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Pancreatic β Cell–specific Expression of Thioredoxin, an Antioxidative and Antiapoptotic Protein, Prevents Autoimmune and Streptozotocin-induced Diabetes

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Summary
The cytotoxicity of reactive oxygen intermediates (ROIs) has been implicated in the destruction of pancreatic β cells in insulin-dependent diabetes mellitus (IDDM). Thioredoxin (TRX), a redox (reduction/oxidation)-active protein, has recently been shown to protect cells from oxidative stress and apoptosis. To elucidate the roles of oxidative stress in the development of autoimmune diabetes in vivo, we produced nonobese diabetic transgenic mice that overexpress TRX in their pancreatic β cells. In these transgenic mice, the incidence of diabetes was markedly reduced, whereas the development of insulitis was not prevented. Moreover, induction of diabetes by streptozotocin, an ROI-generating agent, was also attenuated by TRX overexpression in β cells. This is the first direct demonstration that an antioxidative and antiapoptotic protein protects β cells in vivo against both autoimmune and drug-induced diabetes. Our results strongly suggest that oxidative stress plays an essential role in the destruction of β cells by infiltrating inflammatory cells in IDDM.

Key words: oxidative stress • nonobese diabetic mouse • insulitis • cytokine • apoptosis

Insulin-dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of insulin-producing pancreatic β cells (1). By the clinical onset of this disease, β cells are almost completely gone, requiring affected individuals to inject insulin daily to sustain their lives. Nonobese diabetic (NOD) mice spontaneously develop autoimmune diabetes with remarkable similarity to human IDDM (2; for a review, see reference 3). A common pathological feature is the infiltration of inflammatory cells into the islets, termed insulitis, followed by the selective destruction of β cells. Infiltrating cells are composed of T and B lymphocytes, macrophages, and N K cells, among which T cells and macrophages are believed to play the major role in β cell destruction. However, the molecular mechanisms of β cell destruction by these infiltrating cells are poorly understood. Elucidation of these mechanisms is important not only for understanding the pathogenesis of IDDM and other autoimmune diseases, but also for developing effective means of preventing these diseases. Several reports have suggested that locally produced reactive oxygen intermediates (ROIs) are involved in these effector mechanisms of β cell destruction (4–9). In vitro, ROIs such as nitric oxide (NO), hydroxyl radical (OH·), hydrogen peroxide (H2O2), and superoxide radical (O2−·) were shown to be induced in β cells by cytokines such as IL-1, IFN-γ, and TNF-α, which are known to be secreted by T cells and macrophages that infiltrate the islets (5–9). ROIs, either administered exogenously or induced in β cells by cytokines, have been shown to cause the destruction of β cells in vitro, and apoptosis has recently been suggested to be the mechanism involved (10–14). Among endocrine cells in pancreatic islets, insulin-producing cells appear particularly vulnerable to oxidative stress, probably due to their low levels of key enzymes that scavenge ROIs (15–19). Therefore, the targeted expression of antioxidative and antiapoptotic molecules in pancreatic β cells would be expected to protect them from destruction and to prevent the development of IDDM.

Thioredoxin (TRX) is a low molecular mass (~12 kD) redox (reduction/oxidation)-active protein found in both prokaryotic and eukaryotic cells (for a review, see reference...
This protein has a conserved active site containing cysteine residues that are reversibly reduced by the NADPH-dependent seleno-flavoprotein, thioredoxin reductase (21). 

In both models, indicating the involvement of oxidative stress. (Fig. 1) consisted of the human insulin promoter (35), the rabbit β-globin gene sequences from the second exon to the polyadenylation signal, and human TRX cDNA inserted at the unique EcoRI site in the β-globin sequences.

Generation of NOD Transgenic Mice. The Ins-TRX transgene (Fig. 1) consisted of the human insulin promoter (35), the rabbit β-globin gene sequences from the second exon to the polyadenylation signal, and human TRX cDNA inserted at the unique EcoRI site in the β-globin sequences.

To directly assess the implications of oxidative stress in pancreatic β cell destruction in autoimmune diabetes, we have generated NOD transgenic mice with targeted overexpression of the antioxidative protein, TRX, in pancreatic β cells. The protective effect of TRX against β cell destruction was also tested in experimental diabetes induced by the non-β-cell-generating agent, streptozotocin (STZ). Pancreatic β cells were significantly protected from destruction in both models, indicating the involvement of oxidative stress and apoptosis in the destruction of β cells in autoimmune as well as STZ-induced diabetes.

Materials and Methods

Construction of the TRX Transgene. The Ins-TRX transgene (Fig. 1) consisted of the human insulin promoter (35), the rabbit β-globin gene sequences from the second exon to the polyadenylation signal, and human TRX cDNA inserted at the unique EcoRI site in the β-globin sequences.

Generation of NOD Transgenic Mice. The Ins-TRX transgene cassette was purified and microinjected into the pronuclei of one-cell embryos of NOD mice (CLEA Japan Inc., Tokyo, Japan) as described previously (36). Founders (F0) of generated NOD transgenic mice were identified by PCR analysis of tail DNA. The primers used for transgenic mouse screening were as follows: forward 5'-GCTGGTTATTGTGCTGTCTC'-3' and reverse 5'-TCATCCACATCTCTCAAGGA-3' (see Fig. 1). To standardize the amount of mRNA in each sample, RT-PCR of hypoxanthine-guanine phosphoribosyl transferase (hprt) mRNA was performed in parallel. The primers for hprt mRNA detection were as follows: forward 5'-CTCGAAGTTGTTGATACAGG-3' and reverse 5'-TG GCCCTATAGGCTCATTAGT-3'. These primers were designed to encompass intron sequences so that the possible PCR products from the contaminating genomic DNA could be distinguished.

Isolation of Pancreatic Islets. 8-wk-old (NOD × B6) F1 transgenic mice, their nontransgenic littermates, and BALB/c mice (CLEA Japan Inc.) were anesthetized with an intraperitoneal injection of pentobarbital. Pancreatic islets were isolated by stationary collagenase digestion of the pancreas, followed by ficoll 400 (Amerham Pharmacia Biotech, Uppsala, Sweden) density gradient purification (38).

Western Blotting. Isolated islets and dissected pancreata were lysed in lysisbuffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, and 1% NP-40) with a protease inhibitor, PMSE, and were appropriately diluted with cold PBS for immunoblotting. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The lysates were incubated at 100°C for 5 min with a third volume of the sample buffer (75 mM Tris-HCl, pH 6.8, 6% SDS, 15% glycerol, 15% 2-ME, and 0.015% bromophenol blue). These samples were subjected to electrophoresis on an SDS/15% polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). To assess the levels of human TRX expression, known amounts of recombinant human TRX (>95% pure) prepared from human TRX-producing Esherichia coli (39) were simultaneously loaded on the gel. Mouse anti-human TRX mAb provided by Fujirebio Inc. (Tokyo, Japan) was used as a probe to detect human TRX. Endogenous murine TRX was detected using rabbit anti-murine TRX polyclonal Ab raised against a synthetic polypeptide of the COOH-terminal 10 amino acid residues of murine TRX. This Ab recognized murine TRX but not human TRX (I. Yodoi, unpublished data). The second Ab was rabbit anti–mouse IgG Ab or goat anti–rabbit IgG Ab conjugated with horseradish peroxidase (both from Zymed Laboratories Inc., South San Francisco, CA). Human and murine TRX were visualized using an ECL detection kit (Amerham Pharmacia Biotech).

Insulin Secretion for Islets. Isolated islets of 12-wk-old (NOD × B6) F1 transgenic mice (line 90, female) and their nontransgenic littermates were measured for insulin secretion according to the following method (37).

Figure 1. The construction of the Ins-TRX transgene. The transgene contains the insulin promoter, an intron from the rabbit β-globin gene, human TRX cDNA, and a poly A signal from the rabbit β-globin gene. The DNA positions to which the primers for RT-PCR analysis anneal are shown.
batch incubation method (40). Each batch of 10 islets was incubated at 37°C for 3 h in Krebs-Ringer solution containing 0.3% BSA with 3 different glucose concentrations. Five batches of transgenic and nontransgenic islets were simultaneously tested at each glucose concentration (5, 10, and 25 mM). After removal of the islets, the insulin concentration of each batch was determined using a mouse insulin ELISA kit (Seikagaku Corp., Tokyo, Japan).

Diagnosis of Diabetes. Female NOD transgenic mice (F2, F3) and their nontransgenic littermates were monitored from 12 wk of age with weekly measurements of urine glucose using TesTape (Eli Lilly and Company, Indianapolis, IN). When mice were glucosuric for two consecutive weeks, they were diagnosed as diabetic.

Evaluation of Insulitis. Pancreata were removed from 12-wk-old female NOD transgenic mice (line 90, n = 10) and their TRX-negative littermates (n = 8), and were fixed with 20% formalin. After paraffin-embedded fixation, 3-μm slices of each pancreas were prepared for hematoxylin and eosin staining. 25 islets of each pancreas were randomly chosen for microscopic examination of insulitis. According to the classification system of Kurasa et al. (41), each islet was scored as follows: 0, an islet without infiltration; 1, <25% area of an islet was occupied by immunocytic infiltrates; 2, 25% to <50%; 3, ≥50%.

Production of STZ-induced Diabetic Mice. STZ (Wako Pure Chemical Industries Ltd., Oaka, Japan) was dissolved in 0.05 M citrate buffer (pH 4.5; reference 42), and immediately injected intraperitoneally into 8-wk-old (NOD × B6) F1 transgenic mice and their TRX-negative littermates at a dose of 250 mg/kg. These islets had been starved for 18 h before STZ treatment, and were then fed ad libitum.

Measurements of Blood Glucose Levels and Pancreatic Insulin Content. 7 d after STZ injection, blood sugar levels of (NOD × B6) F1 transgenic mice and their TRX-negative littermates were measured with Glucotest (KD Korp., Kyoto, Japan), whose range of measurable blood glucose concentrations is between 40 and 500 mg/dl. 10 d after STZ treatment, pancreata were removed from 6-h-starved (NOD × B6) F1 transgenic mice and their TRX-negative littermates. Pancreatic insulin was extracted according to the acetic ethanol method (43), and the extracted insulin was dissolved in cold PBS. Insulin concentration was determined using a mouse insulin ELISA kit. Insulin content was indicated as the amount of pancreatic insulin (in nanograms) divided by the pancreatic wet weight (in milligrams).

Statistical Analysis. Data were expressed as mean ± SD or mean ± SEM. Statistical analysis was performed by Mann-Whitney U test, unpaired t test, and Kaplan-Meier methods using StatView version 4.5 for the Macintosh (Abacus Concepts Inc., Berkeley, CA).

Results

Transgenic Mice Overexpressing Human TRX. The Ins-TRX transgene containing a human TRX cDNA under the human insulin promoter (Fig. 1) was microinjected into fertilized eggs of NOD mice to generate NOD transgenic mice. In the F1 generation, PCR analysis of tail DNA showed that the Ins-TRX transgene was inherited by three NOD transgenic strains, lines 21, 48, and 90. The expression of human TRX mRNA was detected exclusively in the pancreas of transgenic mice using RT-PCR analysis (Fig. 2). For the quantification of human TRX expression, islets were isolated from (NOD × B6) F1 transgenic mice, which were produced by intercrossing female B6 mice with male NOD transgenic mice of each strain. Western blot analysis using a human TRX-specific mAb as probe showed a high level of human TRX expression in the transgenic islets of lines 21 and 90 (Fig. 3, top). For this analysis, 15 μg of islet protein was loaded on each lane. By comparing the densities of these bands with those of known amounts of recombinant human TRX (Fig. 3, top), the ratio of human TRX to total protein in the islets was estimated to be 1.7 ng/μg in both lines 21 and 90. Al-
though human TRX was not detectable in the islets of line 48, prolonged exposure of the membrane revealed a low-level expression of human TRX in these mice (not shown). The expression of endogenous murine TRX was also examined by Western blot analysis using rabbit anti-mouse TRX serum as the probe, which was not reactive with human TRX. The levels of endogenous TRX expression were found to be much lower in the islets than in the pancreas (Fig. 3, bottom).

Insulin Secretory Capacities of Transgenic Islets. We next examined whether transgenic TRX expression in β cells affects glucose-responsive insulin secretion. The insulin secretory capacities of (NOD × B6) F1 transgenic islets at three glucose concentrations (5, 10, and 25 mM) were 0.40 ± 0.05, 1.02 ± 0.08, and 2.76 ± 0.15 ng/h/islet (mean ± SD), respectively. The insulin secretory capacities of their TRX-negative littermates were 0.43 ± 0.03, 1.04 ± 0.07, and 2.83 ± 0.13 ng/h/islet, respectively. There was no significant difference between the results from the islets of transgenic mice and those of their TRX-negative littermates at any glucose concentration (unpaired t test, NS for all), indicating that the overexpression of TRX did not affect the insulin secretory capacity of β cells.

Incidence of Diabetes. To examine the effect of TRX expression on the development of diabetes, female NOD transgenic mice and their female TRX-negative littermates were monitored for glucosuria up to 32 wk of age. In transgenic mice of lines 21 and 90, the cumulative incidence of diabetes was markedly reduced at 32 wk of age (Fig. 4). Statistical analysis demonstrated a significant difference between NOD transgenic mice and their TRX-negative littermates (Kaplan-Meier method, P < 0.01 for both), but not in line 48 (NS).

Insulitis Score. To determine whether TRX overexpression affects the development of insulitis in pancreatic islets, we performed histochemical analyses to determine the degree of the insulitis in NOD transgenic mice and their TRX-negative littermates before the onset of overt diabetes. The average insulitis scores of 12-wk-old female NOD transgenic mice (line 90) and their female TRX-negative littermates were 1.63 ± 0.32 and 1.57 ± 0.26 (mean ± SEM), respectively, indicating that the transgenic TRX overexpression does not attenuate the development of insulitis.

Blood Glucose Levels and Insulin Content after STZ Injection. We next examined whether TRX overexpression confers resistance to STZ-induced diabetes. In this experiment, we used (NOD × B6) F1 transgenic mice and their nontransgenic littermates to avoid the complications in interpretation that could arise from the autoimmune diabetes accompanying the NOD background. No evidence of insulitis was observed in these mice. Before STZ treatment, fasting blood glucose levels were 80.0 ± 5.7 mg/dl for transgenic mice (n = 9) and 77.6 ± 9.7 mg/dl for their TRX-negative littermates (n = 9). 7 d after STZ injection, blood glucose levels were >500 mg/dl in most of the nontransgenic mice, but were only moderately elevated in TRX transgenic mice, as shown in Fig. 5a. There was a significant difference in blood glucose levels between the transgenic mice and their TRX-negative littermates. We

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Cumulative incidence of diabetes in each NOD transgenic strain. Mice were monitored with weekly measurements of urine glucose. The number (n) of animals tested is shown in each figure. Statistical analysis demonstrated a significant difference between transgenic mice and their nontransgenic littermates in lines 90 and 21 (Kaplan-Meier method, P < 0.01 for both), but not in line 48 (NS).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Alteration of blood glucose levels and insulin content after STZ treatment. STZ (250 mg/kg) was injected intraperitoneally into 8-wk-old (NOD × B6) F1 mice after an 18-h starvation period. (a) 7 d after injection, the blood glucose concentration was measured from mice fed ad libitum. When the concentration surpassed 500 mg/dl, the upper limit of measurement, its value was indicated as >500 mg/dl. There was a significant difference between (NOD × B6) F1 transgenic mice (line 90, male; n = 9) and their TRX-negative littermates (male, n = 9) (Mann-Whitney U test, P < 0.05). (b) Pancreata were excised from 6-h-starved (NOD × B6) F1 transgenic mice (line 21, female; n = 5) and their TRX-negative littermates (female, n = 5) 10 d after STZ injection. Pancreatic insulin contents of transgenic mice were significantly higher than those of their TRX-negative littermates (unpaired t test, P < 0.05).
next examined the insulin content of the pancreas of STZ-treated transgenic and nontransgenic littersmates after subjecting them to a 6-h fast (Fig. 5 b). Pancreatic insulin content was significantly higher in the transgenic mice (21.8 ± 9.4 ng/mg pancreatic weight; n = 5) than in their TRX-negative littersmates (8.2 ± 4.2 ng/mg; n = 5). Without STZ treatment, insulin content was the same for the transgenic mice (66 ± 7.0 ng/mg; n = 5) and their TRX-negative littersmates (63.0 ± 8.6 ng/mg; n = 5). These results clearly indicate that human TRX overexpression protects β cells from the cytotoxicity caused by STZ administration.

**Discussion**

IDDM is characterized by the infiltration of lymphocytes into the pancreatic islets followed by the destruction of β cells, which leads to overt diabetes. However, it is not well understood how β cells are destroyed by the immunocytes in islets. The coexistence of various cell types and their complex interactions in the inflammatory islets make it very difficult to study the mechanism of β cell destruction in vivo. To overcome this complexity, we adopted a strategy in which molecules that are assumed to affect β cell destruction are directly tested by overexpressing them specifically in β cells in vivo. In this study, we have generated NOD transgenic mice that express human TRX, which has strong antioxidative and antiapoptotic activity (20, 26). Analysis by RT-PCR demonstrated that human TRX was expressed exclusively in the islets of our transgenic mice. Immunoblot analysis showed that the ratio of human TRX to total protein of the transgenic islets was ∼1.7 ng/µg (Fig. 3, top). The endogenous TRX levels of normal organs are less than ∼0.3 ng/µg (I. Yodoi, unpublished data). Therefore, the TRX levels in transgenic islets are considerably higher than those of normal tissues. An excessive expression of TRX may be toxic to cells, as pointed out by Baker et al. (44). However, we consider this an unlikely problem in our transgenic mice because endogenous TRX expression is highly susceptible to oxidative stress. Recent studies have indicated a stepwise development of autoimmune diabetes, with insulitis the first phase and massive destruction of β cells the second (48). We showed that there was no significant difference in the severity of insulitis between NOD transgenic mice and their TRX-negative littersmates, indicating that overexpression of TRX did not prevent insulitis, but rather attenuated the cytotoxicity of the infiltrating immunocytes in islets. This result is consistent with the function of TRX as an antioxidative protein. It has also been reported that IDDM develops without insulitis in transgenic mice overexpressing inducible NO synthase (iNOS) in β cells (49), probably through the increased oxidative stress due to NO production. This report and our present study together suggest that oxidative stress is one of the major effector mechanisms of β cell destruction by infiltrating inflammatory cells.

Recently, apoptosis has also been suggested as a pivotal mechanism of β cell destruction in autoimmune (10–13) and drug-induced diabetes (14, 50). The Fas/FasL system has been implicated in β cell apoptosis. NOD transgenic mice that express FasL in their β cells were reported to be highly susceptible to autoimmune diabetes (12), whereas Fas-deficient NOD−/−lpr/lpr mice were resistant to autoimmune diabetes (12, 13). Recombinant TRX has been reported to protect cells against apoptosis mediated by both TNF and anti-Fas agonistic Ab (51). Very recently, marmalian TRX has been shown to function as a direct inhibitor of apoptosis signal-regulating kinase (ASK)-1 (52). These results suggest that the antiapoptotic function of TRX may also participate in the protection of β cells after immunocytic infiltration.

Although β cell–targeted overexpression of TRX effectively suppressed the incidence of autoimmune diabetes in NOD mice, a low percentage of transgenic mice still developed diabetes with significantly later onset. At present, we can only speculate on the molecular mechanisms causing diabetes in these mice. It is probable that TRX could not confer complete resistance to the oxidative stress and β cell death caused by infiltrating cells. Alternatively, other secondary mechanisms for β cell destruction, such as the perforin-dependent cytotoxicity of CD8+ T cells (53), might have led to the autoimmune diabetogenic β cell loss.

In human IDDM, overt diabetes develops when β cells are almost completely destroyed. Subjects at high risk for the future development of IDDM can only be identified by the presence of autoantibodies, which indicate the presence of β cell destruction.
of autoimmunity and probable ongoing β cell destruction. Therefore, these effects of TRX overexpression suggest a new approach to protecting subjects in the second phase of IDDM or to delaying the development of overt diabetes.

This study provides the first direct evidence that an antioxidative and antiapoptotic protein has protective effects on autoimmune diabetes. Our results strongly suggest that oxidative stress plays an essential role in β cell destruction caused by inflammatory cells in islets. The results also suggest an antioxidative and antiapoptotic strategy as a promising approach for the prevention of and intervention in IDDM in particular, as well as other autoimmune diseases in general, in subjects with ongoing autoimmunity.

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