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Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients

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Abstract

Aims/hypothesis. We examined the pancreatic islet lesions in Japanese patients with Type II diabetes mellitus to determine if the damage was related to oxidative stress.
Methods. Morphometric analyses were performed on immunostained sections of the tail portion of the pancreas from 14 diabetic and 15 non-diabetic patients. Amyloid deposition and oxidative stress-induced tissue damage were evaluated by Congo-red staining and immunostaining. Resistance to oxidative stress was assessed from immunostaining results for Cu, Zn-superoxide dismutase (SOD). Expression of (pro)insulin mRNA was assessed by in situ hybridisation.
Results. The pancreas from diabetic patients had amyloid deposition in about 15 % of the islets, intensified reactions of 8-OHdG and HNE, as well as reduced expression of SOD. Islet volume density of beta cells and total beta-cell mass in the pancreas from diabetic patients were reduced by 22 % ($p < 0.001$) and 30 % ($p < 0.05$). Islet volume density and total mass of (pro)insulin mRNA-positive cells were similarly reduced in diabetic patients by 22 % ($p < 0.001$) and 39 % ($p < 0.05$), respectively. Islet volume density of A cells was increased by 20 % ($p < 0.001$) but total mass did not change. There were no changes in volume densities of islet, D and PP cells. Reduced beta-cell volume density correlated with increased positive staining of 8-OHdG.
Conclusion/interpretation. Japanese Type II diabetic patients show a reduction of beta-cell mass and evidence of increased oxidative stress-related tissue damage that is correlated with the extent of the beta-cell lesions. [Diabetologia (2002) 45: 85–96]

Keywords Type II diabetes, islet pathology, morphometry, oxidative stress, islet amyloid.

Impaired insulin secretion and insulin resistance are a characteristic feature of Type II (non-insulin-dependent) diabetes mellitus [1, 2]. In spite of extensive studies on the pathophysiology of diabetes, islet pathology and its pathogenesis remain controversial. Classical studies using histochemical methods to identify endocrine cells have reported varying results, including the severe loss of beta cells [3], modest changes with amyloid deposition [4, 5] and even no change of islet beta-cell population [6]. Immunohistochemical techniques for the identification of specific populations of islet endocrine cells, showed a nearly 50 % reduction in beta-cell volume density in European Type II non-obese diabetic patients compared with non-diabetic control subjects [7]. Other studies have reported a 24 % reduction of islet beta-cell area density, a 58 % increase in A cell area density and a deposition of amyloid that correlated with the severity of islet pathology [8]. In another study of European Type II diabetic patients, a separate research group has reported

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Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HNE, 4-hydroxy-2-nonenal-modified proteins; SOD, superoxide dismutase; HE, hematoxylin-eosin; ISH, in situ hybridisation; Dg, digoxygenin; Ig, immunoglobulin

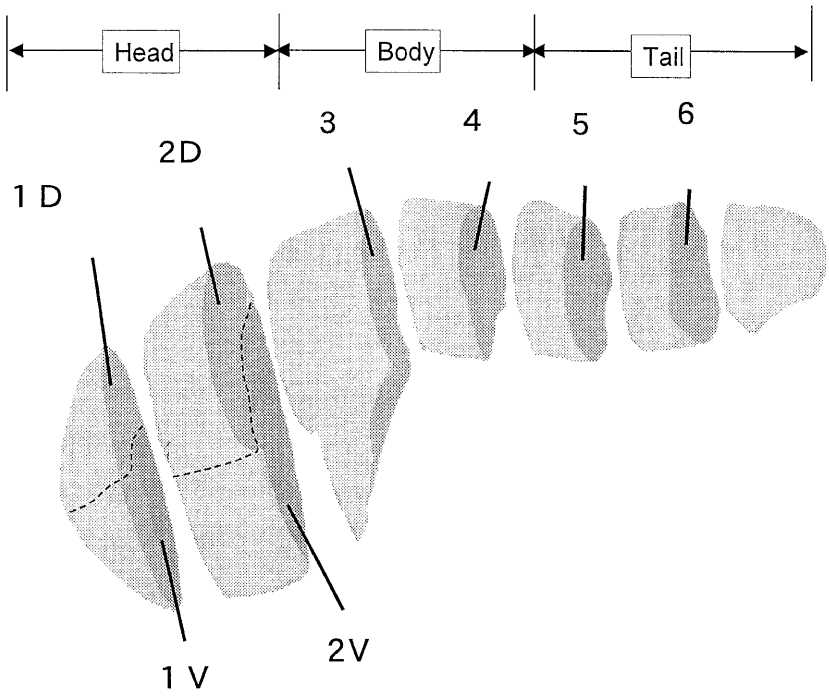

Fig. 1. Schematic view of section sampling for morphometric analysis from various portions of the pancreas from non-diabetic subjects. Two sections each from the ventral portion of the pancreas head (1 V, 2 V) and the dorsal portion of the pancreas head (1D, 2D) in addition to four other (3–6) sections from the body and tail of the pancreas were subjected to analysis

no changes in the volume of beta cells, D, and PP cells but an increase in the A cell population [9].

In Japanese Type II diabetic patients, one classic study [6], using sections with Gomori's histochemical staining, reported a 38% reduction of islet volume with nearly identical decreases of 38, 39, 28% in the total volume of A, beta and D cells, respectively. Conversely, a recent immunohistochemical study [10] reported increased beta-cell volume in Type II diabetic patients. These conflicting results could be due in part to differences in the subjects examined or the methods employed for the morphometric analysis. Heterogeneity of Type II diabetes among races or differences in the extent of disease progression with the study populations could have also influenced the results of these morphometric analyses. Japanese, in the absence of marked obesity, are prone to diabetes when they immigrate to Western countries [11] and in the last 20 years there has been an explosive increase in the diabetic population in Japan [12, 13]. It therefore could be important to characterise changes in the endocrine cell population of the islet in Japanese Type II diabetic patients because this could assist our understanding of the pathophysiology and pathogenesis of diabetes.

Recent studies on Type II diabetic animal models have reported that the progressive reduction of islet beta cells is associated with excessive oxidative stress [14, 15]. In these animal models when hyperglycaemia is allowed to continue, a so-called "glucotoxicity" to beta cells impairs insulin secretion [2, 16, 17] and eventually causes fatal islet cell injury, accelerating beta-cell loss [2, 17]. Aggravated hyperglycaemia with sucrose feeding provokes excessive oxidative stress that has been associated with an increased rate of apoptosis in pancreatic beta cells in Type II diabetic animal models [18, 19]. It is not known, however, whether excessive oxidative stress is also associated with the islet pathology in human Type II diabetic patients. We therefore investigated islet pathology of Japanese Type II diabetic patients and examined whether their islet cells show oxidative-stress-related DNA damage, altered expression of Cu, Zn-superoxide dismutase (SOD) and apoptotic cell death.

Materials and methods

Subjects. To examine islet volume and the volume density of each endocrine cell type, morphometric analyses were performed on five whole pancreases obtained at autopsy from non-diabetic subjects with no history of glucose intolerance. To obtain the pancreas free from autolytic changes, tissues were collected from subjects only when the autopsy was initiated within 2 h of death. The volume of the pancreas was measured by Archimedes' principle and the whole pancreas was then fixed in 10% formalin. For the morphometric analysis of pancreatic islets, six sections of the dorsal pancreas and two sections of the ventral pancreas, collected from six different sites within the pancreas were selected for analysis (Fig. 1).

To characterise islet pathology, morphometric analyses on the volume densities of the islet, volume density of each endocrine cell type and the total mass of beta and A cells were quantified on autopsy material from 14 Type II diabetic pa-

Table 1. Clinical data of the non-diabetic (NDM) and diabetic (DM) subjects used in the study

Case No.		Sex (M/F)	Age (years)	BMI	Major cause of death	Pancreatic weight (g)	Duration of diabetes	Treatment[b]	Complications
NDM	-1	M	68	24.1	Cerebral hemorrhage	110			
	-2	M	61	15.9	Malignant mesothelioma	160			
	-3	F	52	20.6	Cerebral infarction	140			
	-4	F	62	20.6	Ovarian cancer	170			
	-5	M	62	17.9	Remnant gastric cancer	110			
	-6	F	27	18.5	Breast cancer with bone metastasis	130			
	-7	M	54	21.4	Lung cancer	75			
	-8	M	65	22.1	Cholangiocarcinoma	100			
	-9	M	45	22.9	Acute myocardial infarction	149			
	-10	F	54	22.7	Breast cancer with bone metastasis	140			
	-11	F	59	26.7	Breast cancer	160			
	-12	M	69	19.2	Pyothorax, Bronchopneumonia	81			
	-13	M	69	24.2	Lung cancer	90			
	-14	M	61	20.3	Ureter cancer	115			
	-15	M	48	22.5	Acute myocarditis	102			
Mean ± SD			51.7 ± 10.7	21.3 ± 2.7		122 ± 29			
DM	-1	M	46	23.4	Diabetes, Multiple cerebral infarction	115	13	Diet	Kidney
	-2	M	60	17.5	Diabetes, Lung cancer	120	10	Diet	Kidney
	-3	M	69	22.9	Diabetes, Myocardial infarction,	110	20	OHA[a]	Kidney, Nerve
	-4	M	66	16.4	Diabetes, Gastric cancer	120	16	Insulin (3 years)	Kidney
	-5	M	64	21.6	Diabetes, Lung cancer	100	8	Insulin (2 years)	Nerve
	-6	M	59	16.6	Diabetes, Cerebral infarction	110	12	Diet	Nerve, Eye
	-7	M	57	17.2	Diabetes, Liver cirrhosis	75	14	Insulin (4 years)	Kidney, Nerve
	-8	M	60	16.8	Diabetes, Myocardial infarction	140	7	OHA[a]	Nerve, Eye
	-9	F	47	24.1	Diabetes, Kimmelstiel-Wilson syndrome	135	16	Insulin (6 years)	Kidney, Nerve, Eye
	-10	F	68	23.0	Diabetes, Kimmelstiel-Wilson syndrome	125	21	Insulin (10 years)	Kidney, Nerve, Eye
	-11	F	59	26.5	Diabetes, Mucormycosis, Renal failure	85	18	Insulin (6 years)	Kidney, Nerve, Eye
	-12	M	69	19.8	Diabetes, Cerebral infarction	98	12	OHA[a]	Kidney, Nerve
	-13	M	66	21.0	Diabetes, Renal cancer	110	14	Insulin (5 years)	Kidney, Nerve
	-14	F	62	23.6	Diabetes, Breast cancer	120	11	OHA[a]	Nerve
Mean ± SD			60.9 ± 7.0	20.7 ± 3.2		112 ± 17	13.7 ± 4.0		

[a] OHA: oral hypoglycemic agents, [b] Parenthesis is a total duration of insulin treatment

tients and 15 non-diabetic patients. Clinical data and major causes of death are listed in Table 1. All diabetic patients requiring insulin treatment in this study were negative for islet cell antibody and for GAD antibody. Additionally, the pancreatic pathology in these diabetic subjects was distinct from the late-onset Type I (insulin-dependent) diabetes mellitus which is characterised by about 10% residual islet beta cells, marked atrophy of exocrine pancreas, and an infiltration of CD8 (+) T cells into the exocrine pancreas [20, 21]. Furthermore, none of the subjects had a family history of muscle disorders or signs of mitochondrial diabetes. Autopsy material was accepted only from subjects younger than 70 years of age. Because the morphometric data on islet endocrine cells was constant throughout the dorsal pancreas, 2 paraffin blocks of tissues from the pancreatic tail were used for the comparisons between diabetic and non-diabetic subjects. The tissue sampling procedure followed the guideline of the ethical committee of Hirosaki University School of Medicine.

Immunohistochemistry. Hematoxylin-eosin (HE) stained sections were used to measure islet area. Immunohistochemistry was performed on serial sections to identify endocrine cell populations within the islet. The immunostaining procedures used streptavidin-biotin (SAB) methods with antibodies to human insulin (1:2000, produced in our laboratory), synthetic human glucagon (1:2000, produced in our laboratory), synthetic somatostatin (1:3000, produced in our laboratory) and pancreatic polypeptide (PP) (1:2000, donated by Dr. RE Chance, Eli Lilly Lab., Indianapolis, Ind., USA). To identify islet amyloid, Congo-red staining and immunostaining with rabbit antibody to human amylin (Peninsula, Belmont, Calif., USA) were performed. Only the islet with amyloid deposition in the extracellular matrix was defined as an amyloid-positive islet. Amyloid deposition was confirmed by the positive apple-green birefringence colour of Congo-red stained sections observed with polar microscopy and by the positive green fluorescence of Thioflavin T-stained sections on fluorescent microscopy. For the immunostaining of oxidative-stress–related proteins, monoclonal antibodies to 8-hydroxy-2'-deoxyguanosine (8-OHdG; 1:100 dilution; Nihon Yushi, Jika, Shizuoka, Japan) a marker of oxidative stress-induced DNA damage [22, 23]; 4-hydroxy-2-nonenal-modified proteins (HNE; 1:250 dilution; Nihon Yushi), a marker of lipid peroxidation products [24, 25] and Cu, Zn-SOD (donated from Professor N. Taniguchi, Osaka University, Osaka, Japan; 1:200 dilution) a representative anti-oxidative enzyme [26, 27] were used.

Immunostaining involved removing the paraffin from the sections, followed by treatment with methanol containing 0.3 % hydrogen peroxide, added to inhibit endogenous peroxidase activity. After incubation with normal goat serum for 30 min to block non-specific reactions, each primary antibody was applied overnight at 4 °C. For the detection of 8-OHdG, HNE and SOD, the tissue sections were treated with microwave irradiation for 15 min (3 times for 5 min) in 0.01 mol/l citric buffer (pH 6.0) for antigen retrieval, before the application of the primary antibodies. Biotin-labelled anti-rabbit-immunoglobulin (Ig) or anti-mouse Ig was then applied to the sections for 30 min at room temperature. To detect the binding sites of antigen to antibodies, peroxidase-labelled streptavidin was applied to the sections and finally the reaction products were visualised with 3, 3'-diaminobenzidine. The nuclei were lightly counterstained with hematoxylin. The specificity was confirmed by, firstly, blocking the primary antibody reaction with the addition of excessive antigens, when antigen was available; secondly, by the replacement of the primary antibodies with non-immune sera; and thirdly, by the omission of the primary antibodies.

The detection of apoptotic cells was based on the nick-end labelling (TUNEL) method using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, Md., USA) [18, 28]. As a positive control for this method, lymphnode samples were stained concurrently.

The intensity of the immunoreactions of HNE and SOD within the islets was graded semi-quantitatively, as follows: negative (score 0); weakly positive (lightly stained but clearly differentiated from negative background; score 1); moderately positive (between weak and strong) (score 2); and strongly positive (dark brown with high contrast; score 3). Scores from a minimum of 20 islets were averaged for the evaluation of tissues from each subject. Agreement among the two observers was greater than 85 %. Islets with discordant decisions were omitted from the analysis. Cells positive for 8-OHdG staining were quantified by the presence of a dark brown nuclear stain. Observations were collected from a minimum of 10 islets and when quantified, were expressed as a percentage of the total number of islet cells.

In situ hybridisation of (pro)insulin mRNA. To detect the transcript expression of (pro)insulin, in situ hybridisation (ISH) was performed on pancreatic tissues from 6 diabetic and 6 non-diabetic subjects using a previously described method [29]. In brief, 4 μm thick paraffin sections were deparaffinized with xylene, ethanol and air-dried. The sections in 0.01 mol/l citric buffer, pH 6.0 were then treated with microwave irradiation for 10 min. Following this procedure the sections were treated with proteinase K (Sigma, St. Louis, MO, USA) 1 μg/ml for 15 min at 37 °C. They were washed 3 times with phosphate buffered saline for 5 min and dried with ethanol. After heating to 99 °C for 1 min then cooling in ice, the sections were hybridised with digoxygenin (Dg)-labelled oligoprobe (1 μg/ml) overnight at 37 °C. The Dg-labelling kit (SP6/T7) (Boehringer Mannheim, Mannheim, Germany) was used for the labelling of Dg with oligoprobe. The oligoprobe was a 24 mer sequence of 5'-TTG TTC CAC AAT GCC ACG CTT CTG-3'. The hybridisation buffer consisted of a 70 μl hybridisation cocktail (50 % formamide, 20 mmol/l TRIS-HCl [pH 7.6], 1 mol/l EDTA, 0.3 mol/l NaCl, 0.1 mol/l dithiothreitol, 0.5 μg/ml yeast tRNA, 0.1 μg/ml poly-A-RNA, 1xDenhardt's solution, and 10 % dextran sulphate). After hybridisation, the slides were washed twice for 10 min in 1 x standard saline citrate (SSC) containing 0.1 % sodium dodesyl sulphate (SDS) and then in 0.2 · SSC/0.1 % SDS for 37 °C for 10 min at 37 °C. The slides were then treated with 0.1 mol/l TBS (0.1 mol/l NaCl, 0.002 mol/l $MgCl_2$, 0.05 % Triton X, pH 7.6) for 10 min. A blocking solution (Dig Nucleic Acid Detection Kit, Boehringer Mannheim) for eliminating non-specific reactions was then applied for 5 min. Next the hybridised RNA was made to react with sheep anti-Dg antibody IgG (Fab)-alkaline phosphatase (1:500) (Boehringer Mannheim) for 1 h at room temperature. The slides were then washed three times for 5 min in 0.1 mol/l TRIS-buffered saline. Finally, the reaction products were coloured with NBT/BCIP (Dig Nucleic Acid Detection Kit). For the controls, sense-probe instead of anti-sense was used for the hybridisation.

Morphometric analysis of islet. Islet morphometry was performed with a computer-assisted point counting method on an Olympus AX80 microscope connected to an Apple Macintosh G3 computer system using NIH Image (version 1.56) (National Institutes of Health, Bethesda, MD, USA). Calculation of the volume densities of islet area, A, beta, D and PP cells with the point counting method was based on a previously described method [30]. Briefly, the image of a pancreas section stained with HE was visualised under a 4× objective (× 57 on monitor) and positioned under a regular lattice. Following this procedure, 3600 to 10000 (mean 5400) lattice points positioned above the pancreas were counted. First, volume density of islet per pancreatic parenchyma ($V_{I/P}$) was obtained as follows. On each section, the total number of the points above each islet was counted and designated as P_I. Then, the total number of points above the pancreas parenchyma (excluding the area of vessels, as well as connective and fatty tissues) was counted and was expressed as P_P. Then $V_{I/P}$ was calculated by the formula $V_{I/P} = P_I/P_P$. To evaluate the volume densities of each individual endocrine cell type per islet, a high-magnification (× 200) image of the immunostained section was overlaid with a grid, consisting of 875 points. For each cell type, 25 to 80 fields (average 45) were analysed. The total number of the points above each islet was expressed as Pi, and total number of the points above each endocrine cell, A, beta, D or PP cell in the whole section was expressed as Pa, Pb, Pd, Ppp, respectively. Then islet volume densities for each endocrine cell (per islet) was expressed as Vb/i (= Pb/Pi), Va/i (= Pa/Pi), Vd/i (= Pd/Pi) and Vpp/i (= Ppp/Pi), respectively. The volume density of beta cells per pancreatic parenchyma was expressed

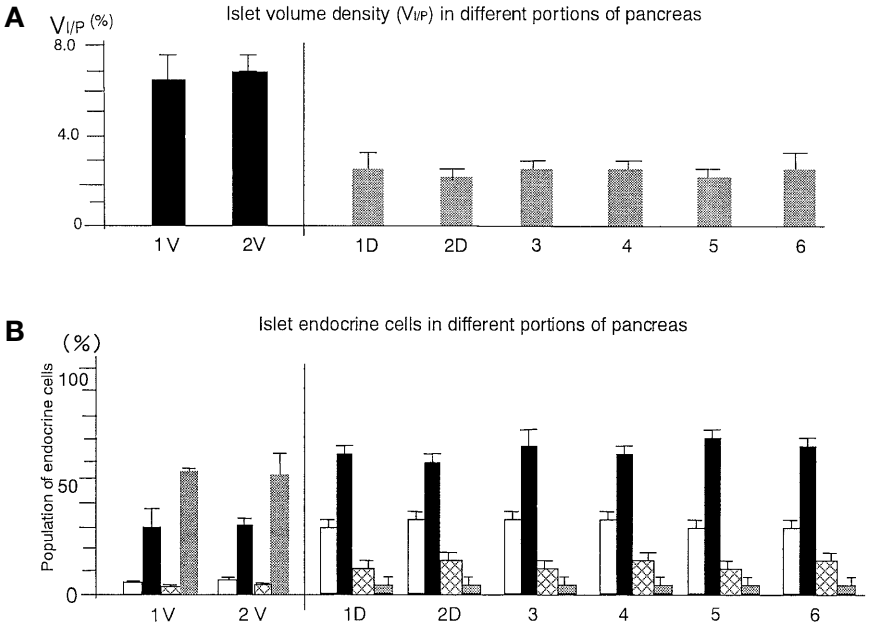

Fig. 2 (A, B). Volume density of islets from various portions of the pancreas (**A**). The islet volume density was 2.4-fold greater in the head compared with that in the body and tail. Population of each endocrine cell type in various portions of pancreas (**B**). The islet in the ventral head contained predominantly PP cells, whereas beta cells were the prevalent cell type in the islet of the body and tail. Bar stands for SD. □, A cell (Va/i), ■, B cell (Vb/i), ▨, D cell (Vd/i), ▩, PP cell (Vpp/i)

by Vb/p, which was obtained by the formula Vb/p = (Vb/i)x (VI/P) = (Pb/Pi)x(Pi/Pp). Finally, total mass of the islet and endocrine cell was obtained by multiplying the pancreas weight with each volume density.

Volume density of (pro)insulin-mRNA positive cells was similarly obtained by counting the number of the points above the cells positively stained with (pro)insulin mRNA. These values were expressed per islet as VbmRNA/i and then as VbmRNA/p per pancreatic parenchyma. The percentage of islets with amyloid deposition was measured by calculating the amylin-positive islets among 150 ~ 200 islets within the tail sections.

All the morphometric analysis and evaluation of immunoreactions were done by observers who did not know the source of the tissue sections, i. e. diabetic or non-diabetic subject.

Statistical analysis. Results are expressed as means ± SD. Comparisons of the mean values between diabetic and non-diabetic subjects were performed with the Mann-Whitney's U test. For the correlation analysis of two parameters, Spearman's rank test was used. For all of these analyses, a p value of 0.05 or less was considered to be statistically significant.

Results

Distribution and islet cell composition in non-diabetic subjects. In the ventral pancreas, large islets with irregular contour were often encountered, whereas islets in the dorsal pancreas were mostly round or oval. The volume density of islets per pancreatic parenchyma (VI/P) was 6.09 ± 1.99 % in the ventral pancreas and 2.56 ± 0.61 % in the dorsal pancreas (Fig. 2A). The density of islet in the ventral pancreas was 2.4-fold greater than that in the dorsal pancreas.

The volume densities of A, beta, D, and PP cells per islets (Va/i, Vb/i, Vd/i, Vpp/i) were 7.1 ± 1.7, 32.7 ± 7.8, 3.6 ± 1.4, 56.4 ± 8.3 % in the ventral pancreas and 27.2 ± 5.9, 65.5 ± 4.9, 7.5 ± 2.0, 1.3 ± 1.1 % in the dorsal pancreas, respectively. The per cent composition of A, beta, D, PP cell density was consistent throughout the dorsal pancreas (Fig. 2B). The PP cells formed the densest areas in the islet of ventral pancreas and the beta cells formed the densest areas in the islets of the dorsal pancreas.

Immunohistochemistry of amylin and oxidative stress-related substances. Most islets were round to oval in tissues from non-diabetic subjects, while an irregular contour was conspicuous in tissues from diabetic subjects. Amyloid deposition was frequently encountered in tissues from diabetic subjects (0 ~ 15 % of islets) (means ± SD, 6.2 ± 4.6 %) but rare in non-diabetic subjects (0 ~ 2 %). Nuclear staining of 8-OHdG was only found infrequently in acinar and ductal cells and in a few islet cells in non-diabetic subjects (Fig. 3A), while islets in pancreatic tissues from diabetic subjects contained many positive cells (Fig. 3B). Islets with amyloid deposits showed strongly positive reactions of 8-OHdG (Fig. 3C). The positive cells were more numerous in tissues from diabetic subjects than in non-diabetic subjects ($p < 0.002$). Cells undergoing apoptosis by the ApopTag test were not evident in tissues from either group of subjects (not shown). However, diabetic islets were positively stained with HNE, which was not evident in the islets of non-diabetic subjects (Fig. 4A, B).

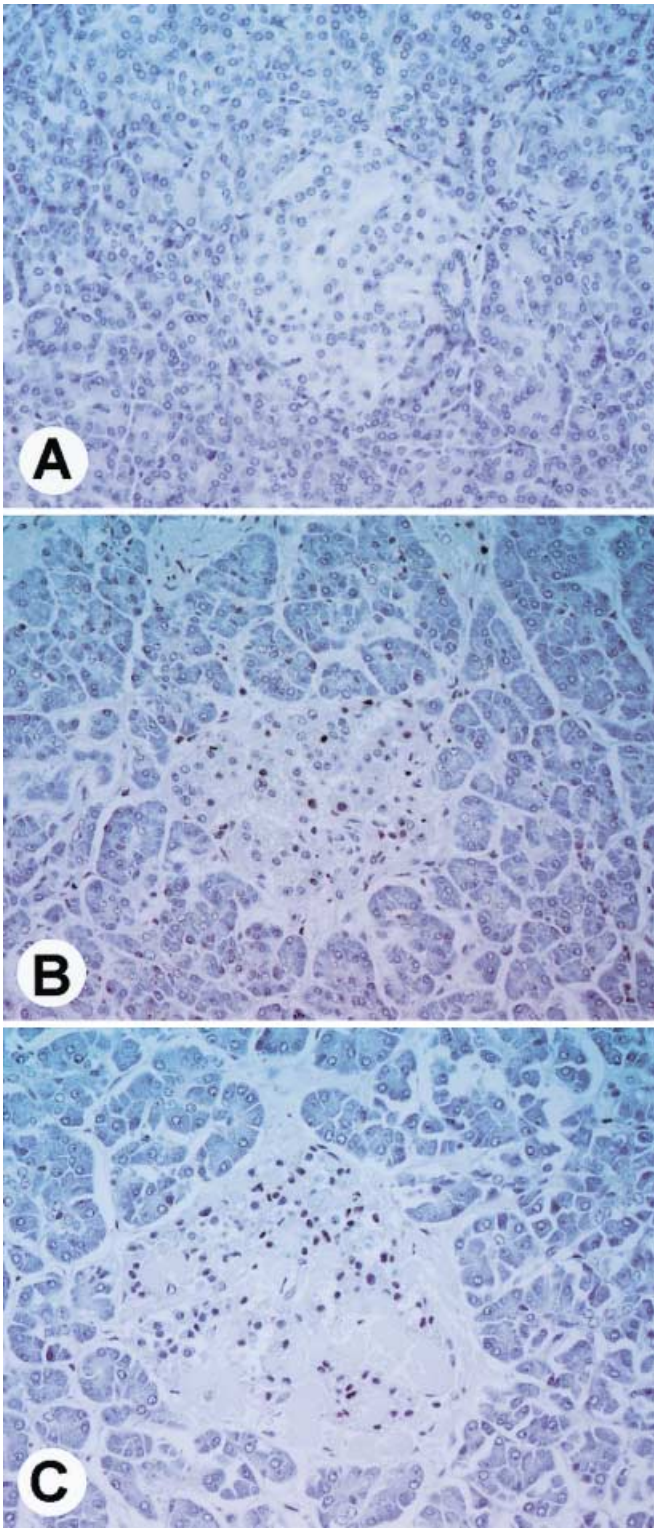

Fig. 3 (A–C). Immunoreactive 8-hydroxy-2'-deoxyguanosine (8-OHdG) reactions in the pancreatic islet. Islets in non-diabetic patients did not show positive reactions (**A**, × 120, non-diabetic case; NDM-1). In contrast, many islets in the diabetic subjects showed conspicuous staining of many endocrine cells (**B**, × 120, diabetic subject; DM-10). When the islets exhibited amyloid deposition, the remaining islet cells demonstrated strong 8-OHdG-reactions (C, × 120, diabetic case; DM-11)

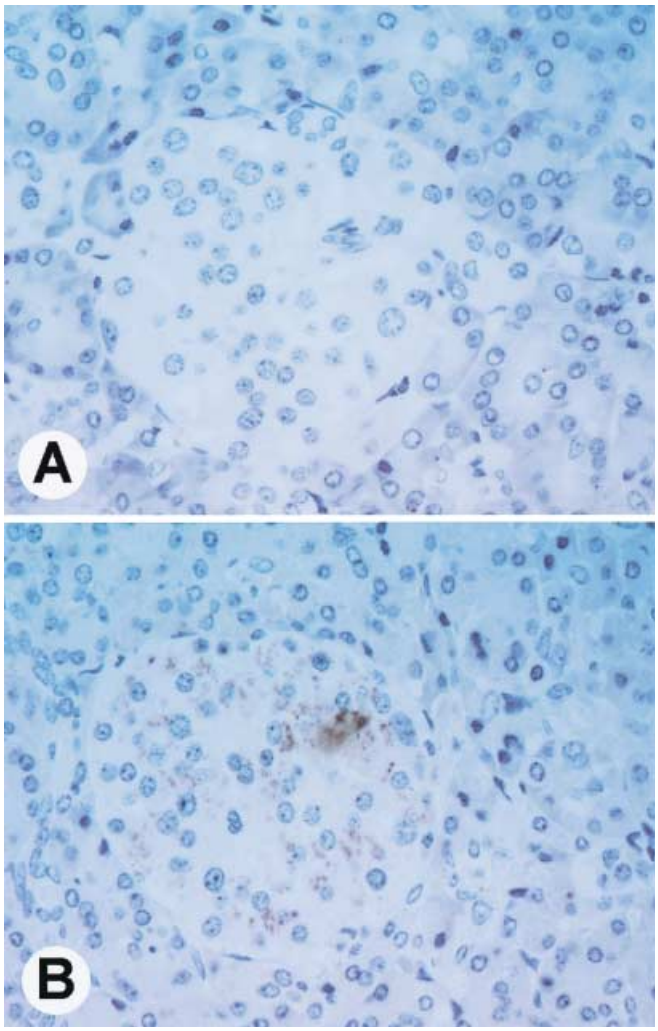

Fig. 4 (A, B). Expression of immunoreactive 4-hydroxy-2-nonenal (HNE)-modified proteins in the islet. Only faint reactions were detected in islets of non-diabetic patients (**A**, × 240, non-diabetic case; NDM-3). In contrast, islets in diabetic subjects had positive reactions (**B**, × 240, diabetic case; DM-3)

Distinct immunoreactions of Cu, Zn-SOD within islet cells were detected in non-diabetic subjects (Fig. 5A). Ductal cells and some acinar cells were also positive. In contrast, islets in diabetic subjects showed only weak immunoreactions as long as reactions of ductal or centroacinar cells were still present (Fig. 5B). Semi-quantitative evaluations of the scores of HNE and Cu, Zn-SOD expression showed increased expression of HNE and reduced immunoreactions of Cu, Zn-SOD in tissues from diabetic subjects compared with those from non-diabetic subjects ($p < 0.01$ for both) (Table 2).

Islet morphometry on diabetic and non-diabetic groups. Although there was no significant difference in the volume density of islet ($V_{I/P}$) or islet mass between tissues from diabetic and non-diabetic groups

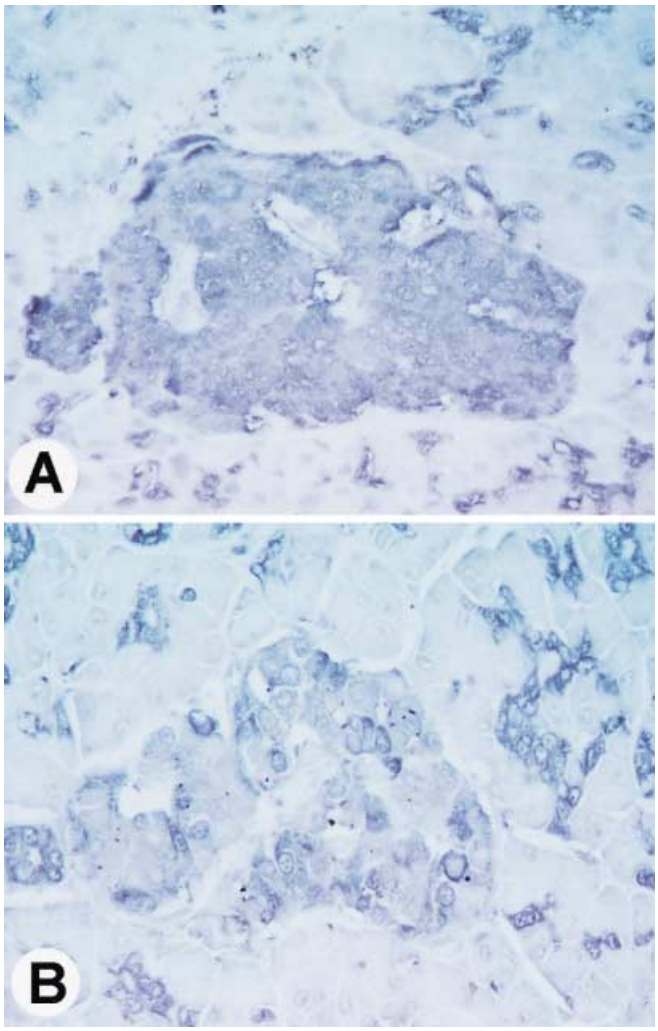

Fig. 5 (A, B). Expression of immunoreactive Cu, Zn-superoxide dismutase (SOD) in pancreatic islets. Non-diabetic patients had strong reaction in the whole islet and in some ductal cells, as well as centroacinar cells (**A**, × 240, non-diabetic case; NDM-12). In contrast, islets in diabetic patients showed equivocal or no positive reactions, although some ductal cells were positive (**B**, × 240, diabetic case; DM-3)

($p > 0.1$), there was a trend for smaller average values in the diabetic group (Table 3). The mean value of islet beta-cell volume density (Vb/i) in the diabetic group was about 22 % less than that in the non-diabetic group ($p < 0.001$). The volume density of beta cells per pancreatic parenchyma (Vb/p) in diabetic subjects was further reduced to 66 % of that of the non-diabetic group ($p < 0.002$). Additionally, the total beta-cell mass was 30 % smaller in the diabetic group than in the non-diabetic group ($p = 0.043$).

The mean value of islet A cell volume density (Va/i) in the diabetic group was 1.4-fold greater than that in non-diabetic group ($p < 0.01$). However, there was no difference in the pancreatic A cell volume density (Va/p) between diabetic and non-diabetic groups. There was also no difference in the D cell density

Table 2. Immunoreactivities of 4-hydroxy-2-nonenal-modified proteins (HNE) and Cu, Zn-superoxide dismutase (Cn, Zn-SOD) in pancreatic islets of diabetic and non-diabetic patients

HNE score		Negative		Positive		Mean ± SD
		0	1	2	3	
Diabetic patients	$n = 8$	0	1	4	3	2.3 ± 0.7[a]
Non-diabetic patients	$n = 10$	2	6	2	0	1.0 ± 0.6
Cu, Zn-SOD score		**Negative**		**Positive**		**Mean ± SD**
		0	1	2	3	
Diabetic patients	$n = 8$	1	5	2	0	1.1 ± 0.6[a]
Non-diabetic patients	$n = 10$	0	1	5	4	2.3 ± 0.6

Immunoreactions were graded as 0, unstained; 1, weakly positive or partially positive in some islets,; 2, positive in entire islets; 3, strongly positive in entire islets. [a] $p < 0.01$ vs non-diabetic patients

(Vd/i) or PP cell density (Vpp/i) between the diabetic and non-diabetic groups.

Reduced islet beta-cell volume density in tissues from diabetic subjects correlated well with the incidence of 8-OHdG positive cells (Fig. 6).

In situ hybridisation of (pro)insulin mRNA. ISH demonstrated the presence of (pro)insulin mRNA-positive cells in 57 to 64 % of the islet area within the pancreas of non-diabetic subjects. The (pro)insulin mRNA positive cell area (VbmRNA/i) per islet was 22 % less in the pancreas of diabetic subjects ($p < 0.001$) (Fig. 7). The volume density of (pro)insulin mRNA cell area per pancreas (VbmRNA/p) and the total mass were similarly reduced in the diabetic patients (40 % and 39 %) ($p < 0.001$, $p < 0.05$, respectively). In diabetic patients, the expression of (pro)insulin transcripts in beta cells was well preserved in islets that showed extensive amyloid depositions (Fig. 8).

Discussion

This study demonstrates a 33 % reduction of pancreatic beta-cell volume density and a 22 % reduction of islet beta-cell volume density in Japanese Type II diabetic patients in tissues at autopsy following 7 to 21 years of diabetes. The difference in the beta-cell volume density per islet and per pancreatic tissue between the diabetic and non-diabetic groups was highly significant ($p < 0.002$). In contrast, the reduction in total beta-cell mass (30 % average decrease in the diabetic group) was only marginally significant ($p = 0.043$) due to the large individual variations of pancreatic weight in the diabetic subjects. In fact,

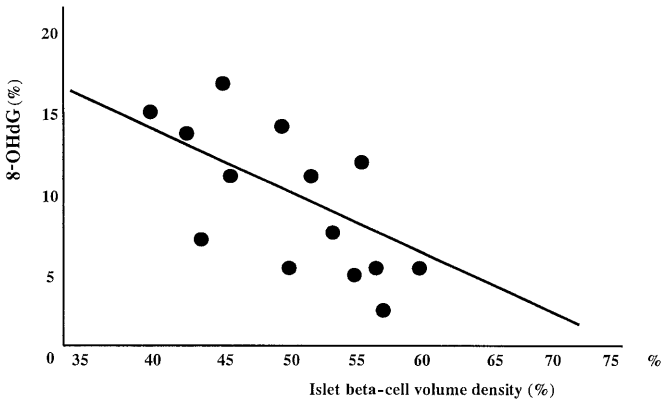

Fig. 6. Relation between islet beta-cell volume density and the percentage of cells with percentage positivity 8-OHdG reactivity in the islet in diabetic subjects. There was an inverse correlation between 8-OHdG positive cells and beta-cell volume density. Y = 31.3–0.41X, $r^2 = 0.39$ ($p < 0.01$)

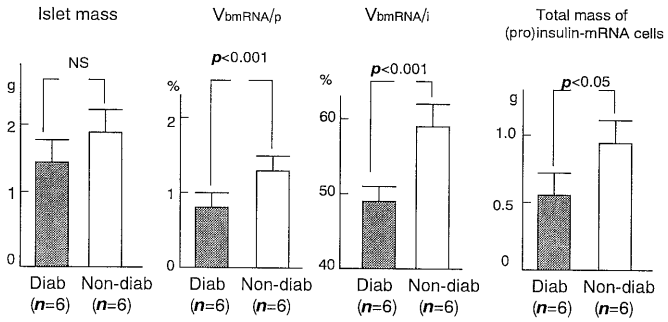

Fig. 7. Morphometric analysis of (pro)insulin-mRNA positive cells in six diabetic (Diab) and six non-diabetic (Non-diab) subjects. The mass of the PP lobe amounting to 10% value of the total pancreatic weight was excluded from the total islet mass in each case. The islet mass was not different between two groups. Volume densities of (pro)insulin-mRNA cells in the pancreatic parenchyma (Vbmrna/p) and in the islet (Vbmrna/i) were reduced in the diabetic group compared with the non-diabetic group (40%, 22%, $p < 0.001$ for both). The total mass of (pro)insulin-mRNA positive cells was similarly reduced by 22% in the diabetic group. Bar stands for SD. Closed column represents the diabetic group and the open column represents the non-diabetic group

beta-cell mass in 3 out of the 14 (21%) diabetic subjects exceeded the average value for non-diabetic subjects illustrating the heterogeneity of disease progression in this population of patients. Recent morphometric studies, where specific endocrine cell types were identified by immunostaining, have also shown a reduction in the volume densities of beta cells per islet or pancreatic parenchyma in European Type II diabetic patients [5, 7, 8, 31]. A similar reduction as in these studies was found in our study, showing a 30 to 50% reduction of beta-cell volume density. A report on two American Type II diabetic patients [32] also found about a 50% decrease in beta-cell volume density. In most of these studies, it is not known whether the total beta-cell mass was also reduced because the pancreatic volume or weight was not provided. Measurements of total beta-cell mass are particularly useful in evaluating the insulin content or insulin secretion capacity of the pancreas.

In a recent study of a large series of European Type II diabetic subjects in whom beta-cell mass was measured [33], the investigators did not observe a significant reduction of total beta-cell mass except for a group of subjects who required insulin treatment [34]. These results are not contradictory to our current results. In the 50% of our diabetic patients who were treated with insulin, the beta-cell mass was 40% less than that of non-diabetic patients ($p < 0.01$). Our results are also consistent with the previous finding by other investigators [35] who observed that insulin-treated Type II diabetic subjects contained lower beta-cell volume (mean: 0.41 ml) than subjects treated with oral compounds (0.52 ml) and diet (0.8 ml). Massive loss of beta cells such as that observed in a 95%-partial pancreatectomy in rodents [36] or a 50% reduction of beta-cell mass in streptozotocin-injected baboons [37] is required to induce hyperglycaemia. It is not therefore likely that the reduction of beta-cell mass found in our study could, by itself, account for the onset of diabetes. It is more likely that the reduction of beta-cell mass merely reflects the severity of diabetes incurred after long-term hyperglycaemia. Based on these data, it is

Table 3. Volume density (VD) of islet and each endocrine cells and total B and A cell mass in diabetic and non-diabetic subjects

		Islet VD ($V_{I/P}$) (%) (Islet mass[a]) (g)	Islet beta cell VD ($b_{b/i}$) (%) (Pancreas beta cell VD) ($V_{b/p}$) (%)	Total beta-cell mass[a] (g)	Islet A Cell VD ($V_{a/i}$) (%) (Pancreas A cell VD) ($V_{a/p}$) (%)	Total A cell mass[a] (g)	Islet D Cell VD ($V_{d/i}$) (%) (Pancreas D cell VD) ($V_{d/p}$) (%)	Islet PP cell VD ($V_{pp/i}$) (%) (Pancreas PP cell VD) ($V_{pp/p}$) (%)
Diabetic patients	$n = 14$	2.24 ± 0.86 (1.70 ± 0.87)	48.9 ± 6.2[b] (1.09 ± 0.42[c])	0.82 ± 0.44[d]	38.7 ± 8.0[b] (0.88 ± 0.40)	0.69 ± 0.38	9.84 ± 3.78 (0.22 ± 0.19)	2.52 ± 1.51 (0.06 ± 0.05)
Non-diabetic patients	$n = 15$	2.58 ± 0.52 (2.03 ± 0.56)	62.7 ± 5.6 (1.64 ± 0.38)	1.14 ± 0.37	28.4 ± 5.5 (0.71 ± 0.15)	0.50 ± 0.15	7.41 ± 2.62 (0.19 ± 0.07)	1.58 ± 1.11 (0.04 ± 0.03)

Values are mean ± SD (%). [a] The mass of the PP lobe amounting to 10% of the total pancreatic weight was excluded. [b] $p < 0.001$, [c] $p < 0.002$ and [d] $p < 0.05$ vs non-diabetic patients

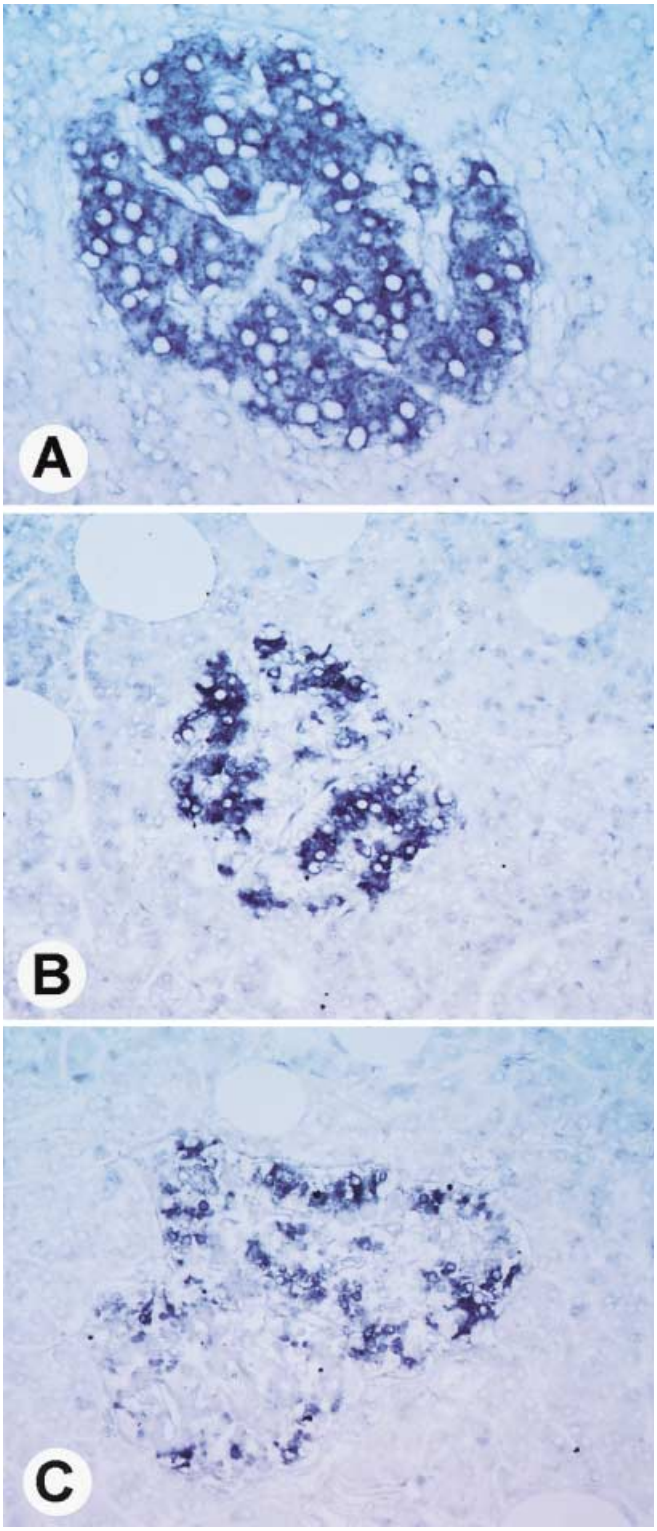

Fig. 8 (A–C). In situ hybridisation of (pro)insulin-mRNA on the pancreatic sections. Tissue samples from representative non-diabetic subject (NDM-12) show strong positive expression of transcripts in the central area of the islet (**A**, × 240), whereas in the sections from a representative from the diabetic subject (**B**, DM-13, × 120) positive area of (pro)insulin-mRNA positive cells appears to be slightly reduced even though the intensity of the reaction was well preserved. In an islet with severe amyloid deposition, (pro)insulin-mRNA appeared to be well preserved in areas near the amyloid deposition (**C**, DM-14, × 120)

conceivable that an improvement of impaired beta-cell function rather than increasing beta-cell mass should be the primary therapeutic target for Type II diabetes [38].

In this study, most of the diabetic pancreas had well preserved (pro)insulin productivity, reflected by the strong expression of (pro)insulin transcripts. Although the volume density of (pro)insulin-mRNA positive cells in the islet was reduced in the diabetic group, the total islet mass was not different between the diabetic and non-diabetic subjects. Even in islets with severe amyloid deposition, the expression of (pro)insulin mRNA was maintained (Fig. 8C). These findings indicate that defects in the processing from transcript to secretion could cause lowered insulin secretion in Japanese Type II diabetic patients. Others have shown that areas of proinsulin-positive cells were larger in islets from obese Type II diabetic subjects than in non-diabetic subjects [39] suggesting that the processing from (pro)insulin to insulin was impaired, resulting in lowered insulin secretion in these patients [39]. Because we did not stain islet for proinsulin, it is not clear whether the processing of insulin at this step is also disturbed in Japanese Type II diabetic patients.

The number of islets with amyloid deposition was much greater, ranging from 0 ~ 15 % (6.2 ± 4.6 %) in tissues from our diabetic subjects compared with non-diabetic subjects (0 ~ 2 %). However, our observed frequency of amyloid-laden islets was considerably less than that observed in previous studies of European Type II diabetic patients [5, 40, 41]. It has been noted that with increasing age, the number of islets with amyloid deposition increases in the pancreases of non-diabetic subjects but not in those of diabetic subjects [42]. After the age 65 to 70, some diabetic subjects have islet amyloid deposition in as many as 30 % of their islets [42]. The lower percentage of amyloid-laden islets in our study could result from our inclusion of younger subjects (< 70 years old).

In contrast to our observations, two studies, one in European subjects [5] and one in Japanese subjects [6], showed a reduction of islet volume in Type II diabetes. In both of these studies a considerable number, if not the majority, of subjects were over 70 years of age. In our study the average age of the diabetic subjects was about 61 and none of our diabetic subjects was older than 69. This difference in age, which could indicate less disease progression, could explain why we did not observe a reduction in islet volume in our diabetic subjects.

In animal models of Type II diabetes, both impaired proliferative activity and reduced survival have been shown to be the cause for the beta-cell loss [18, 19, 43]. Once animals mature, beta-cell replication ceases and any further depletion of beta cells could be dependent on decreased activities of cell sur-

vival [44, 45]. Although we were not able to demonstrate the presence of apoptotic beta cells in our diabetic subjects, reduced beta-cell mass is probably due to the premature death of beta cells. Because depletion of beta-cell mass is prevented in genetically-determined Type II diabetic rats by metabolic control [45], or aggravated by sucrose feeding [14, 18], exaggerated glucotoxicity or lipotoxicity is believed to initiate accelerated beta-cell loss in the advanced stage of Type II diabetes [1, 2, 46–48]. Recent studies of animal models have shown that accelerated beta-cell loss is associated with excessive oxidative stress [14, 18], identified by increased expression of HNE and 8-OHdG in the islets of sucrose-fed diabetic rats.

HNE-modified protein is a tissue marker of lipid peroxidation products and is commonly found in injured hepatocytes of subjects with alcoholic liver diseases [49], neuronal cells of Alzheimer's disease [25, 50] and Parkinson's disease [51] and in macrophages from atherosclerotic lesions [52]. In a variety of tissues, 8-OHdG is used as a marker of oxidative stress-related DNA injury to the nuclei or mitochondria [22, 23]. Diabetic patients have been shown to have higher concentrations of 8-OHdG in blood cells [53] and in the urine [54], as well as in muscle tissue [55]. In several studies the rise of 8-OHdG has been correlated with HbA_{1C} values suggesting that increased 8-OHdG expressions predict the progression of diabetic microvascular complications [56, 57]. Deposition of HNE and the increased number of 8-OHdG-positive cells in the islets from our diabetic subjects suggest that a prolonged exposure to oxidative stress contributes to the islet pathology in Japanese Type II diabetes. Direct evidence that exposure to oxidative stress causes beta-cell loss still needs to be provided but the expressions of these markers in islet tissues and a significant correlation of reduced beta-cell volume density with the number of 8-OHdG positive cells support this contention.

Although it is not yet clear how excessive entry of glucose or lipid elicits beta-cell injury, increased non-enzymatic glycation of cellular proteins has been identified in animal studies [58, 59]. The presence of HNE-modified proteins suggests that oxidative stress with lipid peroxidation also contributes to islet-cell injury [60]. Islets have relatively low contents of anti-oxidative enzymes and are therefore weak in scavenging hydroxide and oxygen-free radicals [61]. A scarcity of anti-oxidants together with decreased blood concentrations of reduced glutathione could lead to an accumulation of free radical products in the islet during diabetes [62]. We observed reduced expression of Cu, Zn-SOD immunoreactivity in the islets of the pancreas from our diabetic subjects. In chronic pancreatitis, a disease in which islets are well preserved, intensified expression of this enzyme in islets has been reported [63]. Because anti-oxidants inhibit beta-cell death in vitro [15] and in Type II diabetic animal model [15], further analysis on the enzymatic activity and transcript expression of SOD in the pancreas of diabetic subjects elucidate the significance of our current results. Of note, 8-OHdG positive cells were numerous in the area of amyloid deposition. Amylin protein has been shown to be cytotoxic to beta cells [64], and therefore the presence of amyloid proteins could be an additional factor accelerating beta-cell loss in the islets.

In conclusion, this study provides evidence that selective beta-cell depletion in Japanese Type II diabetic patients is associated with increased oxidative stress.

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