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Molecular and Histological Evaluation of Pancreata from Patients with a Mitochondrial Gene Mutation Associated with Impaired Insulin Secretion

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A mutation in mitochondrial DNA, which was originally identified in patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), can be associated with a subtype of diabetes mellitus. To determine the molecular and histological basis of impaired insulin secretion in the subjects with this mutation, we studied autopsy pancreata specimens from eight subjects diagnosed as having MELAS. The 3243 bp mutation was identified in seven out of eight pancreata examined. Immunohistochemical studies demonstrated a reduction in total islet mass, and in the numbers of both B and A cells. No evidence of insulitis or apoptosis was found. These data suggested that the 3243 bp mutation may cause the reduction of islet cells, mainly through mechanisms other than autoimmune destruction. © 1999 Academic Press

A subtype of diabetes mellitus associated with a mutation in mitochondrial DNA (an adenine to guanine transition at position 3243 in transfer RNA $(tRNA)^{Leu(UUR)}$ mutation; herein referred to as the 3243 bp mutation) appears to constitute approximately 1% of diabetic patients in Japan and Western countries (1-6). The term MIDD (maternally inherited diabetes and deafness) has been proposed for this subtype of diabetes mellitus because of its characteristic features (7,8). Initially, it was supposed that the functional defect of mitochondrial oxidative phosphorylation in pancreatic B cells may blunt glucose-regulated insulin secretion. However, many of the patients carrying the mutation show a progressive decrease in insulin secretion and some are clinically diagnosed as having secondary failure to sulfonylurea or slowly progressive insulin-dependent diabetes mellitus (IDDM) (5), suggesting the reduction of B cell mass. To examine this idea, we performed molecular and histological analysis of pancreata from diabetic subjects with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), most of whom are expected to have this mutation.

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MATERIALS AND METHODS

Specimens of pancreata from patients with a clinical diagnosis of MELAS. Fixed and embedded pancreas samples were obtained from eight autopsied patients with a clinical diagnosis of MELAS randomly selected from the 1990-1993 annuals of pathological autopsy cases in Japan. The control blocks of postmortem pancreata were obtained from three non-diabetic subjects in their thirties and from an IDDM patient with the duration of four months who died from hypoglycemia at the age of 35.

Brief clinical profiles of these subjects are shown in Table 1. All the eight patients were clinically diagnosed as having MELAS from neuromuscular symptoms and some from muscle biopsy. In patient 2 and 3, the 3243 bp mutation had been detected in DNA from muscle tissue; however, it was not detected in DNA from circulating blood of patient 2. Other patients had not been investigated for the existence of the 3243 bp mutation before they died. Five of the eight patients had been treated with insulin and one patient had impaired glucose tolerance on a 75 g oral glucose tolerance test according to the World Health Organization criteria (9). Three diabetic patients were investigated for insulin secretory capacity, all of whom had low levels of serum C-peptide, and patient 3 was clinically diagnosed as having IDDM.

Pathological examination of pancreata from patients with the 3243 bp mutation. Five micron serial sections were cut from all the formalin fixed paraffin embedded blocks of pancreata. Each section was stained by hematoxylin and eosin. Immunohistochemical studies were performed by the avidin-biotin peroxidase method (10) using the following antisera: anti-insulin, anti-glucagon, and anti-leukocyte common antigen supplied by LIPSHAW, Pittsburgh, Pa.

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TABLE 1

Features of the Eight MELAS Patients

	Age (yr)	Sex	Height (cm)	wt (kg)	Family history	Duration of diabetes	Therapy		Postprandial 0′	Postprandial 120′	Hearing loss	Ragged-red fiber	Clinical findings of brain (Image of CT*)		Lactate‡ (mmol/l)	Pyruvate§ (mmol/l)	Clinical findings of heart
Patient 1	37	M	161	35	brother (diabetes mellitus)	>2 wk	insulin	S-CPR‖ (nmol/L)	0.4	1.7	(+)	(+)	low density areas in right temporal, occipital and parietal lobe	blood liquor	3.4 5.5	0.16 0.21	hypertrophy
Patient 2	55	F	146	30	unknown	>4 mo	insulin	BG¶ (mmol/L)	31.3		(+)	(−)	all ventricles were markedly wide	blood liquor	1.9 4.0	0.14 0.22	hypertrophy
Patient 3	31	F	140	27	unknown	8 yr	insulin	BG¶ (mmol/L) S-CPR‖ (nmol/L)	14.7 0.1	13.0 0.5	(+)	(+)	normal	blood liquor	1.6 3.9	0.14 0.18	sinus tachycardia subendocardial ischemia
Patient 4	17	F	163	42	aunt (cardiomyopathy)	>4 d	insulin	BG¶ (mmol/L)	26.1		(−)	(+)	low density area in the right occipital lobe	blood liquor	4.7 4.3	0.22 0.18	normal
Patient 5	57	F	140	34	mother, aunt (diabetes mellitus)	6 yr	insulin	BG¶ (mmol/L) S-CPR‖ (nmol/L)	17.1 0.3	 0.6	(+)	—	remarkable brain atrophy	blood liquor	4.7 4.2	0.32 0.16	hypertrophy diffuse hypokinesis
Patient 6	37	M	156	32	mother (cardiomyopathy)	unknown	diet	BG¶ (mmol/L)	6.7		(+)	(+)	diffuse cerebral and cellebellar atrophy	blood	2.9	0.17	hypertrophy
Patient 7	49	M	170	47	unknown	—	—	BG¶ (mmol/L)	5.5	6.6	(−)	(+)	mild crebral and cellebellar atrophy	blood liquor	2.3 5.0	0.14 0.12	sick sinus syndrome hypertrophy
Patient 8	66	M	162	34	unknown	—	—	BG¶ (mmol/L) insulin** (pmol/L)	4.5 12.6		(−)	(+)	cerebral atrophy and mild dilatation of ventricles	liquor	2.3	0.15	normal

* CT: computed tomography.
‡ Normal range of blood: 0.5–1.8 mmol/l and liquor: 1.1–1.5 mmol/l.
§ Normal range of blood: 0.03–0.08 mmol/l and liquor: 0.03–0.06 mmol/l.
¶ BD: blood glucose.
‖ S-CPR: serum C peptide, normal range of fasting blood: 0.3–0.9 mmol/l.
** Normal range of fasting blood: 18–120 pmol/l.

TABLE 2

Histopathology of Pancreas of the Eight MELAS Patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Heteroplasmy of whole pancreas (%)	72	32	53	63	48	25	45	not detected

For quantification of the islets, the total section area of 1 cm^2 was investigated for each individual block, and the number of islets was counted and the largest diameter of each islet was determined under a microscope. The numbers of A cells and of B cells were estimated by counting hormone-positive cells per 1 cm^2 area. The existence of apoptosis was investigated by TUNEL method using an Oncor Apop Tag In situ Detection kit (Oncor, Inc. Gaithersburg, Germany) as manufactures' instruction. Although it is true that these immunohistochemical methods may sometimes fail on formalin fixed paraffin embedded samples, we have recently applied these methods to the similarly prepared blocks from diabetic OLETF rat (11), in which we successfully demonstrated apoptosis in the pancreas.

Detection of the 3243 bp mutation from total pancreata and islets. DNA was extracted from tissues using a modified phenol-chloroform extraction method (12,13). The segments spanning nucleotides at position 3243 were amplified by PCR using ^{32}P-labeled primers (5′-GCCTTCCCCCGTAAATGATA-3′, 3163-3182; 5′-AGGTTGGCCATGGG-TATGTT-3′, 3323-3304) and resulted in 160 bp fragments. The cycling parameters were: denaturing for 2 min at 94°C; annealing for 2 min at 45°C, and extension for 2 min at 72°C for 35 cycles, with a final extension for 7 min at 72°C. PCR products with the 3243 bp mutation were digested into two 80 bp fragments with Apa I at 37°C overnight. The reaction products were electrophoresed on 8% polyacrylamide gel and the radiolabeled bands of 160 bp and 80 bp were counted in a Fuji BAS 2000. We used the binomial correction factor determined by Schoffner et al. (14) to avoid underestimation of the proportion of mutant mitochondrial DNA. Islets were picked up from a section of patient 7 by microdissection under a microscope (Fig. 1a), and DNA was extracted separately from the islets and the remaining exocrine pancreas and subjected to mutation detection.

RESULTS

Molecular Evaluation of the 3243 bp Mutation in Pancreata Specimen

PCR fragments from pancreata blocks generated 80 bp fragments of DNA after ApaI digestion, indicating that a portion of mtDNA contained the 3243 bp mutation. Sequencing of the amplified fragments confirmed the presence of this mutation in these cases (data not shown). The degree of heteroplasmy was then determined for each pancreas (Table 2). In patient 7, the degree of heteroplasmy in acinar cells (exocrine) and islet cells (endocrine) was 45% and 43%, respectively (Fig. 1b), suggesting that there may not be big difference between these two cell types in this regard.

Pathological Findings

Pathological findings of each pancreas in hematoxylin-eosin and immunohistochemical staining are

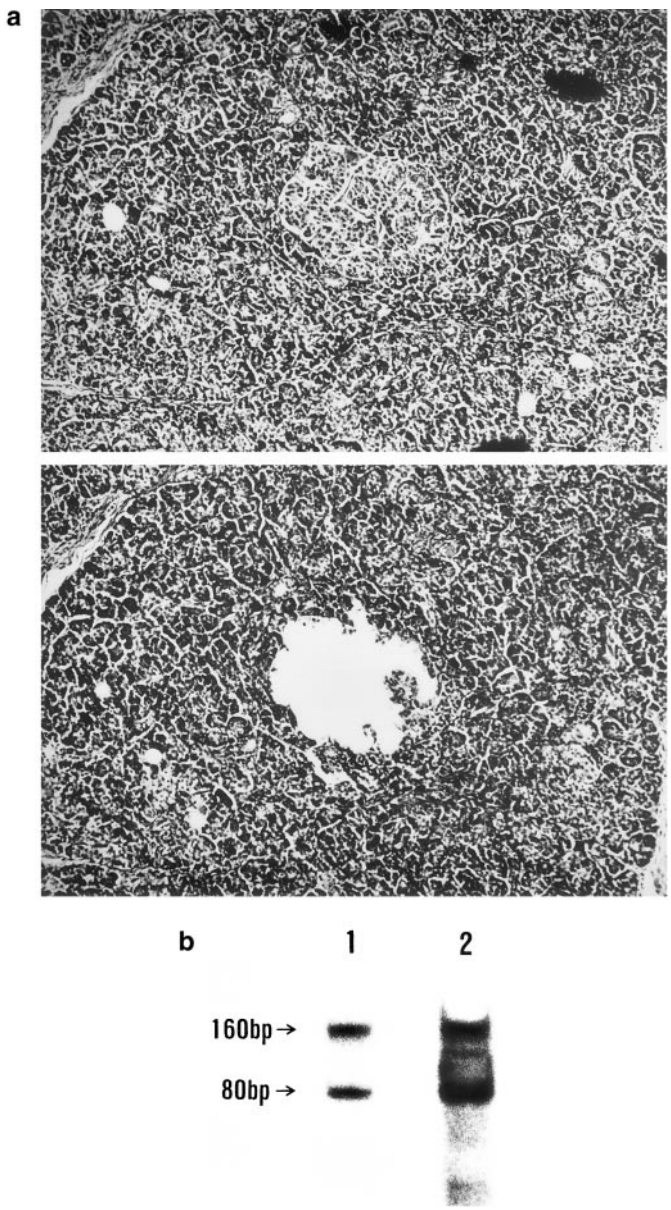

FIG. 1. (a) The islet of patient 7 was stained with hematoxylin and eosin staining (above). The islet was picked up by a microdissection method (below). (b) Identification of the 3243 bp mutation from the pancreas specimen. Lanes 1 and 2 represent islets and remaining exocrine cells, respectively. ^{32}P-labeled PCR products (160 bp) is digested with Apa I. The presence of mutant mitochondrial DNA resulted in the formation of 80 bp fragment. The heteroplasmy rate was 43% (lane 2) and 45% (lane 2), respectively.

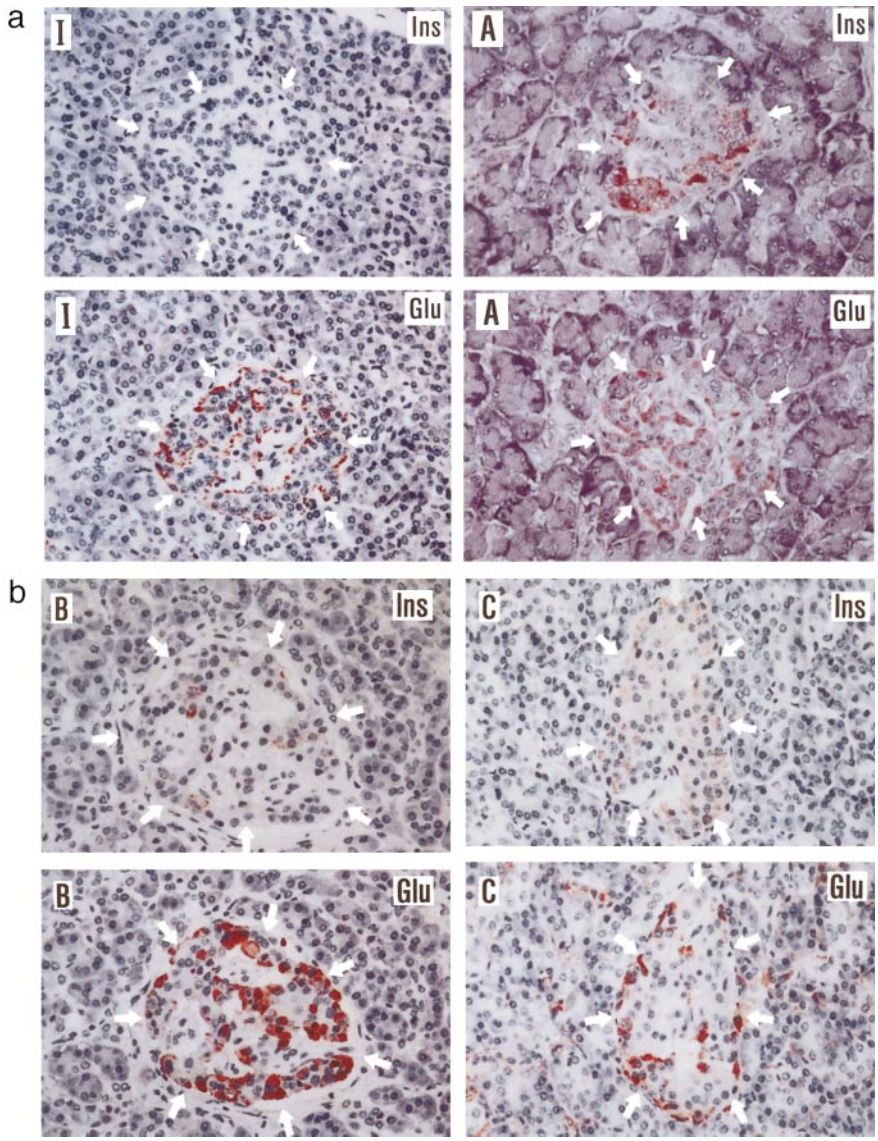

FIG. 2. (a) Immunohistochemical staining for insulin (ins) and glucagon (glu). An IDDM patient (I) (x200) who died at the age of 35, with a four-month duration of diabetes mellitus as a control subject. Patient 1 (A) (x400). (b) Immunohistochemical staining for insulin (ins) and glucagon (glu). Patient 6 (B) (x200) and patient 7 (C) (x200).

summarized in Figs. 2a, 2b, 3, and 4. Figures 2a and 2b demonstrated the atrophy or degeneration of islets. The number of islets per 1 cm^2 area of each section was decreased in all the MELAS patients in comparison with the non-diabetic subjects (Fig. 4). It seems that islets of larger size were relatively preserved (Fig. 3). The total number of B cells in any of the MELAS patients, as well as the IDDM patient, was reduced. The number of A cells was also reduced in all the MELAS patients (Fig. 4). No cells were stained with anti-leukocyte antigen sera, suggesting the absence of lymphocyte infiltration, and TUNEL method did not demonstrate the existence of apoptosis.

DISCUSSION

A relatively high proportion of MELAS patients studied here (five of the eight) had definite diabetes mellitus treated with insulin, and four of the five with diabetes had suffered from hearing loss. Although MIDD patients with this mutation are not generally accompanied by MELAS, we could well expect that the results shown here could be extrapolated to MIDD patients, since the patients with MELAS and diabetes mellitus shown here shared almost all the clinical features of patients with MIDD (5, 7, 15).

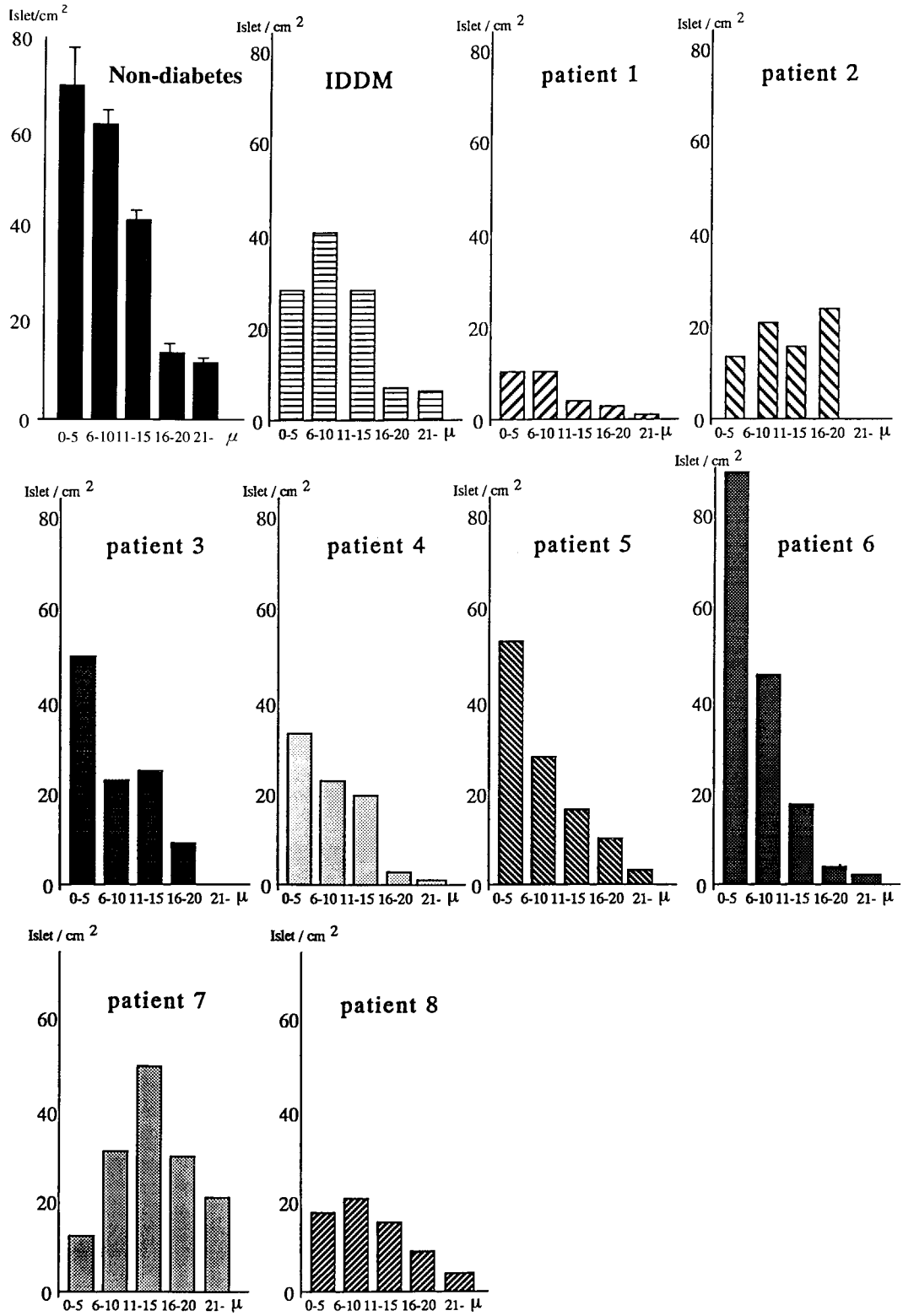

FIG. 3. The number of islets per 1 cm^2 area of each section. The longest distance of each islet was measured, and all islets were classified according to the following sizes: 0-5, 6-10, 11-15, 16-20, and 21- μm. The squared area demonstrated the number of islets.

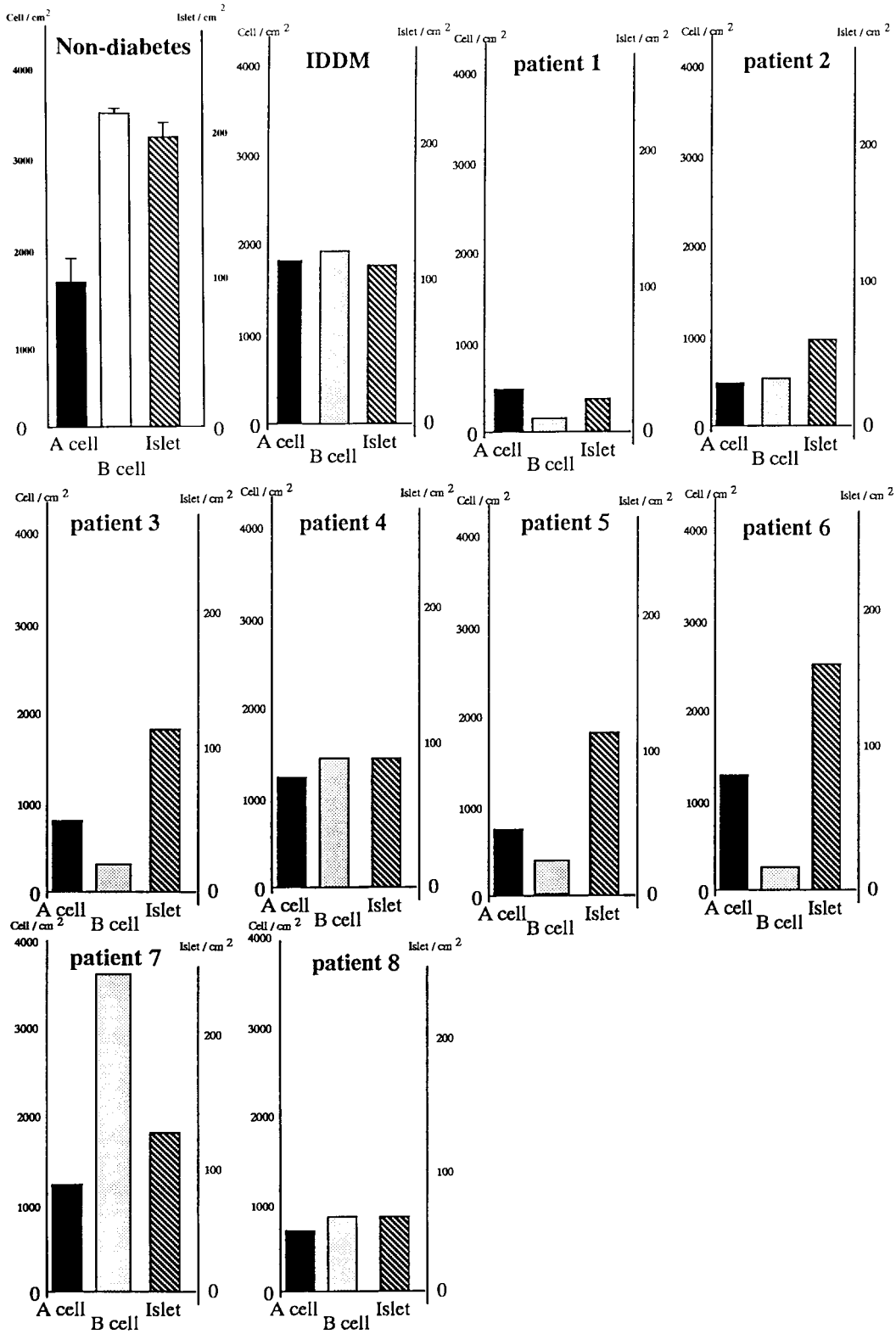

FIG. 4. The total numbers of A cells (black), B cells (white), and islets (shadow) per 1 cm^2 area of each section.

The pancreatic islets from general NIDDM patients showed a variety of histological features (16), and the most striking finding of these pancreata with the 3243 bp mutation was the markedly reduced number of insulin-positive cells. The reason for this is unclear. It is possible that pancreatic B cells are highly sensitive to mitochondrial dysfunction, which we have recently suggested in a mouse pancreatic B cell line, βHC9, using ethidium bromide to inhibit mitochondrial function (17). Glucose not only regulates insulin release (18), but is also known to stimulate the growth of B cells (19,20). Mitochondrial dysfunction may blunt trophic effects of glucose or its metabolites (including ATP) and lead to the reduction in the number of B cells (insulin-positive cells).

We also observed a decrease in A cells, which might differentiate this subtype of diabetes from IDDM caused by autoimmunity against B cells. This is consistent with the report by Kishimoto et al. (21) which suggested a defect in A cell function in diabetic patients with this mutation. Our results are also consistent with another recent paper (22), which reported reduced number of both B cells and non-B cells in autopsy pancreata of one clinically insulin-dependent patient harboring the 3243 bp mutation.

It was originally hypothesized that mutations in mitochondrial DNA in the pancreatic B cells could trigger autoimmune destruction of these cells, resulting in reduced insulin secretory capacity (23,24), but subsequent reports have been rather controversial on the involvement of autoimuunity (4,25). In this study, we did not get any evidence that autoimmune destruction of pancreatic islets plays a major role in the pathogenesis of diabetes with this mutation.

For mitochondrial mutations, it is well known that the degree of heteroplasmy is different among tissues (26). Indeed in patient 2, we could demonstrate the existence of the 3243 bp mutation in pancreas, while it was not detected from peripheral blood. It has been suggested that pancreatic islets, like neuronal cells or muscle, contain a higher rate of mutated DNA. In patient 7, the degree of heteroplasmy in islets was almost the same as that in exocrine cells (43% vs. 45%). Although we were unable to pick up atrophic islets from other subjects, this suggests that pancreatic islets are indeed rich in mutated DNA and that there may be little, if any, difference in the degree of heteroplasmy between islets and exocrine cells.

In this study, there was no clear correlation between the percentage of heteroplasmy in total pancreas and clinical features or histological findings. This apparent discrepancy suggested that insulin secretory capacity may not be determined solely by the number of B cells. The reason for the discrepancy might also be due to other genetic backgrounds or environmental factors, as suggested recently for diabetes mellitus associated with mitochondrial mutation (27).

In conclusion, we have successfully demonstrated the 3243 bp mutation in the formalin fixed paraffin embedded blocks, and performed molecular and pathological studies on pancreas and pancreatic islets with this mutation. Identification of the mechanism leading to the reduced number of islet cells would open the door to a new therapy for subjects with the 3243 bp mutation, which may consist approximately 1% of diabetic patients (5,6,8).

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