EXPERIMENTAL STUDY

Prevention of lymphocytic thyroiditis in iodide-treated non-obese diabetic mice lacking interferon regulatory factor-1

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Abstract

Objective: Interferon regulatory factor-1 (IRF-1) is a critical regulator of interferon- γ (IFN γ)-mediated immune responses. To determine whether IRF-1 is involved in the pathogenesis of thyroiditis in animal models, we evaluated the incidence of iodide-induced lymphocytic thyroiditis (LT) in non-obese diabetic (NOD) mice lacking IRF-1 as well as IRF-1 +/+ and +/- mice.

Design: IRF-1 +/+, +/- and -/- NOD mice at 6 weeks of age were fed water (group 1) or iodide water (group 2) for 8 weeks.

Methods: Thyroids were examined histopathologically and intrathyroidal lymphocytic infiltration was arbitrarily graded. Serum thyroxine (T₄) and anti-mouse thyroglobulin antibody (anti-mTgAb) levels were measured. Spleen cell population was analyzed by flow cytometry, and IFN γ and interleukin-10 produced by splenocytes were measured by enzyme-linked immunosorbent assay.

Results: In group 1, only 4.3% of NOD mice developed LT. In contrast, 67.6% of mice in group 2 developed the disease. Iodide treatment induced LT in more than 80% of IRF-1 +/+ and +/- mice. However, no IRF-1 -/- mice in group 2 developed LT. There was no difference in both serum anti-mTgAb and T₄ levels among the three IRF-1 genotypes of NOD mice. Numbers of splenic CD8⁺ T cells and IFN γ production by Concanavalin A-stimulated splenocytes were markedly decreased in IRF-1-deficient NOD mice.

Conclusions: IRF-1 is involved in the development of iodide-induced LT in NOD mice.

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Introduction

Hashimoto's thyroiditis (HT) is an autoimmune disease characterized by a lymphocytic infiltration in the thyroid and the presence of autoantibodies to thyroid peroxidase and thyroglobulin (Tg), resulting in the destruction of follicles and loss of thyroid function (1). A series of studies has revealed aberrant expression of class II major histocompatibility complex (MHC) antigens as well as enhanced expression of a variety of cytokines such as interferon (IFN)- γ and adhesion molecules in the thyroids of patients with HT (2–4). It has been clearly shown that IFN γ induces class II MHC antigens and intercellular adhesion molecule-1 (ICAM-1) in cultured human thyrocytes and FRTL-5 rat thyroid cells (5–8). In addition, IFN γ upregulates class II MHC antigens on antigen-presenting cells (APC) and potentiates antigen presentation to T cells by APC (9, 10). Taken together, IFN γ may play an important role in the development of autoimmune thyroid disease (AITD) including HT.

Transcription factor interferon regulatory factor-1 (IRF-1) regulates the expression of IFN-inducible genes such as MHC class II antigens, ICAM-1, class II transactivator (CIITA) and inducible nitric oxide synthase (11–14). In addition, IRF-1 is involved in the differentiation of CD8⁺ T lymphocytes (15, 16) and development of the T helper 1 (Th1) immune response (17, 18). These findings suggest that IRF-1 is a critical regulator of IFN γ -mediated immune responses and thus may be involved in the pathogenesis of autoimmune diseases. In fact, targeted disruption of the IRF-1 gene in mice results in reduced incidence and severity of collagen-induced arthritis (19). Recently, we have shown that IFN γ induced IRF-1 gene expression in FRTL-5 rat thyroid cells (20). Taken together, IRF-1 may play a role in the pathogenesis of AITD. We have recently consistently demonstrated that IRF-1-deficient non-obese diabetic (NOD) mice did not develop insulinitis and diabetes (21). However, it remains unknown as to whether IRF-1 is involved in the development of AITD.

NOD mice spontaneously develop lymphocytic thyroiditis (LT) as well as insulinitis, thus they are often utilized as a model of HT in the human (22). Iodide treatment results in an increase in the severity and frequency of LT in NOD mice (23). In the present study, we examined the role of IRF-1 in the development of LT using iodide-treated IRF-1 $+/+$, $+/-$ and $-/-$ NOD mice.

Materials and methods

Mice

NOD mice lacking the IRF-1 gene were generated and characterized as previously described (21). Mice were genotyped by PCR analysis of tail DNA. IRF-1 $+/+$ and $-/-$ mice are fertile and grow as normally as IRF-1 $+/+$ mice. They were bred and maintained in our animal facility under specific pathogen-free conditions. This study was approved by the institutional review board of Tohoku University School of Medicine.

Iodide treatment

Mutant IRF-1 heterozygous and homozygous NOD mice as well as wild-type mice were randomized into two groups at 6 weeks of age and received tap water (group 1) or 0.05% iodide water (0.64 g NaI/l; group 2) for 8 weeks (24).

Histopathology

Thyroids were removed, fixed in 4% phosphate-buffered paraformaldehyde solution, and embedded in paraffin. Six to ten 4 μ m-thick sections were prepared in a non-contiguous way and stained with hematoxylin and eosin. Lymphocytic infiltration was arbitrarily graded as previously described, based on the percentage of thyroid infiltrated (25): 0 = normal thyroid; 1 = less than 10% lymphocytic infiltration of the thyroid; 2 = 10–30% lymphocytic infiltration; 3 = 30–50% lymphocytic infiltration; 4 = greater than 50% lymphocytic infiltration. The histological specimens were interpreted by two investigators in a blind fashion. The total score for each mouse was divided by the number of observations for that mouse.

Anti-mouse thyroglobulin antibody

Mouse Tg (mTg) was obtained and purified as previously described (26, 27). Serum anti-mTg antibody (Ab) was measured in duplicate in one assay by a modification of an enzyme-linked immunosorbent assay (ELISA) as previously described (25, 28). In brief, 96-well plates (Becton Dickinson and Co., Oxnard, CA, USA) were coated with mTg at a concentration of 10 μ g/ml in 50 mM carbonate buffer, pH 9.6, overnight at 4 °C. Wells were blocked by a 30-min incubation at

room temperature with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (BSA-PBS). Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated at 4 °C overnight with serum samples diluted 1:100 in BSA-PBS. Plates were again washed with PBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham Pharmacia Biotech, Tokyo, Japan) diluted 1:5000 in BSA-PBS. Plates were washed with PBS-T and color reaction was developed with 3,3',5,5'-tetramethylbenzidine solution (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was stopped by the addition of 0.5 mol/l H_2SO_4 . Optical density (OD) was read at 450 nm. Results are expressed as absorbancy minus the reagent blank.

Serum thyroxine concentrations

Serum thyroxine (T_4) was measured by enzyme immunoassay with reagents supplied by Dainabot Co. (Tokyo, Japan).

Flow cytometry

Spleen cells were analyzed for surface expression of CD3, CD4, CD8 and B220 by flow cytometry using a FACSCalibur and the CellQuest software (Becton Dickinson, Mountain View, CA, USA) as previously described (21). Ten thousand cells were counted. FITC-conjugated antibodies to CD3 and B220 were obtained from Caltag (South San Francisco, CA, USA) and APC-conjugated anti-mouse CD4 and PerCP-labeled anti-mouse CD8 were obtained from Pharmingen (San Diego, CA, USA).

In vitro proliferation and cytokine assay

As previously described (29), spleen cells were cultured in flat-bottomed, 96-well tissue culture plates at a concentration of 5×10^5 cells/well in the presence or absence of mTg (40 μ g/ml) or Concanavalin A (Con A; 1 μ g/ml; Sigma Chemical Co., St Louis, MO, USA). Culture medium consisted of RPMI 1640 (ICN Biochemicals, Inc., Aurora, OH, USA) with 10 mmol/l HEPES buffer supplemented with 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 50 μ mol/l 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum. Following 66 h of incubation, 1 μ mol/l bromodeoxyuridine (BrdU; Amersham Pharmacia Biotech) was added, and cells were further incubated for 6 h. BrdU incorporated into spleen cells was determined using an ELISA kit (Amersham Pharmacia Biotech). IFN γ and interleukin-10 (IL-10) levels in 72-h supernatants were measured using ELISA kits (Bio-source International, Camarillo, CA, USA).

Statistical analysis

The incidence of LT was tested by Fisher's exact probability test. The severity of LT was compared by the Mann–Whitney U test. Serum anti-mTgAb levels are shown as the median (range) and were compared by one-way ANOVA followed by the Kruskal–Wallis test. The other data are shown as the mean $\pm$ S.D. and were compared by unpaired Student's *t*-test or one-way ANOVA, followed by Bonferroni test. A level of $P < 0.05$ was considered statistically significant.

Results

As summarized in Table 1, only one IRF-1 +/– mouse developed LT at 14 weeks of age in group 1 (1/23, 4.3%). In contrast, iodide administration for 8 weeks induced LT in 23 of 34 mice (67.6%) in group 2 ($P < 0.01$ vs group 1). While more than 80% of IRF-1 +/+ and +/– NOD mice developed LT, –/– mice did not develop LT ($P < 0.01$; Fig. 1). The severity of LT was not significantly different between IRF-1 +/+ and +/– mice (Fig. 2). There were no significant differences between males and females in the incidence and severity of LT (data not shown).

Iodide treatment did not affect serum anti-mTgAb levels (Table 1). In contrast, it led to a significant decrease in serum T_4 levels in group 2 (Table 1; $P < 0.05$). There was no difference in both serum anti-mTgAb and T_4 levels among three IRF-1 genotypes of NOD mice (Table 1).

Since IRF-1 plays an important role in the differentiation of CD8⁺ T cells and development of the Th1 immune response (15–18), we analyzed the spleen cell population in IRF-1 +/+ and –/– NOD mice by flow cytometry. As shown in Table 2, there was a selective reduction of CD8⁺ T cells in the spleen of IRF-1-deficient mice, resulting in an increase in CD4⁺ T cell number and a marked increase in CD4/CD8 ratio. Lack of IRF-1 did not lead to a change in B cell (B220-positive cell) numbers. Iodide treatment did not affect the splenocyte populations in both IRF-1 +/+ and –/– NOD mice. There was no difference in total

Figure 1 Hematoxylin and eosin staining of NOD mouse thyroids. (A) Thyroid with no lymphocytic infiltration (IRF-1 –/– in group 2) and (B) thyroid with lymphocytic infiltration (IRF-1 +/+ in group 2). Original magnification $\times 200$.

spleen cell numbers between IRF-1 +/+ and –/– NOD mice (data not shown).

ConA, but not mTg, stimulated proliferation of splenocytes from both IRF-1 +/+ and –/– mice (data not shown). In IRF-1-deficient NOD mice, IFN γ production by ConA-stimulated splenocytes was significantly reduced compared with that in IRF-1 +/+

Table 1 Incidence of lymphocytic thyroiditis and serum levels of anti-mTgAb and T_4 of NOD mice in groups 1 (water) and 2 (iodide water). Data shown are means $\pm$ S.D. for T_4 or median (range) for mTgAb.

Group		IRF-1 +/+	IRF-1 +/-	IRF-1 -/-
1	No.	4	15	4
	Incidence of thyroiditis (%)	0	6.7 (1/15)	0
	mTgAb (OD: 450 nm)	0.24 (0.14–0.28)	0.24 (0.08–0.86)	0.18 (0.15–0.49)
	T_4 (μ g/dl)	4.7 $\pm$ 0.3	4.2 $\pm$ 0.5	4.4 $\pm$ 1.0
2	No.	8	19	7
	Incidence of thyroiditis (%)	87.5 (7/8)	84.2 (16/19)	0*
	mTgAb (OD: 450 nm)	0.26 (0.10–0.84)	0.20 (0.07–2.24)	0.27 (0.04–0.46)
	T_4 (μ g/dl)	3.7 $\pm$ 0.6**	3.7 $\pm$ 0.7**	3.7 $\pm$ 0.5**

The incidence of thyroiditis was tested by Fisher's exact probability test (* $P < 0.01$ vs IRF-1 +/+ or +/-). Serum mTgAb and T_4 levels were compared using one-way ANOVA, followed by Kruskal–Wallis test (mTgAb) or Bonferroni test (T_4) (** $P < 0.05$ vs group 1).

Figure 2 The severity of LT in each IRF-1 genotype of NOD mice treated with iodide (group 2). The severity of LT was classified as previously described (25), and compared by the Mann-Whitney U test. Each bar shows $\pm$ S.D. * $P < 0.05$ vs IRF-1 $-/-$ mice.

mice ($P < 0.05$; Fig. 3). Spleen cells did not produce IFN γ in response to mTg in both IRF-1 $+/+$ and $-/-$ NOD mice (data not shown). IL-10 production by ConA-stimulated splenocytes was not significantly different between IRF-1 $+/+$ and $-/-$ NOD mice (data not shown).

Discussion

In the present study, we have clearly demonstrated that iodide ingestion induces LT in IRF-1 $+/+$ and $+/-$ NOD mice but not in IRF-1 $-/-$ mice, indicating the involvement of IRF-1 in the development of iodide-induced LT in NOD mice. These findings are essentially identical to those in our previous study showing that

IRF-1 $-/-$ NOD mice do not develop autoimmune insulinitis and subsequent diabetes (21). Further, our results are consistent with a report by Tada *et al.* (19) demonstrating that targeted disruption of IRF-1 gene in mice results in reduced incidence and severity of collagen-induced arthritis. Taken together, IRF-1 plays an important role in the development of autoimmune diseases such as LT, insulinitis and arthritis.

Recent studies have demonstrated the presence of CD4 $^{+}$ T cells and CD8 $^{+}$ T cells in the thyroid of iodide-treated NOD mice (29, 30) and prevention of iodide-induced LT by anti-CD4 or anti-CD8 Ab in NOD mice (23, 29). Taken together, these results suggest that both CD4 $^{+}$ T cells and CD8 $^{+}$ T cells are required for the development of iodide-induced LT in NOD mice. In addition, a series of studies has suggested that predominance of Th1 immune reaction is associated with the development of autoimmune diabetes in NOD mice (31). Previous studies have demonstrated that lack of IRF-1 results in a marked decrease in the number of CD8 $^{+}$ T cells in the thymus and peripheral lymphoid organs and strongly impaired Th1 immune response (15–18). Consistently, we found a selective decrease in numbers of CD8 $^{+}$ T cells in the spleen of IRF-1-deficient NOD mice. Further, we have observed a marked decrease in IFN γ production, but not in IL-10 production, by ConA-stimulated splenocytes in IRF-1 $-/-$ mice. Taken together, the possibility that a decrease in the number of CD8 $^{+}$ T cells and impaired Th1 immune reaction may be associated with the prevention of iodide-induced LT in IRF-1-deficient mice cannot be excluded. However, further studies are

Figure 3 IFN γ produced by ConA-stimulated spleen cells of NOD mice. IFN γ levels in culture medium were evaluated using ELISA kits, and compared by the unpaired Student's *t*-test. Each bar shows $\pm$ S.D. * $P < 0.05$ vs IRF-1 $+/+$ mice.

Table 2 Splenic cell population proportions in IRF-1 $+/+$ ($n = 5$) and $-/-$ ($n = 3$) NOD mice analyzed by flow cytometry. Values are means $\pm$ S.D.

Group	IRF-1	CD4	CD8	CD4/CD8	B220
1	$+/+$	73.3 ± 2.4	18.1 ± 1.9	4.1 ± 0.4	27.4 ± 4.0
	$-/-$	$84.9 \pm 5.3^*$	$5.1 \pm 1.6^*$	$17.4 \pm 4.1^*$	27.9 ± 6.3
2	$+/+$	77.3 ± 4.0	17.9 ± 3.1	4.4 ± 0.8	30.8 ± 2.7
	$-/-$	$91.3 \pm 3.1^*$	$4.4 \pm 1.1^*$	$21.7 \pm 5.4^*$	28.7 ± 3.0

* $P < 0.01$ vs $+/+$ mice (unpaired Student's *t*-test).

clearly required to elucidate the involvement of CD8⁺ T cells and Th1 immune reaction in iodide-induced LT in NOD mice.

IFN γ is an immunomodulatory cytokine which is suggested to be involved in the pathogenesis of AITD. For instance, the onset of experimental autoimmune thyroiditis (EAT) was prevented by the treatment of mice with neutralizing anti-IFN γ Ab (32). However, recent studies demonstrated that mice lacking either IFN γ or IFN γ receptor gene can develop EAT (33, 34). Thus, the importance of IFN γ in the pathogenesis of AITD remains a matter of controversy. In addition to IFN γ and its receptor, signal transducers involved in the IFN γ -activated pathway must be examined to obtain further insight on the role of IFN γ in AITD. Using NOD mice, we have now clearly demonstrated that IRF-1, an IFN-inducible transcription factor, plays an essential role in the development of iodide-induced LT for the first time. Similarly, NOD mice lacking CIITA, a transcription factor involved in MHC class II expression and peripheral CD4⁺ T cell development, develop pancreatic infiltrates, but do not develop diabetes (35). Thus, NOD mice lacking IFN γ -activated transcription factors seem to be quite useful in elucidating the role of the IFN γ -activated signaling system in the pathogenesis of iodide-induced LT.

In accordance with previous studies in NOD mice and BioBreeding/Worcester rats (23, 25), iodide ingestion did not affect serum anti-mTgAb levels. In contrast, iodide treatment resulted in a decrease in serum T₄ levels in NOD mice. Our results are quite different from previous studies showing that serum T₄ levels are not affected by iodide administration in NOD mice (28). The reason for such a discrepancy is unclear. Development of LT is unlikely to be involved in our findings since lowered T₄ was demonstrated even in IRF-1-deficient mice which did not develop LT. There was no difference in both serum anti-mTgAb and T₄ levels among IRF-1 +/+ , +/- and -/- NOD mice, suggesting that IRF-1 deficiency neither induces anti-mTgAb production nor affects thyroid hormone production and/or metabolism in NOD mice.

In summary, we have demonstrated for the first time that IRF-1 plays a critical role in the development of iodide-induced LT in NOD mice.

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