Transgenic Mice Overexpressing Type 2 Nitric-oxide Synthase in Pancreatic β Cells Develop Insulin-dependent Diabetes without Insulitis*

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We generated transgenic mice carrying the mouse type 2 nitric-oxide synthase (NOS2) cDNA under the control of the insulin promoter. Western and immunohistochemical analyses revealed that NOS2 was expressed abundantly in transgenic islets but not in control islets. When islets were isolated and cultured, high levels of nitrite were released from the transgenic islets. In transgenic mice, the β cell mass was markedly reduced without the infiltration of macrophages or lymphocytes, and extensive DNA strand breaks were detected in the islets by in situ nick translation. All the transgenic mice developed hypoinsulinemic diabetes by 4 weeks of age, and treatment with an inhibitor of NOS2, aminoguanidine (200 mg/kg body weight every 12 h), prevented or delayed the development of diabetes. The present study shows that the production of nitric oxide by β cell NOS2 plays an essential role in the β cell degeneration.

Insulin-dependent diabetes mellitus (IDDM) is caused by the degeneration of insulin-producing β cells in pancreatic islets (1–4). Nitric oxide (NO), first identified as a physiological signaling molecule, has been also shown to be a cytotoxic effector molecule when generated in high concentrations by type 2 NO synthase (NOS2) (5). In the process of IDDM, activated macrophages produce NO, which is thought to be cytotoxic to β cells, and NO, which is produced by β cell NOS2 induced by macrophage-derived cytokines such as interleukin-1β (IL-1β), is also thought to be involved in β cell degeneration (6). Although many in vitro studies (7–13) suggest that NO produced by cytokine-induced NOS2 can cause the degeneration of β cells, no in vivo study has clearly demonstrated the pathological significance of NO produced within β cells in the development of IDDM, because an infiltration of macrophages in islets always occurred in animal models of IDDM (6, 14). In this study, we produced transgenic mice expressing NOS2 constitutively in pancreatic β cells and found that the transgenic mice developed severe IDDM without macrophage or lymphocyte infiltration in and around islets.

EXPERIMENTAL PROCEDURES

Construction of Rat Insulin II Promoter/Mouse NOS2 Hybrid Gene—Islets were isolated from ICR mice by the collagenase digestion method (15) and cultured for 12 h in the presence of 150 units/ml IL-1β (Sigma). A mouse NOS2 cDNA was cloned by polymerase chain reaction (PCR) of reverse-transcribed RNA from IL-1β-stimulated islets. Primers used in the PCR reaction were 5′-TTCCGGAGACGAGCTCGAGCTCA-GAC-3′ and 5′-AAAGATGGCGGTCTCAGGCTCCTGCGCTT-3′; these sequences correspond to the nucleotides −25 to −1 and 3418 to 3444 of mouse NOS2 cDNA (16) and contain XmaI and BglII sites (underlined sequences), respectively. The cloned cDNA sequence was determined and was found to be exactly the same as the reported NOS2 sequence (16). To express NOS2 in β cells, the 0.7-kbp BamHI-XmaI fragment of the rat insulin II promoter (17), the 3.5-kbp XmaI-BglII fragment of mouse NOS2 cDNA containing the entire coding region, and the 1.6-kbp BglII-EcoRI fragment of the SV40 intron/polyadenylation signal (18) were ligated at the XmaI and BglII sites in the correct orientation. The resultant hybrid gene (5.8 kbp) separated from the pBlueScript SK(−) (Stratagene) by XpnI and NotI was used for microinjection.

Generation of Transgenic Mice—To generate transgenic mice, a DNA solution (2 µg/ml) was microinjected into the male pronuclei of fertilized eggs from BDF1 females as described (17, 19). Identification of transgenic mice was performed by PCR on genomic DNA. In the present study, the two diabetic transgenic lines, 31 and 40, were established. Maintenance on CD-1 mouse background, and analysis. Because the diabetes occurred in two independent lines of transgenic mice, the pathology was assumed to have resulted from the transgene expression rather than from the positional or insertional effects of the transgene.

Western Blot and Immunohistochemical Analyses—Pancreatic islets were isolated from ICR mice by the collagenase digestion method (15) and homogenized under nonreducing conditions (21). Insulin staining was carried out as described (17) using a mouse anti-insulin antibody (Novo, Bagsvaerd, Denmark) and rat insulin standards. The insulin levels in the islets were determined by using an insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards.

Measurement of Nitrile Release from Isolated Islets—Islets (50–200 islets, obtained from the male Sprague-Dawley rats) were isolated and cultured in medium containing 150 µl of RPMI 1640 medium without phenol red (Sigma) containing 16% fetal calf serum (JRH Biosciences, Lenexa, KS) and 11.1 mM glucose. Medium samples (50 µl) were removed at 12 and 24 h. Nitrate levels in the medium were analyzed by using Griess reagent (22).

Measurements of Glucose and Insulin—Blood and urinary glucose levels were determined by using Advantage equipment (Boehringer Mannheim) and Tes-tape assay (Lilly, Indianapolis, IN), respectively. Pancreatic extracts were obtained from the entire pancreas by acid–ethanol extraction (23). The insulin levels in the serum and pancreatic extracts were determined by using an insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards.

Pancreatin with Aminoguanidine—Aminoguanidine hemisulfate salt (Sigma) at 12 mg/ml in 0.9% NaCl solution was administered intraperitoneally at a dose of 200 mg/kg body weight every 12 h, beginning at 1 day of age.

Analyses of DNA Strand Breaks by Nick Translation—Pancreata
from 4-week-old mice were fixed in 4% paraformaldehyde and embedded in paraffin. In situ nick translation reaction was performed as described (9, 24), and the incorporated biotin-dUTP was visualized by peroxidase reaction. For analysis of DNA strand breaks by non-denaturing gel electrophoresis (9), genomic DNA was extracted from isolated islets by phenol-chloroform extraction. The DNA (25 ng) labeled by nick translation (25) using \([\alpha-32P]dCTP\) (Amersham Corp.) was electrophoresed on 1.2% agarose gel and autoradiographed. The incorporation of \([\alpha-32P]dCTP\) into the DNA was determined as described (25).

RESULTS AND DISCUSSION

Western blot analysis using an NOS2-specific antibody showed that the transgenic mouse (lines 31 and 40), but not the nontransgenic control mice, expressed NOS2 in the pancreatic islets (Fig. 1A). The NOS2 expression was not detected in other tissues such as brain, liver, kidney, and small intestine of the transgenic mice (Fig. 1B). NOS2 mRNA was detected only in the transgenic islets by Northern blot analysis (data not shown). In immunohistochemistry, islets of transgenic mice were stained for NOS2 (Fig. 2, B and C). The NOS2 expression in transgenic islets was observed from 1 week of age (data not shown). The proportion of insulin-producing cell mass to total pancreatic cell mass was markedly reduced in the transgenic mice (Fig. 2, D–G). On the other hand, islets of the control mice showed no immunoreactivity for NOS2, and the pancreatic exocrine cells showed no detectable staining for NOS2 in the transgenic and nontransgenic mice (Fig. 2, A–C).

We next incubated islets from transgenic and control mice and measured the nitrite content in the incubation medium, which is indicative of NO release (16). As shown in Fig. 3, high levels of nitrite were detected in a time-dependent manner in the medium containing transgenic islets, whereas the nitrite content in the control islet medium was under the limit of detection. The amount of nitrite in the medium of transgenic line 31 was almost equivalent to that of the medium containing IL-1β-stimulated control islets.

In the transgenic lines 31 and 40, the mice developed hyperglycemia from 1 week of age (Fig. 4A) and exhibited profound polydipsia/polyuria by 4 weeks of age. The blood glucose levels of both lines of transgenic mice were over 400 mg/dl, and the urine tested strongly positive (250-500 mg/dl) for glucose. The nontransgenic control mice had normal glucose levels (less than 200 mg/dl) and never showed glycosuria. The serum insulin levels of both lines of transgenic mice were over 400 ng/ml, and the urine tested strongly positive (250-500 mg/dl) for ketones; the presence of ketonuria indicated the severity of the diabetes. We followed the diabetes development every week from the first week of age by monitoring the blood glucose levels and pancreatic insulin contents. As shown in Fig.

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**Fig. 1.** Western blot analysis of homogenates from islet (A) and various tissues (B). A. Pancreatic islet homogenate (25 μg of protein/lane) from control nontransgenic mice (lane C) and transgenic lines 31 (lane 31) and 40 (lane 40) were subjected to Western blot analysis using anti-mouse NOS2 antibody. The positions of molecular mass markers are shown at the left in kDa. B. Lanes 1–3, brain; lanes 4–6, liver; lanes 7–9, kidney; lanes 10–12, small intestine; lane 13, colon from nontransgenic mouse 6 h after 10 mg/kg lipopolysaccharide injection (25 μg of protein/lane). Lanes 1, 4, 7, and 10, control; lanes 3, 5, 8, and 11, transgenic line 31; lanes 3, 6, 9, and 12, transgenic line 40.

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**Fig. 2.** Immunohistochemical detection of NOS2 in mouse pancreas (A–C), insulin staining of representative sections of pancreas (D–F), and islet cell mass of transgenic mice (G). The pancreata from mice at 4 weeks of age were fixed in 4% paraformaldehyde and embedded in paraffin. A–C, the sections were cut and stained with anti-mouse NOS2 antibody. NOS2 protein was detected in islets of a mouse of transgenic lines 31 (B) and 40 (C) but not in those of the control mouse (A). D–F, the sections from control mice (D) and those from transgenic lines 31 (E) and 40 (F) were stained with the antiserum against insulin and then counterstained with methyl green. Magnification, ×200 (A and D) and ×400 (B, C, E, and F). Bars, 50 μm. The transgenic islet cell mass was markedly reduced without insulinitis, as compared with the control islet cell mass. G, the proportions of insulin-producing cell mass to total pancreatic cell mass were evaluated morphometrically after insulin staining of the pancreata from mice of control (C) and transgenic lines 31 (31) and 40 (40) at 4 weeks of age, according to the method described previously (26). Values are the means ± S.E. Statistical analyses were performed using Student’s t test. *p < 0.05 (different from control).
4, the transgenic mice developed diabetes from as early as 1 week of age. The diabetes was clearly insulin-dependent, because the blood glucose level of diabetic mice injected with 4 units of exogenous insulin (Humulin U, Lilly) was reduced to below 200 mg/dl (data not shown). These results indicated that the diabetes observed in the transgenic mice was characteristic of IDDM (type 1 diabetes, ketosis prone) (27). At 1 week of age, NOS2 was immunohistochemically detected in the islets of the transgenic mice, suggesting that NOS2 expression was correlated with the development of diabetes. There was no evidence of transient insulitis such as infiltration of macrophages or lymphocytes at any time from the first to the eighth week of age (Fig. 2).

It has been reported that aminoguanidine, an inhibitor of NOS2 (28), was effective in reducing NO produced by IL-1β-treated islets in vitro (29). To demonstrate that the diabetes in the transgenic mice was dependent on NO production, we undertook a prophylactic intervention with aminoguanidine. The elevation of blood glucose levels as well as the reduction of pancreatic insulin contents in the transgenic mice were prevented (line 31) or delayed (line 40) by aminoguanidine treatment (Fig. 4). In control mice, the intraperitoneal administration of aminoguanidine did not affect the body weight, blood glucose levels, or serum insulin levels during the 8 weeks. Furthermore, the reduction of the insulin-producing cell mass was also prevented or improved by aminoguanidine (Fig. 2G), indicating that the transgenic model represents the effect of NO produced by NOS2 in β cells. Immunohistochemical staining of NOS2 showed that the transgenic mice treated with aminoguanidine did express NOS2 (data not shown), indicating that the effects of aminoguanidine were not due to the loss of expression of specific transgenes under the control of the insu-
lin promoter. It has been reported that aminoguanidine treatment has no effect on the appearance of insulin or the incidence of diabetes in animal models of immune-mediated IDDM (14, 30, 31). It is reasonable to assume that not only NO but also other β cytotoxic factors such as hydroxyl radicals and direct cytotoxic actions by cytotoxic T lymphocytes can be involved in the development of the immune-mediated model (3, 4).

Because high concentrations of exogenous NO have been shown to cause islet strand breaks in vitro (8, 9), we examined DNA strand breaks in transgenic and control islets. DNA was labeled by nick translation and analyzed by gel electrophoresis. As shown in Fig. 5A, [32P]dCTP was incorporated into transgenic islet DNA but not into control islet DNA. The estimated incorporation of [32P]dCTP into transgenic islet DNA (line 31, 27.2 ± 0.98 cpm/pg DNA; line 40, 45.1 ± 1.58 cpm/pg DNA) was much higher than that into control DNA (0.66 ± 0.06 cpm/pg DNA). We further confirmed the presence of DNA strand breaks by in situ nick translation. Transgenic islet cells showed extensive DNA strand breaks (Fig. 5, C and D), but nontransgenic islet cells did not (Fig. 5B). We and others have reported that when NO is produced in β cells upon cytokine stimulation (4, 6–12), extensive DNA strand breaks can occur to initiate a “suicidal” response; once β cell DNA strand breaks occur, nuclear poly(ADP-ribose) synthetase is activated, causing the depletion of intracellular NAD+. The depletion of NAD+ severely impairs β cell functions and ultimately evokes β cell death (3, 4, 15, 32–35).

We have already proposed that although IDDM can be caused by many different agents such as immunologic abnormalities, inflammatory tissue damage, and β cytotoxic chemical substances, the final pathway for the toxic agents is the same (3, 4, 12, 15, 32–35). This pathway involves DNA damage by free radicals such as NO and hydroxyl radicals, poly(ADP-ribose) synthetase activation, and NAD+ depletion. Therefore, IDDM is theoretically preventable by suppressing immune reactions, scavenging free radicals, and inhibiting poly(ADP-ribose) synthetase by its inhibitors. The mechanism of β cell death and its prevention was confirmed by using poly(ADP-ribose) synthetase gene disrupted mice (13). In the present study, we showed that NOS2 transgenic mice developed IDDM with β cell DNA damages by NO produced in the cells. Moreover, it was recently reported that NO-mediated poly(ADP-ribose) synthetase activation plays an essential role in ischemic neuronal cell death (36). It is thus reasonable to assume that not only β cell death in IDDM but also many other cell deaths such as ischemic brain injury can be explained by the pathway described above.

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