Apoptosis and remodelling of beta cells by paracrine interferon- γ without insulinitis in transgenic mice

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Abstract

Aims/hypothesis. To examine whether interferon- γ destroys islet beta cells directly or indirectly through lymphocyte activation, or whether direct action of interferon- γ on beta cells by itself induces diabetes without insulinitis.

Methods. To avoid possible nonspecific breakdown of beta cells by transgenic overexpression of interferon- γ by the insulin promoter, we generated transgenic mice expressing interferon- γ under the control of rat glucagon promoter (RGP-IFN- γ -Tg mice).

Results. The absence of insulinitis in RGP-IFN- γ -Tg mice enabled us to investigate the direct effects of paracrine interferon- γ . In RGP-IFN- γ -Tg mice, serum concentrations of interferon- γ and tumour necrosis factor- α (TNF- α) were 50 and 6 times higher than those in their littermates, respectively, and glu-

cose-responsive insulin secretion decreased to one-half the level of that in the littermates. Transgenic interferon- γ induced remodelling of beta cells where apoptosis of many beta cells was compensated by their vigorous regeneration and diabetes did not occur in most of the RGP-IFN- γ -Tg mice.

Conclusion/interpretation. Interferon- γ alone is insufficient for the complete destruction of beta cells in vivo, and factors other than interferon- γ including activated lymphocytes or other cytokines, are necessary in addition to interferon- γ for the development of Type I (insulin-dependent) diabetes mellitus. [Diabetologia (1999) 42: 566–573]

Keywords Type I diabetes, interferon- γ , transgenic mice, apoptosis, insulin secretion, tumour necrosis factor.

Received: 16 November 1998 and in final revised form: 11 January 1999

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Abbreviations: NOD, non-obese diabetic; BB, biobreeding; BDF1, C57BL/6 x DBA2; F1, F1 of founder mice; HE, haematoxylin and eosin; HIP-IFN- γ -Tg mice, transgenic mice expressing IFN- γ driven by human insulin promoter; IFN- γ , interferon- γ ; RGP-IFN- γ -Tg mice, transgenic mice expressing IFN- γ driven by rat glucagon promoter; Th1, T helper type 1; Th2, T helper type 2; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; GAD, glutamic acid decarboxylase.

Interferon- γ (IFN- γ) has been thought to have a major role in the onset of Type I (insulin-dependent) diabetes mellitus [1]. Recent studies in patients with Type I diabetes [2, 3] and animal models for it such as non-obese diabetic (NOD) mice [4] and biobreeding (BB) rats [5] have shown that insulinitis in Type I diabetes is mediated by T-helper type 1 (Th1) cells producing IFN- γ and interleukin (IL)-2 and not by T-helper type 2 (Th2) cells producing IL-4 and 10. Moreover, the reported prevention of insulinitis in NOD mice by oral treatment with insulin [6, 7], glutamic acid decarboxylase (GAD)-derived peptide [8] or IL-12 antagonist [9] has been associated with a balance shift from a Th1 to a Th2 pattern of cytokine expression in pancreatic islets [10]. Despite the suggestion that IFN- γ contributes to autoimmune insulinitis, it has not been clarified whether IFN- γ destroys

islet beta cells directly, or indirectly through the activation of other cells such as cytotoxic T-cells or the up-regulation of other cytotoxic factors. It is also not known how far IFN- γ contributes to insulinitis and beta-cell destruction compared with other cytokines. The transgenic overexpression of IFN- γ under the control of human insulin promoter (HIP-IFN- γ -Tg) has been associated with an inflammatory destruction of pancreatic islets [11], suggesting IFN- γ has a causal role in Type I diabetes. The genetic inactivation of IFN- γ in NOD mice has been found, however, to prevent neither insulinitis nor diabetes but only delay their onset [12]. This paradox suggests the possibility that pancreatic beta cells are nonspecifically destroyed by the transgenic overexpression of a protein driven by the insulin promoter. Indeed, transgenic mice expressing H-ras [13], calmodulin [14], MHC class I [15] or class II [11, 16] by the insulin promoter have been found to develop Type I diabetes without insulinitis. To circumvent this issue, we generated transgenic mice expressing IFN- γ under the control of rat glucagon promoter (RGP-IFN- γ -Tg mice). None of these mice showed insulinitis despite massive expression of IFN- γ in islet alpha cells. Thus, we were able to investigate the direct effects of paracrine IFN- γ on beta cells in vivo, without the influence of inflammatory cells including lymphocytes and macrophages.

Materials and methods

Generation of transgenic mice. Mouse IFN- γ cDNA was a kind gift from Dr. W. Fiers (Laboratory of Molecular Biology, Gent University, Belgium). The expression vector that included a rat glucagon promoter for transgenic mice has been constructed previously [17] and mouse IFN- γ cDNA was inserted into its *Eco* R I cloning site flanking the exon-intron organization and a polyadenylation signal of the rabbit β -globin gene. An *Xho* I fragment of this transgene was purified by agarose-gel electrophoresis and the glass-powder method with Gene Clean II Kit (Bio 101, Vista, Calif., USA), and resuspended in 10 mmol/l TRIS-Cl (pH 7.4) and 0.1 mmol/l EDTA at the concentration of 500 copies/pl. The transgene was microinjected into the male pronuclei of fertilized eggs obtained from superovulated BDF1 (C57BL/6 $\times$ DBA2 F1) female mice crossed with male BDF1. Injected embryos were implanted in the oviducts of pseudopregnant female mice and allowed to develop [18]. Subsequently DNA was extracted from tail snips of live offspring by the proteinase K/SDS method [19]. The integration of the transgene into the mouse genome was detected by polymerase chain reaction with 4 pairs of primers, a sense primer in exon 1 of the rat glucagon promoter (5'-TTGGGCGCAGAACACACTCA-3' or 5'-CTCAAAGTTCCCAAAGGAGCTC-3') and an antisense primer in exon 2 (5'-ACTCACCCCTGAAGTTCTCAG-3') or in intron 2 (5'-GAAAGAACAATCAAGGTCC-3') of the rabbit β -globin gene, and also by Southern blot analysis. An *Eco* R I fragment of the transgene, i.e. mouse IFN- γ cDNA, was used as a probe. The number of copies of integrated transgenes was determined from the intensity of each radioactive band in Southern blot analysis compared with indicator bands of 1, 10 and 100 copies of the transgene, or the band for the intrinsic

IFN- γ gene. We crossed RGP-IFN- γ -Tg founders with BDF1, and their F1 and F2 mice of 20 to 60 weeks of age were used for comparison with sex-matched littermates. Body weight was determined every week. The spleen and gonadal adipose tissue were weighed at the age of 45 weeks. To examine whether the onset of Type I diabetes in NOD mice is affected by the overexpression of IFN- γ in alpha cells, all lines of RGP-IFN- γ -Tg mice were repetitively crossed with NOD mice through several generations.

Histopathological examinations. Pancreata of adult mice were fixed with 20% formalin in phosphate-buffered saline, embedded in paraffin, sectioned and stained with haematoxylin and eosin (HE), aldehyde fuchsin for beta cells and Grimelius' stain for alpha cells. For immunohistochemistry, guinea-pig polyclonal antibodies against porcine insulin and glucagon (DAKO Japan, Kyoto, Japan), rat monoclonal antibodies against mouse MHC class II antigen (I-A) (BMA Biomedicals, Rheinstrasse, Switzerland), and a mouse monoclonal antibody against mouse MHC class II antigen (I-E) (PharMingen, San Diego, Calif., USA) were used.

Culture of mouse islets. Islets were obtained from mouse pancreata by the collagenase method [20]. To measure insulin secretion, five islets were preincubated in 1 ml of Krebs-Ringer bicarbonate buffer containing 115 mmol/l NaCl, 5 mmol/l KCl, 2.2 mmol/l CaCl₂, 1 mmol/l MgSO₄, 1 mmol/l Na₂HPO₄, 24 mmol/l NaHCO₃, 10 mmol/l HEPES/NaOH (pH 7.4), and 0.3% bovine serum albumin with 2.8 mmol/l glucose at 37°C in an atmosphere of humidified air:CO₂ (95:5) for 60 min and stimulated by an increase in glucose concentration of 16.7 mmol/l. To ascertain IFN- γ production, ten islets of transgenic mice were incubated in 1 ml of Dulbecco's modified Eagle's medium with 5.5 mmol/l glucose in a CO₂ incubator at 37°C for 3 h.

Measurement of glucose, insulin, IFN- γ , and TNF- α . Blood glucose concentrations were determined by the glucose oxidase method with a Diasensor (Kyoto Daiichi Kagaku, Kyoto, Japan). Concentrations of insulin, IFN- γ , and TNF- α were assayed by enzyme-linked immunosorbent assay kits for insulin with a mouse insulin standard (Seikagaku Kogyo, Tokyo, Japan), for mouse IFN- γ (ENDOGEN, Woburn, Mass., USA), and for rat TNF- α with high sensitivity and cross-reactivity to mouse TNF- α (BioSource International, Camarillo, Calif., USA), respectively.

Glucose tolerance test and insulin tolerance test. After overnight fasting, 1 mg/g body weight of glucose for the glucose tolerance test or 0.75 mU/g body weight of regular insulin in physiological saline for the insulin tolerance test were injected intraperitoneally. At 0, 30, 60, 90, and 120 min after the injection, blood glucose concentrations were determined.

Insulin content in the whole pancreas. Each pancreas removed from an adult mouse was homogenized in 4 ml of ice-cold acid-ethanol solution [21] and insulin was extracted at 4°C overnight. After centrifugation at 2000 $\times$ g at 4°C for 30 min, the supernatant was neutralized and diluted with phosphate-buffered saline. Insulin concentrations were assayed in 1000 $\times$ or 10 000 $\times$ diluted samples.

Detection of apoptosis. To detect apoptotic cells in pancreatic islets, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method was used. The paraffin sections of the mice pancreata were dewaxed by heating at 60°C followed by washing in xylene, rehydrated

through a graded series of ethanols and water and incubated with 20 $\mu\text{g/ml}$ of proteinase K in 10 mmol/l TRIS-Cl (pH 7.4) at 37°C for 30 min. After washing with phosphate-buffered saline, the sections were incubated with TdT from calf thymus and fluorescein-labelled dUTP (Boehringer Mannheim, Indianapolis, Ind., USA) and the fluorescein incorporated into DNA cleavage sites was detected by Fab fragments of anti-fluorescein sheep antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) and its substrates: nitro blue tetrazolium (Wako Pure Chemical Industries, Osaka, Japan) and 5-bromo-4-chloro-3-indolylphosphate (Wako). For a positive control, the section was treated with 1 mg/ml of DNase I (TAKARA SHUZO, Otsu, Japan) in 30 mmol/l TRIS-Cl (pH 7.5), 4 mmol/l MgCl_2 and 0.1 mmol/l dithiothreitol for 10 min just before TdT reaction, to induce DNA strand breaks.

Statistical analysis. All data are presented as means $\pm$ SEM. For comparison of two means, Student's unpaired *t* test was used.

Results

Generation of transgenic mice. Although four founders of RGP-IFN- γ -Tg mice were produced, one female founder mouse of line 4 died of unknown causes at the age of 15 weeks before breeding a large number of offspring. Therefore, F1 and F2 of lines 1, 2 and 3 were mainly used in this study. The numbers of copies of transgenes integrated into the mouse genome were 10, 2 and 5 in RGP-IFN- γ -Tg lines 1, 2 and 3, respectively. After crossing with BDF1 mice, 70 RGP-IFN- γ -Tg mice and their 88 littermates were obtained and 56 of the RGP-IFN- γ -Tg mice were killed for the experiments in this study. Of the remaining 14 RGP-IFN- γ -Tg mice, 2 died of malignant lymphoma by 60 weeks of age, 4 of infectious diseases of renal abscess, bacterial colitis, or sepsis of unknown causes by 80 weeks of age and 1 of an unknown cause at the age of 29 weeks. Of the 70 RGP-IFN- γ -Tg mice, bilateral cataracts and microphthalmia were observed in 2 before 6 weeks of age and only 1 with renal abscess suffered from diabetes with a blood-glucose concentration of more than 15 mmol/l. None of the 88 littermates died a natural death nor were cataracts or diabetes observed even at an age of more than 100 weeks. The weekly body weight and the weight of gonadal adipose tissue at the age of 45 weeks were similar in RGP-IFN- γ -Tg mice (1.8 ± 0.2 g, $n = 20$) and their littermates (1.7 ± 0.2 g, $n = 20$), whereas the weight of the spleen in RGP-IFN- γ -Tg mice (900 ± 9 mg, $n = 10$; 3 of line 1, 4 of line 2, and 3 of line 3) was 1.4 times larger than that in their littermates (656 ± 8 mg, $n = 10$) with statistical significance.

In crossing with NOD mice, 90 RGP-IFN- γ -Tg mice and their 178 littermates were examined. The number of births of RGP-IFN- γ -Tg mice gradually decreased as their genetic background approached that of NOD mice through several generations

Fig. 1. Number of births of RGP-IFN- γ -Tg mice after crossing with NOD mice. The RGP-IFN- γ -Tg births gradually decreased as their genetic background approached that of NOD mice through several generations. ○—○, line 1 ($n = 29$); ●—●, line 2 ($n = 49$); △—△, line 3 ($n = 56$); ▲—▲, line 4 ($n = 134$) of RGP-IFN- γ -Tg mice

(Fig. 1). Finally, no transgenic mouse was born by further crossing with NOD mice although pups without the transgene were born and developed normally. Diabetes did not occur in surviving F1 ($n = 66$), F2 ($n = 20$), and F3 ($n = 4$) of RGP-IFN- γ -Tg mice $\times$ NOD mice.

Expression of IFN- γ and TNF- α . The expression of the transgene was ascertained by the production of IFN- γ by 10 islets of RGP-IFN- γ -Tg mice in 1 ml of Dulbecco's modified Eagle's medium with 5.5 mmol/l glucose at 37°C in a CO₂ incubator (Fig. 2). In the culture medium of islets in littermates, no IFN- γ was detected with the enzyme-linked immunosorbent assay kit for mouse IFN- γ with a detection limit of 0.8 pmol/l. Serum concentrations of IFN- γ in RGP-IFN- γ -Tg mice varied from 3 to 85 times those in the littermates and their mean (549 ± 159 pmol/l, $n = 12$; 3 of line 1, 5 of line 2, and 4 of line 3) was 50 times higher than the value for the littermates (11 ± 2 pmol/l, $n = 10$). The serum concentration of TNF- α in RGP-IFN- γ -Tg mice (3.0 ± 0.7 pmol/l, $n = 8$; 1 of line 1, 5 of line 2, and 2 of line 3) was also higher than that of littermates (0.5 ± 0.1 pmol/l, $n = 12$) by a factor of 6.

Histopathological examinations. We used 14 of 70 RGP-IFN- γ -Tg mice and their littermates for histopathological examinations. Despite excessive IFN- γ production, neither insulinitis nor peri-insulinitis were observed in RGP-IFN- γ -Tg mice (Fig. 3). Even in one RGP-IFN- γ -Tg mouse affected with diabetes with renal abscess, no insulinitis was found but the

Fig. 2. Interferon- γ production by islets of RGP-IFN- γ -Tg mice. Ten islets were incubated in 1 ml of Dulbecco's modified Eagle's medium with 5.5 mmol/l glucose at 37 °C in a CO₂ incubator and IFN- γ concentrations in the medium were determined by enzyme-linked immunosorbent assay. ○—○, RGP-IFN- γ -Tg mice ($n = 7$); ●—●, littermates ($n = 3$)

Fig. 3. Histological examinations of islets in RGP-IFN- γ -Tg mice. Neither islet inflammation nor lymphocytic infiltration into and around islets were observed in RGP-IFN- γ -Tg mice. HE, haematoxylin and eosin staining; Insulin, immunohistochemistry with an anti-insulin antibody; Glucagon, immunohistochemistry with an anti-glucagon antibody; Tg, RGP-IFN- γ -Tg mice; LM, littermates

number of islets was remarkably decreased (data not shown). In RGP-IFN- γ -Tg mice, obvious manifestations of islet neogenesis were, however, observed (Fig. 4). Regenerating islets were larger than normal islets (Fig. 4A and 4E) and often adjacent to pancreatic ducts. In anti-insulin immunohistochemistry, regenerating islets showed a lower intensity and a larger heterogeneity of staining than normal islets (Fig. 4B and 4F) and insulin-positive epithelial cells of pancreatic ducts were also observed (Fig. 4D). The results from aldehyde fuchsin staining for beta cells and Grimelius' staining for alpha cells (data not shown) were similar to those from anti-insulin and

Fig. 4A–F. Islet neogenesis in RGP-IFN- γ -Tg mice. A, B, E, and F are at the same magnification. C and D are enlargements of A and B, respectively. Arrows indicate insulin-positive epithelia of a pancreatic duct. HE, haematoxylin and eosin staining; Insulin, immunohistochemistry with an anti-insulin antibody; Tg, RGP-IFN- γ -Tg mice; LM, littermates

anti-glucagon immunohistochemistry, respectively. In immunohistochemistry against mouse MHC class II antigens (I-A and I-E), no positive cell was detected even after microwave treatment in islets of RGP-IFN- γ -Tg mice nor their littermates (data not shown).

Glucose tolerance test. Because all RGP-IFN- γ -Tg mice except for one with renal abscess did not develop diabetes, the glucose tolerance test was carried out. Blood glucose concentrations before and at all time points after glucose treatment in RGP-IFN- γ -Tg mice were higher than those in their littermates (Fig. 5).

Insulin tolerance test. To estimate insulin resistance in peripheral tissues, regular insulin was injected into 20 mice (10 RGP-IFN- γ -Tg mice and 10 littermates) after overnight fasting. The two decreasing curves of blood glucose concentrations were similar and a statistically significant difference for the insulin effect on blood glucose concentration was not found when RGP-IFN- γ -Tg mice were compared with their littermates (data not shown).

Insulin secretion and storage. At 2.8 mmol/l glucose concentration, the insulin secretion rate of pancreatic islets of RGP-IFN- γ -Tg mice was the same as that of their littermates (Fig. 6). At 16.7 mmol/l glucose con-

Fig. 5. Glucose tolerance test. After overnight fasting, 1 mg/g body weight of glucose in physiological saline was intraperitoneally injected. Blood glucose concentrations before and at all time points after glucose treatment in RGP-IFN- γ -Tg mice were higher than those in their littermates ($p < 0.05$). $\circ$ — $\circ$, RGP-IFN- γ -Tg mice ($n = 10$); $\bullet$ — $\bullet$, littermates ($n = 10$)

Fig. 6. Response of insulin secretion to glucose stimulation of cultured islets. Five islets were preincubated in 1 ml of modified Krebs-Ringer bicarbonate buffer with 2.8 mmol/l glucose at 37°C in a CO₂ incubator and stimulated by 16.7 mmol/l glucose. The insulin secretion rate of islets of RGP-IFN- γ -Tg mice was lower at 16.7 mmol/l glucose ($p < 0.05$). Open bars, RGP-IFN- γ -Tg mice ($n = 7$); solid bars, littermates ($n = 7$)

centration, the insulin secretion rates of islets of RGP-IFN- γ -Tg mice and their littermates increased 4.3-fold and 8.7-fold, respectively, compared with the values for 2.8 mmol/l glucose and the insulin secretion rate of islets of RGP-IFN- γ -Tg mice was only

Fig. 7A–D. Apoptotic beta cells detected by the TUNEL method. **A**, **C**, and **D** are of the same magnification. **B** is an enlargement of **A**. White and black arrowheads indicate fragmented and lobulated nuclei, respectively. **D** was treated with DNase I before the TUNEL method as a positive control. Tg, RGP-IFN- γ -Tg mice; LM, littermates

53% of that of the littermates. The insulin content of the pancreas in RGP-IFN- γ -Tg mice (3.3 ± 0.4 nmol/pancreas, $n = 10$: 3 of line 1, 4 of line 2, and 3 of line 3) was 20% lower than that in the littermates (4.1 ± 0.5 nmol/pancreas, $n = 10$), although this difference is not statistically significant.

Detection of apoptosis. The apoptotic cells observed in HE staining of pancreatic islets in RGP-IFN- γ -Tg mice were ascertained by the TUNEL method. In the islets of RGP-IFN- γ -Tg mice, pyknotic, fragmented, lobulated, or amorphous nuclei were stained by the TUNEL method (Fig. 7A and 7B) whereas no staining was observed in the islets of the littermates (Fig. 7C). By superimposing consecutive sections with immunohistochemical staining, almost all the nuclei stained by the TUNEL method were shown to be beta cells (data not shown). In a positive control with DNase I treatment, round nuclei of a relatively similar size were stained both in endocrine and exocrine cells (Fig. 7D). In the islets of littermates after DNase I treatment, the nuclear periphery was preferentially stained by the TUNEL method because highly differentiated cells, such as beta cells and lymphocytes in general, have a large quantity of heterochromatin at the periphery of their nuclei.

Discussion

In addition to impaired glucose tolerance, RGP-IFN- γ -Tg mice developed cataracts, microphthalmia, malignant lymphoma, and bacterial infections including renal abscess and acute colitis. Because cataracts and microphthalmia were reported in transgenic mice expressing IFN- γ driven by α -A-crystallin promoter [22] and rhodopsin promoter [23], excessive

IFN- γ may affect the development and function of ocular tissues. Occurrence of lymphoma and infectious diseases may suggest a disturbed immune regulation due to overproduced IFN- γ . After crossing with NOD mice through several generations, no transgenic mouse was born. Because RGP-IFN- γ -Tg fetuses were not found at 19.5 embryonic days, RGP-IFN- γ -Tg embryos are possibly resorbed after intrauterine fetal death. Indeed, Th1-type immunity producing IFN- γ and TNF- α in response to stimulation by reproductive tissue antigens or sperm in uterus has been associated with unexplained recurrent abortion in humans [24] and the arrest of mouse embryonic development [25]. It is not clear why the genetic background of NOD mice inhibits the development of RGP-IFN- γ -Tg embryos. A high incidence of spontaneous fetal resorption in the placenta of CBA/J $\times$ DBA/2 mice is associated with an increased production of IFN- γ and TNF- α [26] and a decreased production of anti-inflammatory Th2-type cytokines (IL-4 and IL-10). The injection of an anti-IFN- γ antibody, an anti-TNF agent, or IL-10 reduced the fetal resorption in CBA/J $\times$ DBA/2 mice [27]. Thus, the production of anti-inflammatory Th2 type cytokines could be insufficient in the placenta of NOD mice to cancel the fetal toxicity of IFN- γ produced in RGP-IFN- γ -Tg embryos.

Excessive production of IFN- γ in RGP-IFN- γ -Tg mice was ascertained by assaying IFN- γ in the islet culture medium (Fig. 2) and serum. The level of serum IFN- γ in RGP-IFN- γ -Tg mice (50 times higher than that in littermates) was sufficient to inhibit insulin secretion [28, 29] and DNA synthesis [30] and to induce apoptosis of beta cells [29] in *in vitro* studies, although the local concentration of IFN- γ in transgenic islets, *in vivo*, was probably still higher. Impaired insulin secretion (Fig. 6) and apoptosis of beta cells in RGP-IFN- γ -Tg mice (Fig. 7) show a direct toxicity of IFN- γ against beta cells *in vivo* because our transgenic mice did not show insulinitis. Moreover, the 20% reduction in the insulin content of the transgenic pancreas, compared with that in littermates, is consistent with the observation that IFN- γ decreased the insulin content of beta cells in *in vitro* studies [31].

The serum TNF- α in RGP-IFN- γ -Tg mice was 6 times higher than that in their littermates. Because the spleen of RGP-IFN- γ -Tg mice was larger than that of their littermates, the increase in serum TNF- α is probably derived from IFN- γ -activated macrophages or lymphocytes. Despite the supposed mediator function of TNF- α between obesity and insulin resistance [32, 33], the body weight, weight of gonadal adipose tissue and the results of insulin tolerance tests were similar in RGP-IFN- γ -Tg mice and their littermates. The serum TNF- α concentration of 3.0 ± 0.7 pmol in RGP-IFN- γ -Tg mice is much lower than TNF- α concentrations sufficient to induce insulin resistance, which were 42 pmol/l in a systemic infusion

study [34] and 67 pmol/l in an *in vitro* study [35]. Because such high concentrations of serum TNF- α are encountered only in limited conditions including sepsis and cachexia, it is possible TNF- α contributes to the insulin resistance of muscle [36] and adipose tissue [32] in an autocrine or paracrine manner.

We believe the compensation for the loss of beta cells by islet neogenesis is responsible for the absence of diabetes in RGP-IFN- γ -Tg mice. Interferon- γ seemed to be a stimulator of islet neogenesis in our study (Fig. 4) and in HIP-IFN- γ -Tg mice [37]. Immature beta cells in the human fetus have been found to be resistant to the toxic effects of IFN- γ , TNF- α and streptozotocin [31, 38], and their growth was stimulated by IFN- γ and TNF- α [31]. This is consistent with the resistance of beta cells in HIP-IFN- γ -Tg mice to streptozotocin [39]. Biphasic functions of IFN- γ to stimulate the growth of immature islet cells and to induce the apoptosis of adult beta cells strongly support the hypothesis that IFN- γ promotes islet remodelling. Thus, many islet cells including a small amount of insulin in RGP-IFN- γ -Tg mice (Fig. 4B and 4D), are probably immature beta cells in regenerating islets. The IFN- γ -induced expression of reg mRNA [40], which is associated with the regeneration of pancreatic islets [41], is also consistent with the neogenic capacity of IFN- γ for pancreatic islets.

Because of the putative ability of IFN- γ to stimulate islet remodelling, factors other than IFN- γ are thought to be necessary for the onset of diabetes. These probably include activated lymphocytes, in particular, cytotoxic T-lymphocytes against beta cells. The presentation of beta-cell antigens plays a crucial part in the local activation of lymphocytes in pancreatic islets [42]. A vicious circle is formed where the presentation of self-antigens activates lymphocytes and beta cells destroyed by activated lymphocytes provide more antigen to activate lymphocytes. We did not detect MHC class II antigens in the islets of RGP-IFN- γ -Tg mice by immunohistochemistry. Inducibility of MHC class II antigens by IFN- γ is controversial in *in vitro* studies and depends on animal strain [43] or antibodies used to detect MHC class II antigens. In cultured human islet cells, HLA class II antigens have been induced by a combination of IFN- γ of 50 U/ml with TNF- α of 10 U/ml [44]. The serum concentration of TNF- α in RGP-IFN- γ -Tg mice in our study was, however, as low as 3.0 ± 0.7 pmol/l, i.e. about 0.1 U/ml. In another study, in contrast to our RGP-IFN- γ -Tg mice MHC class II antigens were expressed in pancreatic islets of HIP-IFN- γ -Tg mice and an inflammatory destruction of islets and diabetes were observed [11]. We suppose, by comparing these two transgenic strains, that nonspecific destruction of beta cells due to IFN- γ overexpression by the insulin promoter augmented the presentation of beta cell antigens and triggered a vicious circle of a tissue-specific autoimmune mechanism. Interfer-

on- γ -induced apoptotic beta cells in our transgenic mice, however, are rapidly removed by macrophages and the surrounding cells of apoptotic cells and do not lead to autoimmune responses [45]. The difference in phenotype between RGP-IFN- γ -Tg mice and HIP-IFN- γ -Tg mice is not likely to be due to the difference in their genetic backgrounds because in both cases Tg mice were not generated using an inbred strain and because our transgenic mice did not develop diabetes even in 88% genetic background of NOD (Fig. 1).

Even in the absence of insulinitis, RGP-IFN- γ -Tg mice can develop diabetes in association with increased concentrations of other cytokines. Because one RGP-IFN- γ -Tg mouse with renal abscess developed overt diabetes without insulinitis, the increases in TNF- α and IL-1 induced by sepsis could have caused insulin resistance of peripheral tissues and more severe apoptosis of beta cells. Transgenic mice expressing either TNF- α [46] or B7-1 (a costimulator of T-lymphocytes) [47] by the insulin promoter were found never to develop diabetes. Double transgenic mice expressing TNF- α and B7-1 in beta cells resulted, however, in overt diabetes with insulinitis [47]. Thus, several factors for inducing the local activation of lymphocytes or apoptosis of beta cells that exceeds the remodelling capacity of beta cells are possibly necessary for the development of diabetes. The development of diabetes in IFN- γ -null NOD mice [12] is also compatible with a limited role of IFN- γ in the pathogenesis of Type I diabetes.

In our study, transgenic paracrine IFN- γ driven by the glucagon promoter caused remodelling of beta cells without insulinitis, where apoptosis of beta cells was compensated with their vigorous regeneration. We conclude IFN- γ alone is insufficient for the complete destruction of beta cells in vivo and factors other than IFN- γ , including activated lymphocytes or other cytokines, are necessary for the development of Type I diabetes.

Acknowledgements. This study was supported in part by a grant from Otsuka Pharmaceutical Factory Inc. to the Otsuka Department of Clinical and Molecular Nutrition, School of Medicine, The University of Tokushima.

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