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Accumulation of pathogenic ΔmtDNA induced deafness but not diabetic phenotypes in mito-mice

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Abstract

Mito-mice carrying various proportions of deletion mutant mtDNA (ΔmtDNA) were generated by introduction of the ΔmtDNA from cultured cells into fertilized eggs of C57BL/6J (B6) strain mice. Great advantages of mito-mice are that they share exactly the same nuclear-genome background, and that their genetic variations are restricted to proportions of pathogenic ΔmtDNA. Since accumulation of ΔmtDNA to more than 75% induced respiration defects, the disease phenotypes observed exclusively in mito-mice carrying more than 75% ΔmtDNA should be due to accumulated ΔmtDNA. In this study, we focused on the expressions of hearing loss and diabetic phenotypes, since these common age-associated abnormalities have sometimes been reported to be inherited maternally and to be associated with pathogenic mutant mtDNAs. The results showed that accumulation of exogenously introduced ΔmtDNA was responsible for hearing loss, but not for expression of diabetic phenotypes in mito-mice.
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Human mtDNAs carrying point mutations or large-scale deletions have been shown to be closely associated with clinically distinct syndromes of mitochondrial diseases [1,2]. These mtDNA mutations were proved to be pathogenic by co-transmission of the mutant mtDNAs and respiration defects from patients to mtDNA-less human cells [3–6]. However, these findings did not necessarily show that the in vivo occurrence of respiration defects was due to the mutant mtDNAs. Moreover, even when these mutations induced respiration defects in tissues, there was no unequivocal direct evidence that these respiration defects could induce various clinical phenotypes of mitochondrial diseases.

For resolving these problems, we recently generated disease model mice carrying pathogenic mutant mtDNA by introducing mouse mutant mtDNA with a 4696-bp deletion (ΔmtDNA) from somatic cells into mouse zygotes [7]. These mice, named mito-mice [8], showed that exogenous ΔmtDNA accumulated in tissues induced respiration defects and resultant expression of disease phenotypes characteristic of mitochondrial diseases simultaneously [7,9]. Therefore, mito-mice provided

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direct evidence that respiration defects induced by accumulation of mtDNAs carrying pathogenic mutations are alone sufficient for expression of the clinical phenotypes observed in patients with mitochondrial diseases.

On the other hand, it has been reported that the presence of those mtDNA mutations was not restricted to patients with mitochondrial diseases, but extended to normal, aged subjects [10,11] and to pedigrees with maternally transmitted diabetes mellitus and/or deafness [12–15]. There was also a case report showing association of ΔmtDNA and sporadic diabetes [16]. Moreover, an A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene of mtDNA [17], which has frequently been observed in patients with mitochondrial diseases, was identified in more than 1% of diabetic patients [18]. Considering that mammalian mtDNAs are inherited strictly maternally [19,20], these observations suggested that mutations in mtDNAs are also responsible for the pathogenesis of these common disorders [1,2,21].

This study was aimed at providing direct evidence for the involvement of mtDNA mutations in the pathogenesis of these common disorders. For this mito-mice were again generated by introduction of ΔmtDNA from cultured mouse cells into fertilized eggs of B6 strain mice. However, we obtained the unexpected results that mito-mice carrying predominantly ΔmtDNA expressed hearing loss, but did not express diabetic phenotypes. These observations suggest that mtDNA mutations are not responsible for, or alone not sufficient for, the clinical expression of diabetes.

Materials and methods

Mice. Mice carrying ΔmtDNA, mito-mice, were generated by introduction of ΔmtDNA from cultivated cells into fertilized eggs of B6 strain mice using cell fusion techniques as described previously [7]. Male mito-mice carrying various proportions of ΔmtDNA were used for the study. The proportion of ΔmtDNA in mito-mice was deduced from tail DNA samples, because it was very similar in all the tissues of the same individual mice [7,9].

B6 strain mice without ΔmtDNA were used as normal controls in hearing and diabetic analyses. The streptozotocin (STZ)-treated mice (100 mg/kg body weight) and high fat diet mice (40% lipid) were used as disease controls of type I and type II diabetes, respectively.

Quantitative estimation of ΔmtDNA. Southern blot analysis was carried out as described previously [7]. Briefly, total DNA (2.0–3.0 μg) extracted from tail samples was digested with the restriction enzyme *Xho*I. Restriction fragments were separated in 1.0% agarose gel, transferred to a nylon membrane, and hybridized with [α-^{32}P]dATP-labeled mouse mtDNA probes. The membrane was washed and exposed to an imaging plate for 2 h and radioactivities of fragments were measured with a bioimaging analyzer, Fujix BAS 2000 (Fuji Photo Film).

Hearing analysis. Mice were anesthetized with sodium pentobarbital (70 mg/kg) delivered intraperitoneally and maintained in a headholder within an acoustically and electrically insulated and grounded test room. Stainless-steel needle electrodes were placed on the tympanic bulla (positive lead) and scalp vertex (negative lead). The auditory brainstem response (ABR) was measured using an evoked potential recording system (NEC). Acoustic stimuli evoked by a click were delivered to the mice through a loudspeaker. The peak amplitude was measured as the peak-to-trough and the threshold was defined as 1 μV.

Blood glucose and insulin measurements. For determination of fasting blood glucose concentrations, tail vein blood was prepared from male mito-mice carrying various proportions of ΔmtDNA, B6 mice without ΔmtDNA, and diabetic control mice after overnight starvation using an automatic glucometer (DEXTER-Z, BAYER). Non-fasting blood insulin concentrations in tail vein blood prepared from male mito-mice carrying various proportions of ΔmtDNA and B6 mice without ΔmtDNA were determined using rat insulin EIA system (Amersham Biosciences). The blood samples were centrifuged immediately and sera were stored at −20 °C.

Glucose tolerance tests. Male mito-mice with various proportions of ΔmtDNA and B6 mice without ΔmtDNA were starved overnight, and their body weights were measured. Then, glucose (1.5 g/kg body weight) was orally administered to the mito-mice. Blood glucose concentrations in mito-mice and B6 mice were measured with an automatic glucometer (DEXTER-Z, BAYER) on 5, 15, 30, 60, and 120 min after the glucose administration.

Static incubation experiments on islets. Pancreatic islets were isolated from pancreatic tissues of mito-mice carrying 12.9%, 18.1%, 21.2%, 81.2%, 82.6%, and 87.2% ΔmtDNA in their tails and B6 mice without ΔmtDNA using a collagenase digestion technique. Isolated islets were cultured for 12–16 h in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Size-matched islets were preincubated in Krebs–Ringer bicarbonate buffer containing 0.2% BSA (equilibrated in an atmosphere of 95% O_2 and 5% CO_2, pH 7.4) for 60 min at 37 °C. After preincubation, 10 islets were collected and incubated with glucose (2.8, 16.7 or 33.0 mM) or 20 mM KCl. Then, the supernatant was collected and stored at −20 °C until examination of immunoreactive insulin (IRI). The IRI was determined using rat insulin EIA system (Amersham Biosciences).

Determination of mtDNA contents of isolated islets. After static incubation experiments, the islets were collected and the proportions of wild-type and ΔmtDNA were determined by real-time detection PCR (RTD-PCR). RTD-PCR was carried out for quantification of ΔmtDNA and wild-type mtDNA in islets isolated from mito-mice carrying 12.9%, 18.1%, 21.2%, 81.2%, 82.6%, and 87.2% ΔmtDNA in their tails. The primer set and probe specific for ΔmtDNA were nucleotide positions 7668–7697, 12,488–12,464, and 7699–7733, respectively. The primer set and probe specific for wild-type mtDNA were nucleotide positions 11,933–11,952, 12,076–12,047, and 12,012–12,037, respectively. The reporter dye FAM and the quencher dye TAMRA were attached to the 5′ end and 3′ end of each probe. For protein digestion, isolated islets were incubated at 55 °C overnight in 300 μl PCR buffer/non-ionic detergent and proteinase K solution (50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 100 mg/ml proteinase K), and proteinase K was inactivated at 95 °C for 15 min. Volumes of 1 μl of samples were used directly as RTD-PCR templates. For quantification of ΔmtDNA and wild-type mtDNA, RTD-PCR was carried out using a TaqMan PCR reagent kit for the sequence detector system of ABI Prism 7700 under the conditions recommended by the manufacturer (Applied Biosystems).

Immunohistochemical analysis. Pancreatic tissues from mito-mice carrying 15.7%, 20.4%, 52.8%, 54.9%, 85.0%, and 89.5% ΔmtDNA in their tails were fixed in 10% formaldehyde solution. Serial paraffin sections (6 μm) of the pancreatic tissues were stained by indirect immunostaining using guinea pig anti-insulin, rabbit anti-glucagon, and rabbit anti-somatostatin antibodies (Dako) followed by secondary antibodies, the respective animal's rhodamine-conjugated goat anti-IgGs (H + L) (Jackson Immunoresearch Laboratories). Stained specimens were viewed in a Leica HC microscope (Leica). Using the samples stained with anti-insulin antibody, we calculated average diameters of sectioned areas of pancreatic islets.

Biochemical analysis of COX activity. We estimated relative COX activities in cultured cells [7] with various proportions of ΔmtDNA and in livers and kidneys from mito-mice. The COX activity was measured as previously described [22].

Statistical analysis. We analyzed data with the unpaired Student's *t* test. Values with $P < 0.05$ were considered significant.

Results

Analyses of hearing abnormalities in mito-mice

Hearing abnormalities were examined using three groups of mito-mice, groups 1–3, which accumulated different proportions of ΔmtDNA in their tails. Groups 1, 2, and 3 were male mito-mice (6 months old) carrying 5.9%, 6.0%, 12.0%, 14.7%, and 16.2% ΔmtDNA ($n = 5$), 55.0%, 57.9%, 61.5%, 63.7%, and 68.0% ΔmtDNA ($n = 5$), and 81.0%, 84.7%, 85.0%, 86.6%, and 87.4% ΔmtDNA ($n = 5$), respectively, in their tails. The proportions of ΔmtDNA in tissues could be deduced from tail samples without sacrificing the mice, because they were very similar throughout all the tissues of individual mice [7,9].

All mito-mice share the same nuclear background as that of B6 strain mice, and thus the genetic differences of these mito-mice groups were limited to the proportions of exogenously introduced ΔmtDNA. The proportion of ΔmtDNA in group 3 mito-mice was sufficient for induction of respiration defects, and resultant expression of disease phenotypes, whereas those in group 1 and 2 mito-mice were not. Therefore, the mito-mice in groups 1 and 2 could be used as negative controls.

First, we recorded ABR evoked by clicks of 25–100 dB SPL using group 1–3 mito-mice. All mito-mice did not show hearing loss on 3 months after birth (data not shown). Subsequently, expression of hearing loss was exclusively observed in group 3 mito-mice 6 months after birth. Fig. 1A shows the ABR recordings of a group 1 mouse (Mouse 6), group 2 mouse (Mouse 55), and group 3 mouse (Mouse 87) carrying 6.0%, 55.0%, and 87.4% ΔmtDNA, respectively, in their tails. Similar results were obtained with the other mito-mice. These observations suggested that significant hearing loss was limited to group 3 mito-mice carrying a predominant amount of ΔmtDNA.

As shown in Fig. 1B, the average ABR thresholds of mito-mice in groups 1, 2, and 3 were 45.6 ± 5.3, 49.4 ± 6.7, and 82.0 ± 5.7 dB, respectively. These observations suggested that expression of a severe clinical phenotype of deafness was limited to group 3 mito-mice carrying a predominant amount of ΔmtDNA, although we could not precisely determine where the problem was located (the inner ear, the nerve, or the brainstem). Considering that mito-mice possess the same nuclear background, these results provided convincing evidence that accumulation of ΔmtDNA and resultant expression of respiration defects were responsible for phenotypic expression of the significant hearing impairments in group 3 mice.

Analyses of diabetic abnormalities in mito-mice

Since the maternally inherited form of deafness is often associated with diabetes mellitus [12–15], we examined the possibility that accumulation of ΔmtDNA is also responsible for expression of diabetic phenotypes. For examination of this point, we again prepared three groups (groups 1–3) of 6-month-old mito-mice. They carried 12.9%, 15.7%, 18.1%, 20.4%, and 21.2% ΔmtDNA (group 1, $n = 5$), 52.8%, 54.9%, 60.3%, 65.2%, and 66.5% ΔmtDNA (group 2, $n = 5$), and 81.2%, 82.6%, 85.0%, 87.2%, and 89.5% ΔmtDNA (group 3, $n = 5$) in their tails (Fig. 2A). Group 3 mito-mice possessed sufficient ΔmtDNA for induction of respiration defects, but group 1 and 2 mito-mice did not (Fig. 2C). Thus, group 3 was used as the experimental group, and groups 1 and 2 as negative controls.

First, we measured the fasting blood glucose concentration of the three groups ($n = 15$) carrying various proportions of ΔmtDNA in their tails, because its increase is one of the primary symptoms of clinical expression of diabetes mellitus. We furthermore used B6 strain mice as normal controls, whereas STZ-treated mice and high fat diet mice were used as type I and type II diabetic controls, respectively (Table 1). If accumulation of ΔmtDNA and resultant expression of respiration defects were responsible for clinical expression of diabetes mellitus, the fasting blood glucose levels should increase in mito-mice carrying predominant amount of ΔmtDNA. However, the results showed that concentrations of fasting blood glucose unexpectedly decreased in mito-mice carrying higher proportions of ΔmtDNA (Fig. 2A). The same results were also obtained in 12-month-old mito-mice ($n = 8$; Fig. 2B).

Statistic analysis showed that decreased concentrations of fasting blood glucose were exclusively observed in group 3 mito-mice possessing sufficient ΔmtDNA for induction of respiration defects (Table 1). On the other hand, diabetic control mice for types I and II showed progressive increase of the fasting blood glucose levels (Table 1). These results suggested that accumulation of ΔmtDNA induced reduction of fasting blood glucose concentrations.

These observations predicted higher, rather than lower, blood insulin levels in mito-mice carrying predominant amount of ΔmtDNA. As expected, higher levels of the blood insulin concentration were exclusively observed in group 3 mito-mice (Fig. 3A). Then, glucose tolerance tests were carried out in the groups 1–3 to examine the sensitivity of target organs and peripheral tissues to glucose loading. In all groups, blood glucose concentrations were increased by glucose loading and then decreased to fasting levels, and the

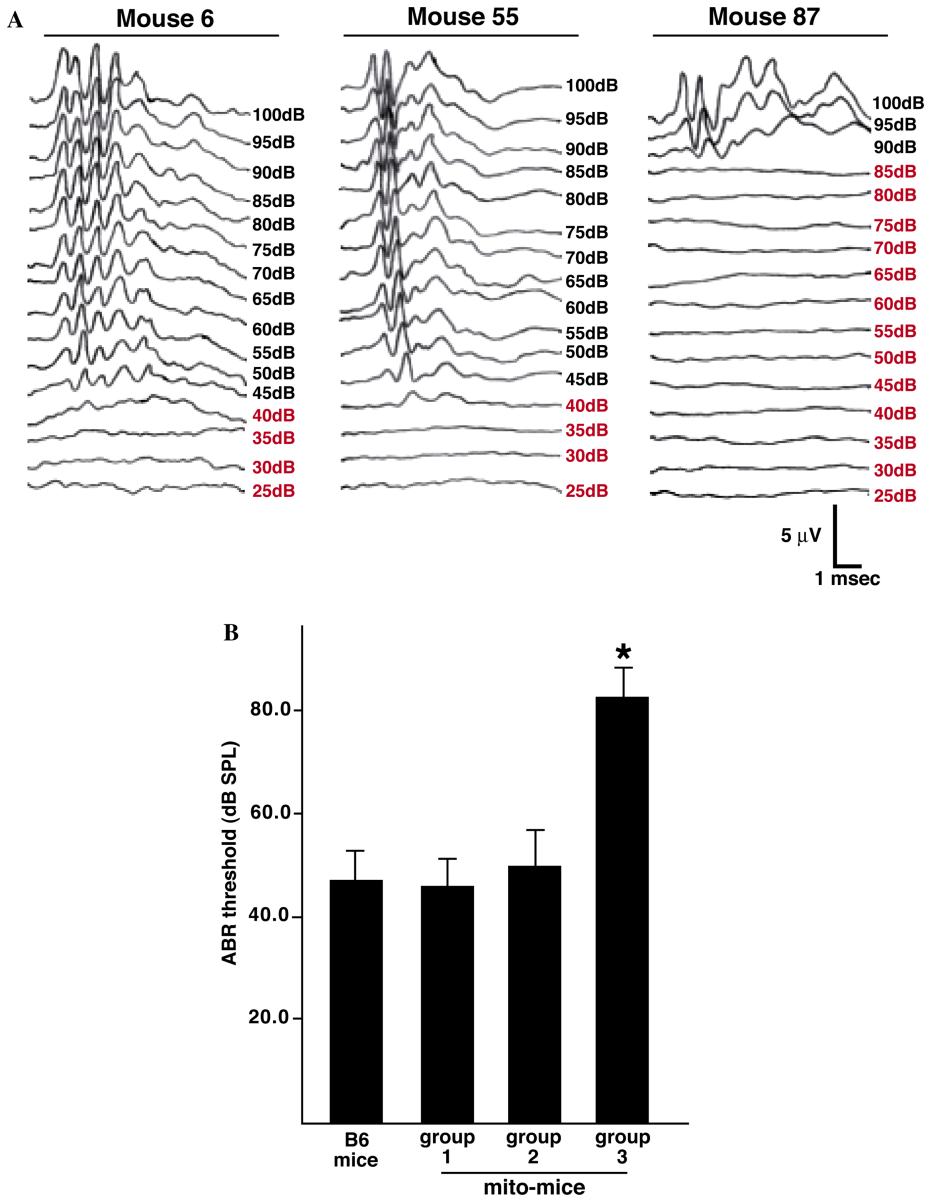

Fig. 1. Analysis of hearing properties in mito-mice. Six-month-old mito-mice with 5.9%, 6.0%, 12.0%, 14.7%, 16.2%, 55.0%, 57.9%, 61.5%, 63.7%, 68.0%, 81.0%, 84.7%, 85.0%, 86.6%, and 87.4% ΔmtDNA in their tails were used in the experiments. (A) ABR recordings of mito-mice carrying 6.0% (Mouse 6), 55.0% (Mouse 55), and 87.4% (Mouse 87) ΔmtDNA in their tails. ABR signals were not obtained at the sound intensities (dB SPL) shown in red. (B) Average ABR thresholds. The mito-mice were divided into three groups, carrying 5.9–16.2% ΔmtDNA (group 1, $n = 5$), 55.0–68.0% ΔmtDNA (group 2, $n = 5$), and 81.0–87.4% ΔmtDNA (group 3, $n = 5$). Their average ABR thresholds were 45.6 ± 5.3, 49.4 ±;6.7, and 82.0 ± 5.7 dB, respectively. The average ABR threshold of B6 mice ($n = 5$) was 47.0 ± 5.7 dB. Asterisks indicate a P value less than 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

group 3 mito-mice showed higher sensitivity to glucose loading (Fig. 3B). These results suggested that increased insulin secretion observed in group 3 mito-mice (Fig. 3A) was not due to insulin resistance of target organs and peripheral tissues. Moreover, our results are consistent with the idea that the low fasting blood glucose concentration (Fig. 2A) and increased sensitivity to glucose loading observed exclusively in group 3 (Fig. 3B) were caused by an increased concentration of blood insulin (Fig. 3A).

Then, we examined whether the higher blood insulin level in group 3 mito-mice (Fig. 3A) was due to higher insulin secretion by individual islets. Pancreatic islets were isolated from group 1 mito-mice carrying 12.9% (Mouse 13), 18.1% (Mouse 18), and 21.2% (Mouse 21) ΔmtDNA and from group 3 mito-mice carrying 81.2% (Mouse 81), 82.6% (Mouse 83), and 87.2% (Mouse 87) ΔmtDNA, respectively, in their tails. By the use of size-matched islets, we quantitatively estimated the amount of insulin released from the isolated islets by glu-

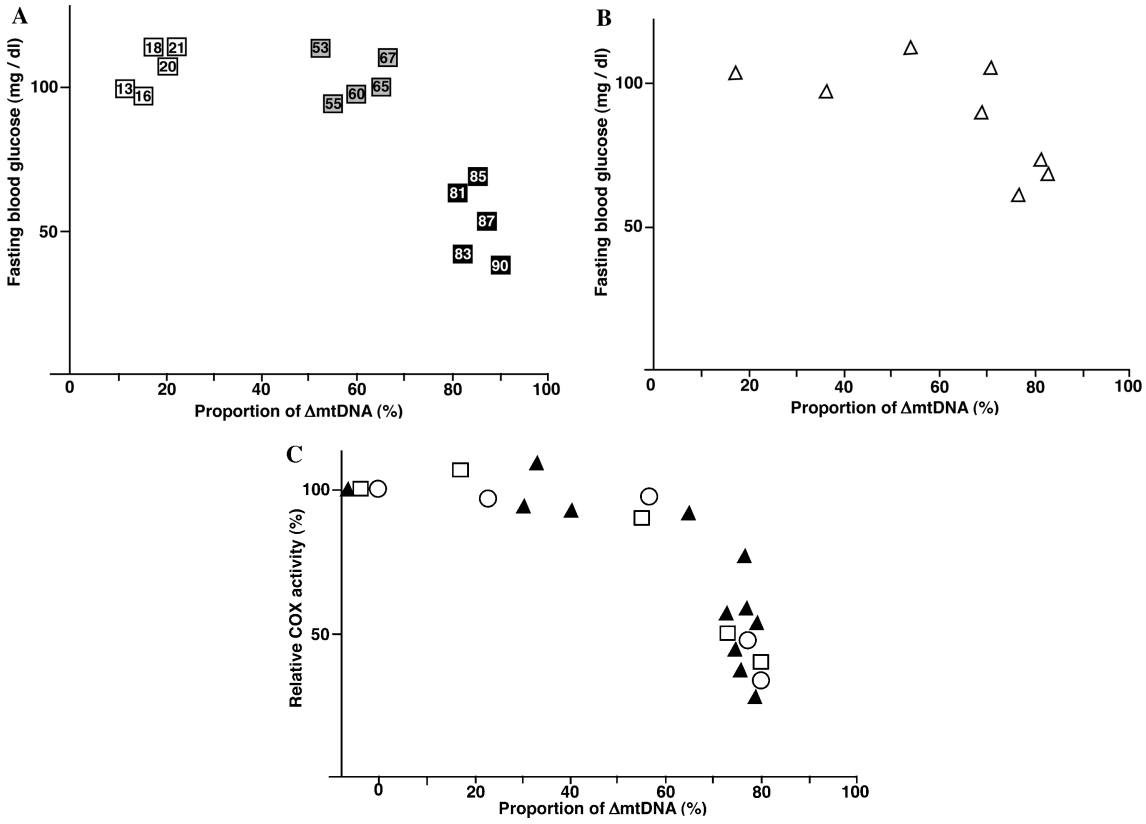

Fig. 2. Fasting blood glucose concentrations in mito-mice. Six- and 12-month-old mito-mice were used in the experiments. (A) Fasting blood glucose concentrations of 6-month-old mito-mice carrying 12.9–89.5% ΔmtDNA in their tails ($n = 15$). Groups 1, 2, and 3 were mito-mice carrying 12.9%, 15.7%, 18.1%, 20.4%, and 21.2% ΔmtDNA (open squares), 52.8%, 54.9%, 60.3%, 65.2%, and 66.5% ΔmtDNA (gray squares), and 81.2%, 82.6%, 85.0%, 87.2%, and 89.5% ΔmtDNA (closed squares), respectively. Numbers in the squares represent the proportion of ΔmtDNA. (B) Fasting blood glucose concentrations of 12-month-old mito-mice carrying 16.2–82.1% ΔmtDNA in their tails ($n = 8$). (C) Effect of the amount of ΔmtDNA on COX activity. ΔmtDNA donor of mouse cell line (closed triangles), livers (open squares), and kidneys (open circles) from mito-mice carrying various proportions of ΔmtDNA were used for biochemical analysis of COX activity. Accumulation of more than 75% ΔmtDNA led to respiration defects irrespective of cells or tissues used for estimation of COX activity.

Table 1
Fasting blood glucose concentrations in mito-mice and control mice

Mouse	Proportion of ΔmtDNA (%)	Fasting blood glucose concentration (mg/dl)
Mito-mice		
Group 1 ($n = 5$)	17.7 (12.9–21.2)	106.4 ± 9.5
Group 2 ($n = 5$)	59.9 (52.8–66.5)	101.8 ± 10.5
Group 3 ($n = 5$)	85.1 (81.2–89.5)	53.6 ± 14.4*
Normal control		
B6 mice ($n = 10$)	0	102.8 ± 12.8
Diabetic control		
STZ-treated mice ($n = 3$) (type I model)	0	413.5 ± 8.5*
High fat diet mice ($n = 3$) (type II model)	0	186.7 ± 22.5*

* P value less than 0.05.

cose stimulation. The results showed that the amounts of insulin released by glucose stimulation increased progressively in the islet prepared from group 3 mito-mice (Fig. 3C). Moreover, the total insulin contents of the islets estimated by addition of 20 mM KCl were also higher in group 3 mito-mice than in group 1 mito-mice (Fig. 3C).

Then, a question is whether pancreatic islets in group 3 mito-mice showed respiration defects or not. Although normal pancreatic islets do not show sufficient respiratory activity to be detectable by currently available procedures, the amounts of ΔmtDNA in the islets were determined by quantitative PCR analysis (Fig. 3D). The results showed that the islets from Mouse 13, Mouse 18, Mouse 21, Mouse 81, Mouse 83, and Mouse 87 contained 9.8%, 12.3%, 19.2%, 76.5%, 78.1%, and 80.4% ΔmtDNA, respectively (Fig. 3D). Moreover, there was no tissue specificity on threshold amount of ΔmtDNA required for induction of respiration defects (75%; Fig. 2C). Therefore, it can be determined that the islets of group 3 mice possessing more than 75% ΔmtDNA expressed respiration defects. Even when 75% ΔmtDNA did not induce respiration defects in the islets, it is certain that 75% ΔmtDNA in the islets is responsible for increased blood insulin contents in

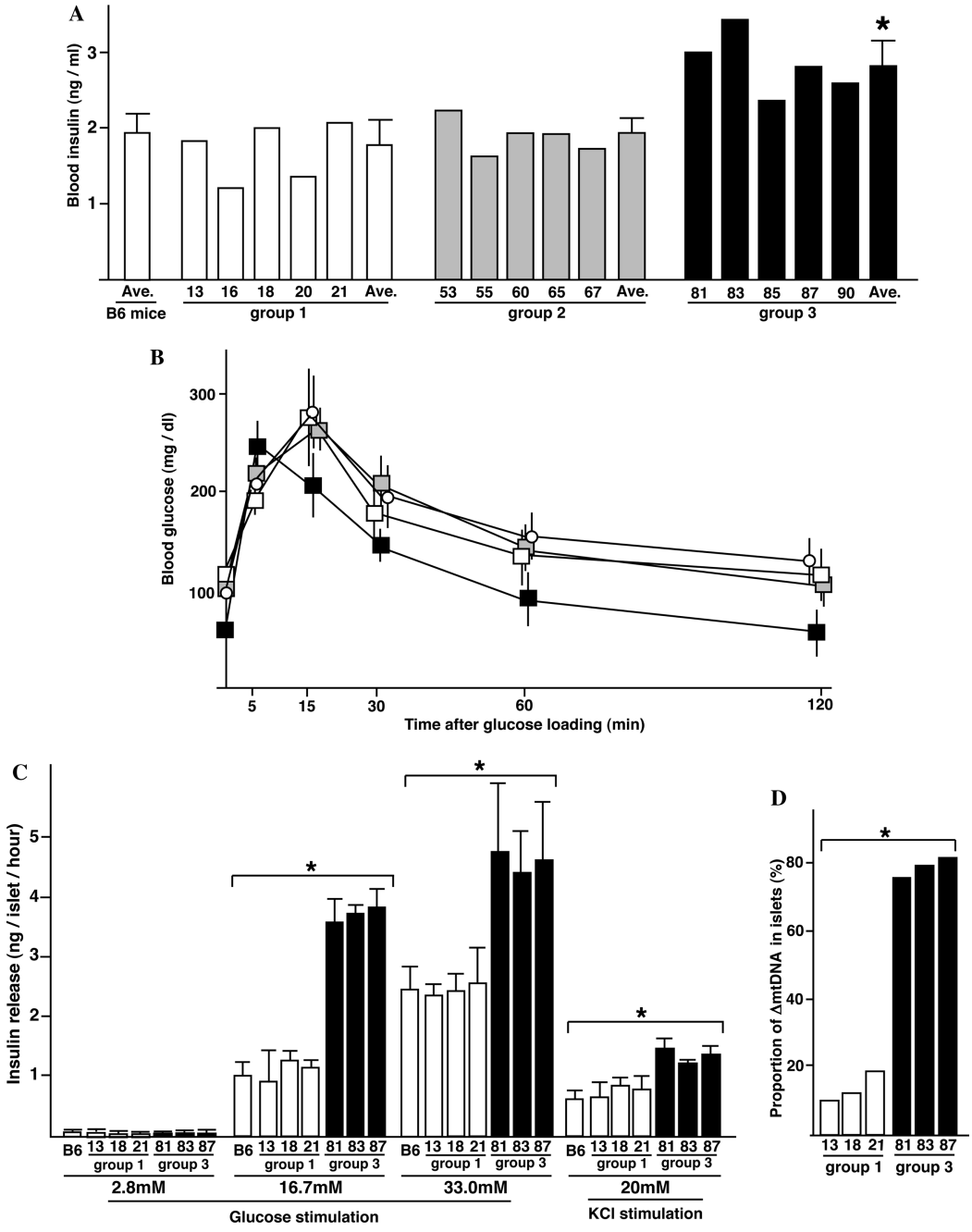

Fig. 3. Analyses of diabetic phenotypes in mito-mice. Three groups of 6-month-old mito-mice carrying 12.9%, 15.7%, 18.1%, 20.4%, and 21.2% ΔmtDNA (group 1, $n = 5$), 52.8%, 54.9%, 60.3%, 65.2%, and 66.5% ΔmtDNA (group 2, $n = 5$), and 81.2%, 82.6%, 85.0%, 87.2%, and 89.5% ΔmtDNA (group 3, $n = 5$) in their tails and B6 mice ($n = 5$) were used in the experiments. (A) Non-fasting blood insulin concentrations in mito-mice groups 1 (open bars), 2 (gray bars), and 3 (closed bars) and B6 mice (open bar, left side). Asterisks indicate a P value less than 0.05. (B) Glucose tolerance tests in groups 1 (open squares), 2 (gray squares), and 3 (closed squares) and B6 mice (open circles). Blood glucose levels were determined 5, 15, 30, 60, and 120 min after glucose injection (1.5 g/kg body weight). (C) Measurements of insulin release in islets isolated from group 1 mito-mice carrying 12.9%, 18.1%, and 21.2% ΔmtDNA (Mouse 13, Mouse 18, and Mouse 21; open bars), from group 3 mito-mice carrying 81.2%, 82.6%, and 87.2% ΔmtDNA (Mouse 81, Mouse 83, and Mouse 87; closed bars), and from B6 mice (open bars). Glucose stimulation was performed at concentrations of 2.8, 16.7, and 33.0 mM. Twenty millimolar of KCl was used to estimate the amount of endogenous insulin in islets. Asterisks indicate a P value less than 0.05. (D) Proportions of ΔmtDNA in islets isolated from group 1 mito-mice (open bars) carrying 12.9% (Mouse 13), 18.1% (Mouse 18), and 21.2% (Mouse 21) ΔmtDNA in their tails and from group 3 mito-mice (closed bars) carrying 81.2% (Mouse 81), 82.6% (Mouse 83), and 87.2% (Mouse 87) ΔmtDNA in their tails. Asterisk indicates a P value less than 0.05. Islets from group 3 mice contained more than 75% ΔmtDNA, which was sufficient for induction of respiration defects, irrespective of whether they included a partially duplicated form or not. Even when partially duplicated form consisting of one wild-type mtDNA and one ΔmtDNA was present in the samples, and gave 25% wild-type mtDNA and 25% ΔmtDNA in this experiment, all remaining 50% should be ΔmtDNA, resulting in expression of respiration defects due to lack of sufficient amount of six deleted tRNAs.

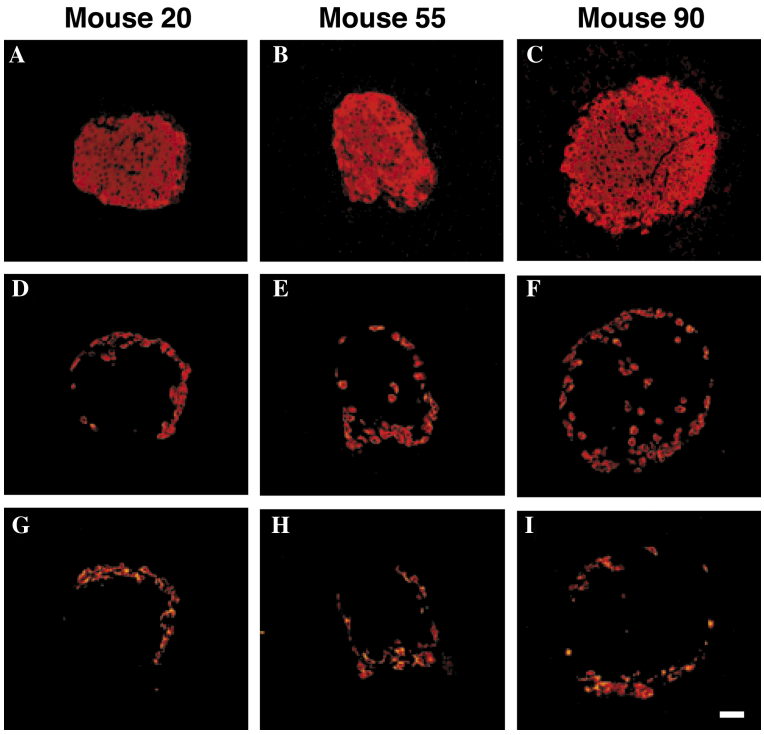

Fig. 4. Immunohistochemical analyses of pancreatic islets from mito-mice. Pancreatic tissues excised from mito-mice carrying 20.4% (Mouse 20) (A, D, and G), 54.9% (Mouse 55) (B, E, and H), and 89.5% ΔmtDNA (Mouse 90) (C, F, and I) in their tails were used for immunostaining with anti-insulin (A, B, and C), anti-glucagon (D, E, and F), and anti-somatostatin antibodies (G, H, and I). The insulin-, glucagon-, and somatostatin-positive cells corresponded to active β-, α-, and γ-cells, respectively. Scale bar, 30 μm.

group 3 mito-mice, considering that all mito-mice share the same nuclear DNA background.

Finally, we used immunohistochemical analyses to examine the amount of β-cells in the islets of mito-mice carrying 15.7% and 20.4% ΔmtDNA (group 1), 52.8% and 54.9% ΔmtDNA (group 2), and 85.0% and 89.5% ΔmtDNA in their tails (group 3). Antibodies against glucagon, insulin, and somatostatin were used for the determination of α-, β-, and γ-cells in the islets, respectively. Typical examples of mito-mice carrying 20.4% (Mouse 20), 54.9% (Mouse 55), and 89.5% ΔmtDNA (Mouse 90) were shown in Fig. 4. In group 3 mito-mice, we observed not only normal islets, but also enlarged islets that showed more than 130 μm in diameter of sectioned area (Figs. 4 C, F, and I). Average diameters of islets of groups 1–3 were 85.3 ± 12.9, 82.9 ± 16.3, and 104.0 ± 31.2 μm, respectively. Probably, slight increase in islet size of group 3 mito-mice was due to increase in the number of insulin-producing β-cells (Fig. 4C). The number of α-cells secreting glucagon was also increased slightly, and some of them became distributed in the central area of the enlarged islets (Fig. 4F), indicating the occurrence of islet hyperplasia in group 3 mito-mice. Since average islet numbers of groups 1–3 were 1.4 ± 0.3, 1.5 ± 0.5, and 1.9 ± 1.2 islets/mm^2, respectively, the immunohistochemical data suggested that the amount of total β-cell mass increased in group 3 mito-mice. The same results were obtained from 12-month-old mito-mice (not shown).

Discussion

Since mito-mice share the same nuclear-genome background, and their genetic variations are restricted to the proportions of pathogenic ΔmtDNA, they could provide unambiguous evidence that the disease phenotypes exclusively observed in the mito-mice carrying accumulated ΔmtDNA are caused by ΔmtDNA-induced respiration defects. Using these mito-mice, we showed that accumulation of ΔmtDNA is responsible for deafness, but not for diabetic phenotypes.

Mito-mice carrying a higher amount of ΔmtDNA did not express diabetic phenotypes as a consequence of higher insulin levels and resultant lower glucose levels in the blood. The number of β-cells in each pancreas was increased in group 3 mito-mice carrying a predominant amount of ΔmtDNA (Fig. 4C), and each β-cell possessed a higher amount of insulin and higher insulin secreting properties on glucose stimulation (Fig. 3C). This led to a higher blood insulin content (Fig. 3A), and increased sensitivity to glucose stimulation (Fig. 3B), followed by decrease in the fasting blood glucose level (Fig. 2A). Therefore, accumulation of the ΔmtDNA

in islets alone is not responsible for expression of diabetic phenotypes at least in mouse species. We cannot yet explain the exact mechanism or biological significance of enhanced insulin secretion from pancreatic β-cells carrying predominant ΔmtDNA. Possibly the increased level of lactic acid in mito-mice expressing respiration defects [7] enhanced insulin secretion, so that accelerated glucose uptake by respiration-deficient target tissues, such as muscle tissues, protected them from progressive glucose deficiency.

These results are unexpected and not consistent with the conventional hypothesis of mitochondrial diabetes, which claims that respiration defects caused by mutant mtDNAs in pancreatic β-cells are responsible for their reduced insulin secretion and resultant expression of diabetic phenotypes [1,2,21]. This concept was based on the following observations. First, a large-scale deletion mutant mtDNA [12], and point mutations in the $tRNA^{Leu(UUR)}$ gene [13,14] and in the $tRNA^{Glu}$ gene of mtDNA [15] were reported in pedigrees with maternally transmitted diabetes mellitus. Second, the point mutation in the $tRNA^{Leu(UUR)}$ gene, A3243G, was identified in more than 1% of diabetes patients [18]. Finally, the ability for glucose-stimulated insulin secretion was totally lost on depletion of mtDNA from pancreatic β-cells both in vitro [23] and in vivo [24].

However, these observations do not necessarily support the hypothesis. An important controversial issue in this hypothesis is that most mtDNA mutations found in pedigrees of maternally transmitted diabetes were not pathogenic. For example, an apparent deletion mutant mtDNA [12] was subsequently found to be identical to one of partially duplicated forms [25], which did not induce respiration defects on their accumulation [26]. A point mutation in the $tRNA^{Glu}$ gene was also shown to be polymorphic by mtDNA transfer from a patient to mtDNA-less human cells [27]. Even the amounts of A3243G mutant mtDNA in autopsy samples of pancreatic islets were less than 63% [28]. This proportion was insufficient to induce expression of diabetic phenotypes, an amount of more than 85% being required to express mitochondrial diseases [29] and to induce respiration defects [5,6]. Then, how could less than 63% A3243G mutant mtDNA induce diabetes in the absence of an affect on respiratory function?

The most important controversial issue in the hypothesis of mitochondrial diabetes is why most maternally transmitted diabetes mellitus is the type II, non-insulin-dependent form [12–15,25]. If clinical expression of diabetes is due to loss of insulin secretion of β-cells or preferential cell death of β-cells by mutant mtDNA-induced respiration defects, the type I, insulin-dependent form, has to be expressed at least in the pedigrees of maternally transmitted diabetes.

Nevertheless, the hypothesis of mitochondrial diabetes appears to be supported by the finding that complete respiration defects in mouse pancreatic β-cells induced diabetic phenotypes. For example, β-cell-specific disruption of the *Tfam* gene, which is required for mtDNA replication, induced mtDNA depletion and a complete respiration defect in mouse pancreatic β-cells followed by expression of diabetic phenotypes [24]. Our previous report also suggested that complete respiration defects induced by mtDNA depletion in cultured mouse β-cells (MIN6 cells) are responsible for the functional absence of their glucose-sensitive insulin secretion [23]. Therefore, a complete respiration defect in β-cells could induce expression of diabetic phenotypes. But there is no report so far of the presence of a sufficient amount of mutant mtDNA for induction of a complete block of respiration in pancreatic islets or β-cells of diabetic patients [12–15,25–28].

The hypothesis of mitochondrial diabetes appears to be also supported by findings that diabetic phenotypes are sometimes inherited maternally, and that family members showed heteroplasmy of wild-type and mutant mtDNA [12–15]. However, it is hard to explain why in most family members pancreatic β-cells consistently accumulate pathogenic mutant mtDNAs, resulting in frequent expression of diabetic phenotypes but not phenotypes associated with mitochondrial diseases. Moreover, the homoplasmic T3394C mutation in the *ND1* gene of mtDNA observed in a patient expressing diabetes and mitochondrial diseases [30] was also observed in a pedigree expressing maternally inherited cardiac abnormalities, but not expressing diabetes [31], suggesting that the specificity of disease phenotypes was determined and controlled by mutations in nuclear genes. In addition, phenotypes that were inherited maternally should not always be due to mutations in mtDNA, because other factors than mtDNA from mothers may be involved in the pathogenesis. In fact, it was recently reported that diabetic phenotypes in non-obese disease model mice were transmitted during pregnancy from mothers to progenies through autoantibodies against β-cells [32], suggesting that apparent maternal inheritance of the disease phenotypes may not necessarily be due to mutant mtDNAs.

None of these observations is inconsistent with our finding that accumulation of the mutant mtDNA is not responsible for, or not sufficient, for clinical expression of diabetes, although we could not completely exclude the possibility that our results were specific cases in mito-mice, and not be generalized to human cases.

Mito-mice consistently express significant hearing impairments on accumulation of ΔmtDNA, providing direct evidence that ΔmtDNA is responsible for the deafness. Recently, Johnson et al. [33] reported that the ABR thresholds of A/J strain mice possessing mutations in the age-related hearing loss (*ahl*) locus were slightly increased by addition of a mutation in

the mtDNA-encoded $tRNA^{Arg}$ gene, suggesting that expression of hearing loss caused by nuclear gene mutation was enhanced by the mtDNA mutation. In this case, however, it was uncertain whether mtDNA mutation in the $tRNA^{Arg}$ gene was pathogenic, and whether the induced respiration defects were responsible for the pathogenesis of hearing loss. Our preliminary experiments, however, showed that the mtDNA mutation in the $tRNA^{Arg}$ gene was polymorphic and not pathogenic, since A/J strain mice carrying the mtDNA mutation in the $tRNA^{Arg}$ gene in homoplasmy did not express respiration defects or clinical abnormalities related to mitochondrial diseases in any tissues examined.

Similar cases of nuclear and mitochondrial cooperation in expression of hearing loss were reported in maternally inherited and non-syndromic congenital deafness [34,35]. The patients carried a pathogenic A1555G mtDNA mutation in the *12S rRNA* gene [35], which we showed to induce very slight respiration defects on aminoglycoside antibiotic treatment [36]. In an Arab-Israeli pedigree, even though the mutant mtDNA was associated with congenital deafness, there were substantial numbers of family members carrying the homoplasmic A1555G mtDNA mutation but lacking the onset of hearing loss, suggesting the possible contribution of environmental factors or mutations in nuclear genes to the pathogenesis [34].

On the contrary, pathogenic mutant mtDNAs, which induced significant respiration defects on their accumulation [3–6], were reported to be responsible for hearing loss as well as mitochondrial diseases [37]. Therefore, all these observations suggested that pathogenic mutations in mtDNA, which induce significant respiration defects, could alone induce hearing loss, whereas some polymorphic or slightly pathogenic mutations in mtDNA require cooperation with nuclear gene mutations for clinical expression of hearing loss.

It should be noticed that the nuclear-genome background of mito-mice is exactly the same as that of B6 strain mice, which possess nuclear gene mutations in the *ahl* locus on chromosome 10, and express hearing loss on aging [38]. In fact, the age-matched ABR thresholds of group 1 and 2 mito-mice carrying insufficient proportions of ΔmtDNA for induction of respiration defects were comparable to those of B6 strain mice, and slightly higher than those of other strain mice without nuclear gene mutations in the *ahl* locus [38]. Therefore, we could not determine whether significant respiration defects caused by ΔmtDNA induced hearing loss in the mito-mice alone, or simply accelerated the age of onset of hearing loss in cooperation with nuclear gene mutations in the *ahl* locus. For study of this problem we are generating mito-mice carrying ΔmtDNA but not nuclear gene mutations that induce hearing loss using nuclear transfer techniques.

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