved in the autoinduction. TNFα did not increase either type 1 or type 2 TNF-R mRNA levels, suggesting that upregulation of TNF receptors is not involved in the autoinduction of TNFα. Although the biological significance of autoinduction of TNFα remains unclear, our results suggest that thyroid epithelial cells may participate in the development of autoimmune thyroiditis through production of TNFα. Furthermore, inhibition of TNFα production in the thyroid may represent a novel approach to mitigating inflammation in autoimmune thyroiditis.


Introduction

Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine that acts as a central regulator of inflammation and immunity. This cytokine is detectable in thyroid tissues obtained from patients with Hashimoto’s thyroiditis (Ajjan et al. 1996, Aust et al. 1996). In accordance, we and others have demonstrated enhanced TNFα gene expression in the inflamed thyroid of BioBreeding/Worcester (BB/W) rats (Mori et al. 1998, Bluher et al. 1999). Furthermore, TNFα augments interferon-γ (IFNγ)-induced class II major histocompatibility complex (MHC) antigen expression (Weetman & Rees 1988, Zakaria et al. 1988). In FRTL-5 rat thyroid cells, TNFα induces interferon regulatory factor-1 (Mori et al. 1999), which plays a role in immune responses (Taniguchi et al. 1997). Taken together, these results suggest that TNFα may be involved in the development of autoimmune thyroiditis such as Hashimoto’s thyroiditis.

While the majority of TNFα detected in inflamed thyroid tissues is produced by infiltrating inflammatory cells (Aust et al. 1996), studies demonstrate the production of TNFα by thyroid epithelial cells (Zheng et al. 1992, Mori et al. 1998). It may be possible that TNFα produced by infiltrating inflammatory cells induces its own expression in thyrocytes in the area of infiltration, since autoinduction of TNFα has been demonstrated in HL-60 human promyelocytic leukemia cells and in rat tracheal epithelial cells (Spriggs et al. 1990, Bader & Netttesheim 1996). If so, thyrocyte-produced TNFα may stimulate infiltrating cells to aggravate inflammation in autoimmune thyroiditis. However, autoinduction of TNFα in thyroid epithelial cells has never been reported. In the present study, we examined whether TNFα induced its own expression in FRTL-5 cells.

Materials and Methods

Reagents

Recombinant rat IFNγ and recombinant mouse TNFα were obtained from Genzyme (Cambridge, MA, USA). Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS; Escherichia coli O55:B5) and amiodarone (AMD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). α-[32P]-dATP was purchased from New England Nuclear Corporation (Boston, MA, USA).

References

35S-methionine (TRAN35S-LABEL) was obtained from ICN Biomedicals (Irvine, CA, USA). Anti-p65 subunit of nuclear factor kappa B (NF-kB) and preimmune rabbit serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies to IKKα and phosphorylated IKKα (Ser 32) were obtained from New England Biolabs (Beverly, MA, USA). The chemiluminescence detection system (ECL) was purchased from Amersham Biosciences (Piscataway, NJ, USA). The Superscript preamplification system containing Superscript II reverse transcriptase was obtained from Gibco/BRL (Grand Island, NY, USA). All other chemicals and reagents were purchased from commercial sources and were of reagent or molecular biology grade.

Cell culture

FRTL-5 cells were obtained from ATCC (Rockville, MD, USA), proliferated in a thyrotropin (TSH)-dependent manner and possessed all the properties previously described (Mori et al. 2001). Cells were grown in Coon’s modified Ham’s F12 medium supplemented with 5% calf serum containing bovine TSH (1 mU/ml), bovine insulin (10 µg/ml), human transferrin (5 mg/ml), glycyl-l-histidyl-l-lysine (2 ng/ml), somatostatin (10 ng/ml) and hydrocortisone (0·36 ng/ml; 6H medium). After cells approached confluence, cells were cultured for 7 days in media devoid of TSH (5H medium). This treatment allowed cells to be quiescent as shown previously (Mori et al. 2001). Human thyroid epithelial cells were obtained and cultured as previously described (Trieb et al. 1992) with modification. Normal thyroid tissues were removed from two patients who underwent thyroidectomy for papillary carcinoma. Informed consent was obtained from each patient and the study protocol was approved by the local ethics committee. The tissues were digested with 1 mg/ml collagenase I (Sigma Chemical Co.) and 2·4 U/ml dispase II (Roche Diagnostics GmbH, Penzberg, Germany) in PBS at 37 °C for 30 min in hypertonc buffer containing 20 mM HEPES-KOH, pH 7·9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM NaF and 1 mM Na3VO4. After incubation on ice for 15 min, cells were vortexed for 10 s in hypotonc buffer containing 0·1% NP-40, followed by incubation on ice for 10 min. Nuclei were pelleted by centrifugation, washed twice with hypotonc buffer and then the nuclear pellets were incubated at 4 °C for 30 min in hypertonc buffer containing 20 mM HEPES-KOH, pH 7·9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM NaF, 1 mM Na3VO4 and 20% glycerol. Supernatants were collected after centrifugation and stored at −80 °C until use. Protein concentrations were measured by the Bradford method (Bradford 1976).

Electrophoretic mobility shift assay (EMSA)

The synthetic oligonucleotides, 5’-CAAAACAGGGGCT TTTCCCCTCCTC-3’ and 5’-GAGGGAGAAAGCCCC CTGTTTG-3’, containing the NFκB (κB) site in rat TNFα gene promoter (Nathens et al. 1997), were used for the detection of the protein–DNA complex by EMSA. The double-stranded probes were end-labeled using Klenow-DNA polymerase and α[32P]-dATP. Nuclear proteins (5 µg) were incubated with 40 fmol 32P-labeled probe at 22 °C for 30 min in buffer containing 10 mM HEPES-KOH, pH 7·9, 100 mM KCl, 0·5 mM EDTA, 0·5 mM EGTA, 0·5 mM DTT, 0·5 mM PMSF, 10% glycerol, 0·05% NP-40 and 2 µg poly(dI-dC)-poly (dI-dC). The nucleoprotein complexes were resolved by nondenaturing electrophoresis in a 5% polyacrylamide gel at 4 °C in buffer containing 45 mM Tris–HCl, pH 8·0, 45 mM boric acid and 1 mM EDTA. Gels were dried and exposed to BioMax MS film (Eastman Kodak, Rochester, NY, USA) at −80 °C. For competition experiments, a 100-fold molar excess of the unlabeled oligonucleotides was added 15 min before incubation of nuclear extract with radiolabeled probes. In supershift assays, nuclear proteins were incubated for 1 h at 22 °C with 1 µl anti-p65 subunit of NFκB or preimmune rabbit serum followed by addition of radiolabeled probes.

Western blot analysis

Cells were washed with ice-cold washing buffer containing 10 mM sodium phosphate, pH 7·4, 137 mM NaCl
and 1 mM Na$_3$VO$_4$ and solubilized for 30 min at 4°C in the lysis buffer containing 50 mM Tris–HCl, pH 7.5, 137 mM NaCl, 2 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na$_3$VO$_4$, 1 mM NaF and 0.1% Triton X-100. Supernatants were collected after centrifugation and aliquots containing 50 µg protein were mixed with Laemmli sample buffer and separated by SDS-PAGE (Laemmli 1970). Proteins were transferred to a nitrocellulose membrane by electroblotting. After blocking with 1% BSA in Tris–buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated with rabbit polyclonal antibody to serine-phosphorylated I$_B$-afii9837I$_B$-afii9825 (1:1000) overnight at 22°C. The membranes were washed and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) for 1 h at 22°C. Blots were visualized using the ECL detection system. After stripping, the membranes were reprobed with rabbit polyclonal anti-I$_B$-afii9837I$_B$-afii9825 (1:1000).

### RT-PCR

Cells were washed twice with ice-cold PBS and total cellular RNA was isolated by the acid guanidinium–thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi (1987), using Trizol (Gibco/BRL). Four micrograms total RNA were reverse transcribed with Superscript II reverse transcriptase according to the manufacturer’s instructions. All PCRs were performed in a 50 µl reaction volume containing 1 µl aliquots from each cDNA reaction, 10 pmol of each upstream and downstream primer, 1·25 units Taq polymerase (Takara, Otsu, Japan), 0·2 mM of each dNTP, and 2 mM MgCl$_2$. Amplification was performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s followed by a 5-min extension at 72°C. The primer sequences, product length and PCR cycles shown in Table 1 were used for the detection of TNF$_a$, two types of TNF receptors, TNF-R1 and TNF-R2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression in FRTL-5 cells. Primer pairs for rat TNF$_a$ span introns, and extra bands suggesting genomic DNA contamination were not seen. Human TNF$_a$ and human $\beta$-actin gene expression was determined using a human TNF$_a$ primer pair (BioSource International, Camarillo, CA, USA) and a human $\beta$-actin

<table>
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**Figure 1** Effect of TNF$_a$ or LPS on nuclear NF-$\kappa$B binding activity in FRTL-5 cells. Nuclear proteins were incubated with $^{32}$P-labeled synthetic oligonucleotides containing the NF-$\kappa$B element (k83) of rat TNF$_a$ gene promoter and NF-$\kappa$B binding activity was analyzed with EMSA. Cells were stimulated with 10 ng/ml mouse TNF$_a$ or 100 µg/ml LPS for the indicated times (0, (unstimulated), 10, 30, 60 min). P, $^{32}$P-labeled probe only and C, a 100-fold molar excess of unlabeled oligonucleotides were included as competitors. The complex formed in response to TNF$_a$ or LPS was supershifted by the anti-p65 subunit of NF-$\kappa$B (p65), but not by preimmune rabbit serum (NS). The data presented are representative of three separate experiments.
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primer transcription were carried out in parallel. Cycle numbers were chosen to lie in the linear range of amplification. Rat GAPDH and human β-actin were used as an internal standard to verify that equal amounts of RNA had been subjected to RT-PCR. PCR products were separated on 1·5% agarose gel and visualized with ethidium bromide. All PCR products were cloned into pGEM-T vector (Promega, Madison, IL, USA) and sequenced by the dideoxy chain termination method.

Metabolic labeling and immunoprecipitation

TNFα protein production in TNFα-stimulated cells was assessed by immunoprecipitation of 35S-labeled TNFα (Zhou et al. 2000). Briefly, cells were washed with PBS and incubated in methionine-free RPMI (ICN Biomedicals) supplemented with 1-glutamine, penicillin-streptomycin, 1% calf serum and 35S-methionine (TRAN35S-LABEL; 100 µCi/ml) for 24 h in the presence or absence of 50 ng/ml TNFα. Cells were washed and lysed in the lysis buffer. Samples were clarified by centrifugation and standardized to protein concentration. Cell extracts were precleared twice by incubating with protein A/G agarose (Santa Cruz Biotechnology). The samples were incubated with 5 µg antibody to TNFα (Genzyme) or normal goat IgG overnight and protein A/G agarose was added for an additional 2 h. The beads were washed 5 times and eluted in Laemmli sample buffer. The proteins were separated by SDS-PAGE and dried gels were subjected to autoradiography.

TNFα ELISA

TNFα released into the culture medium by FRTL-5 cells treated with LPS for the indicated times was measured by a rat TNFα enzyme-linked immunosorbent assay (ELISA) kit (BioSource International), according to the manufacturer’s instructions. The detection limits for rat TNFα were 0·7 pg/ml. Human TNFα was measured by a human TNFα ELISA kit (BioSource International) with the minimum detectable level of 0·1 pg/ml. Data are the means of triplicate culture supernatants ± S.D. and are representative of two separate experiments. Statistical analysis was performed using one-way ANOVA followed by Fisher’s protected least significant difference test. A level of P<0·05 was considered statistically significant.

Results

EMSA was performed to verify that TNFα and LPS activate the transcription factor NF-κB, which plays a central role in the TNFα- and LPS-stimulated intracellular signaling pathway in FRTL-5 cells. No NF-κB binding activity was detected in unstimulated cells (Fig. 1). Mouse TNFα induced a rapid and marked increase in NF-κB binding activity. Addition of a 100-fold molar excess of unlabeled oligonucleotides to the binding reaction completely blocked the binding activity. Furthermore, the complex formed in response to TNFα was supershifted by the anti-p65 subunit of NF-κB, but not by preimmune rabbit serum (Fig. 1), indicating that activated NF-κB containing the p65 subunit was induced in response to TNFα.

Consistently, stimulation of cells with mouse
TNFα resulted in an increase in phosphorylated IkBα levels and a concomitant decrease in total IkBα levels at 10 and 30 mm after stimulation (Fig. 2). Similar to TNFα, LPS activated NF-κB (Fig. 1), consistent with previous studies (Guha & Mackman 2001). Thus, we confirmed that the major signaling molecule, NF-κB, was activated in response to TNFα and LPS in FRTL-5 cells.

TNFα gene expression in FRTL-5 cells was analyzed by semiquantitative RT-PCR. As shown in Fig. 3A, TNFα gene expression was not detected in unstimulated cells. Incubation of FRTL-5 cells with LPS, a very potent inducer of TNFα, resulted in a rapid and marked increase in TNFα mRNA levels. As a result, FRTL-5 cells released very low but detectable levels of TNFα protein in response to LPS (Fig. 3B). Thus, we confirmed that FRTL-5 cells could produce TNFα in response to stimuli such as LPS.

Similar to LPS, TNFα clearly induced its own gene expression in FRTL-5 cells (Fig. 4A). Dose-dependent induction of the TNFα gene was observed in TNFα-treated cells (Fig. 4B). We evaluated TNFα protein production by metabolic labeling and immunoprecipitation of newly synthesized TNFα protein. As shown in Fig. 4C, a modest but apparent increase in the levels of a 26 kDa protein was observed in TNFα-treated cells. Thus de novo TNFα protein biosynthesis was induced in response to TNFα treatment in FRTL-5 cells. We confirmed that induction of TNFα gene was specific for LPS or TNFα since it was not observed over a 24-h incubation period in cells treated with IFNγ or AMD (data not shown), which induces interleukin (IL)-6 gene expression in the thyroid (Nakajima et al. 2001).

To determine whether autoinduction of TNFα gene was specific for FRTL-5 cells, human thyroid epithelial cells were stimulated with LPS or TNFα. As reported previously (Zheng et al. 1992), human thyrocytes produced detectable levels of TNFα protein in response to LPS (Fig. 5B). Thus, we confirmed that autoinduction of TNFα gene expression occurs in human thyrocytes as well as in FRTL-5 cells.

Human TNFα, which does not interact with the TNF-R2 in rodents (Lewis et al. 1991), induced TNFα gene expression (data not shown). We tested whether upregulation of TNF-Rs was involved in autoinduction of TNFα in FRTL-5 cells. As illustrated in Fig. 6, mouse TNFα failed to increase either TNF-R1 or TNF-R2 mRNA levels.
Autoinduction of TNFα has been reported in a variety of cells (Spriggs et al. 1990, Bader & Nettlesheim et al. 1996), but not in thyroid cells. Thus, the present study may be the first to show the autoinduction of TNFα in FRTL-5 cells. This phenomenon is not specific for this rat thyroid cell line since TNFα can induce its own gene expression in human thyroid epithelial cells. Although the biological significance of autoinduction of TNFα in the thyroid remains unclear, these findings suggest that thyrocytes may participate in the development of autoimmune thyroiditis through production of TNFα. Furthermore, it may be possible that TNFα produced by infiltrating inflammatory cells such as activated macrophages and lymphocytes (Sariban et al. 1988, Aust et al. 1996) stimulates thyrocytes to produce TNFα. This cytokine augments IFNγ-induced MHC class II antigen expression (Weetman & Rees 1988, Zakaria et al. 1988) and induces cytokines such as IL-1 and IL-6 (Nawroth et al. 1986, Kikumori et al. 1998). Taken together, TNFα produced by thyroid epithelial cells may stimulate themselves and inflammatory cells in an autocrine and/or paracrine manner to facilitate immune reaction and inflammation in autoimmune thyroiditis. In accordance with this concept, Green et al. (1998) have demonstrated that TNFα produced in islets plays an important role in the development of autoimmune diabetes in nonobese diabetic mice. Accordingly, inhibition of TNFα action and production would be beneficial in order to suppress inflammation in autoimmune thyroiditis. This concept is supported by a recent study by Zaccone et al. (2002) showing that experimental autoimmune thyroiditis induced by immunization with thyroglobulin and LPS is inhibited by soluble TNF-R1.

In the present study, TNFα gene expression was clearly induced in FRTL-5 cells in response to LPS or TNFα, as assessed by RT-PCR. In contrast, LPS- or TNFα-stimulated FRTL-5 cells produced a very small amount of TNFα protein. Taken together, our results suggest that the FRTL-5 cell line may be a suitable model to determine the regulatory mechanism involved in TNFα gene expression, but not in TNFα protein synthesis, in the thyroid. Accordingly, establishment of more sensitive methods such as the immuno-PCR (Sanna et al. 1995) is clearly required to analyze TNFα biosynthesis in FRTL-5 cells. Alternatively, this issue could be addressed by the establishment of thyroid-derived cells that produce much higher amounts of TNFα protein. In this regard, human thyroid epithelial cells may be suitable since LPS-stimulated cells can produce larger amounts of TNFα than can FRTL-5 cells as shown in Fig. 5A.

TNFα exerts its biological effects through binding to two distinct cell surface receptors, TNF-R1 and TNF-R2 (Lewis et al. 1991). Previous studies demonstrated that thyroid cells possess TNF receptors (Pang et al. 1989, 1996). However, TNF-R gene expression remained to be elucidated in TNFα-stimulated thyroid cells. In the present study, we demonstrate detectable levels of

![Figure 5](image1.png)

**Figure 5** (A) Production of TNFα by LPS-stimulated human thyroid epithelial cells. Cells were incubated with 100 μg/ml LPS for the indicated times (h). TNFα levels in culture medium were determined using an ELISA kit. Data are the means of triplicate culture supernatants ± S.D. and are representative of two separate experiments. n.d., not detectable. *P<0.05 compared with unstimulated cells (0 h). (B) Induction of TNFα gene expression in TNFα-stimulated human thyroid cells. Cells were incubated with 10 ng/ml human TNFα for the indicated times (h). The data presented are representative of three separate experiments.

**Discussion**

In the present study, we clearly demonstrate that TNFα induces its own expression in FRTL-5 cells, consistent with a previous observation that TNFα mRNA and protein levels are elevated in thyrocytes of patients with Hashimoto’s thyroiditis (Zheng et al. 1992).

![Figure 6](image2.png)

**Figure 6** TNF-R1 and TNF-R2 gene expression in TNFα-stimulated FRTL-5 cells. Cells were incubated with 10 ng/ml mouse TNFα for the indicated times (h). The data presented are representative of three separate experiments.

TNF-R1 and TNF-R2 mRNA in unstimulated FRTL-5 cells. However, TNFα failed to increase transcript levels. These results suggest that TNFα induces its own expression without upregulating its receptor. The effects of TNFα on its receptor gene expression seem to be tissue-specific. In rat tracheal epithelial cells, TNFα downregulates TNF-R1 mRNA levels (Bader & Nettesheim 1996). In contrast, this cytokine increases TNF-R1 transcripts in rat oligodendrocytes (Dopp et al. 1997). Finally, human TNFα, which binds to murine TNF-R1 but not to TNF-R2 (Lewis et al. 1991), induced TNFα gene expression, indicating that TNF-R1 is involved in autoinduction of TNFα in FRTL-5 cells.

In summary, we demonstrate that TNFα induces its own expression in FRTL-5 cells. Although the biological significance of this phenomenon remains to be elucidated, our results suggest that thyrocyte-produced TNFα may regulate immune and inflammatory reactions in the thyroid and thus may be involved in the development of autoimmune thyroiditis such as Hashimoto’s thyroiditis. Furthermore, inhibition of TNFα production in the thyroid may represent a novel approach to mitigating inflammation in autoimmune thyroiditis.

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