In the present study, we summarize gene mutations of anticoagulant factors in Japanese patients suffering from deep vein thrombosis (DVT). Consistent with our previous report [10], many Japanese DVT patients had gene mutations of the PS/PC anticoagulant system. Among them, 19 and 8 patients had PS gene (*PROS1*) mutations and PC gene (*PROC*) mutations, respectively. The pathological importance of the PS/PC anticoagulation system in the Japanese population will be discussed.

Subjects and methods

Samples

Venous blood samples (9 mL) were collected in 1 mL of 3.2% (0.105 mol/L) sodium citrate, and platelet-poor plasma was prepared by centrifugation at 1500 × *g* for 10 min at 4°C. Leukocytes were obtained from blood samples by hemolysis. Genomic DNA was extracted using a Sepa Gene kit (Sanko Junyaku, Japan) from the leukocyte fraction and stored at 4°C and −80°C. Plasma for functional analyses was either freshly prepared each time or stored in small aliquots at −80°C until ready for analysis [10].

Clinical features of patients examined

Seven hundred and ninety-four (794) patients with a variety of thrombotic episodes were examined in our laboratory from 1994 to 2004. All subjects examined in this study were unselected patients referred to our laboratory to be evaluated for suspected thrombophilia. Out of these 794 patients, 85 subjects (45 men and 40 women) were confirmed to have DVT. Clinical diagnosis of DVT was confirmed by imaging analyses including computerized tomography, magnetic resonance imaging, scintillation analysis, and angiography, in addition to clinical history and laboratory tests [10]. With some of these DVT patients, we could examine the family history and perform further genetic analyses on the patients' families. The age of the subjects ranged from 10 to 79, with peak distribution (n = 52) occurring in the 20–40 year-old group. Types of DVT were DVT alone, DVT with pulmonary embolism, DVT with mesenteric thrombosis, and DVT with portal vein thrombosis.

Strategy for the systematic examination of thrombophilia

The timing of blood collection was principally the time when the patients were first visiting Kyushu University Hospital. Analyses were mainly done either before anticoagulant treatment or at least 8 days after cessation of treatment [10]. In some cases, the analyses were performed after reduction of doses of anticoagulants during treatment, depending on clinical symptoms. In such cases, multiple analyses were performed at the appropriate intervals and the steady state data were used. The general coagulation–anticoagulation/fibrinolysis status and lupus anticoagulants (LA) were examined by basic laboratory tests [13]. Factors showing reduced activity were assayed by special laboratory tests, and the plasma concentrations of anticoagulation factors (antithrombin (AT), protein C (PC), Protein S (PS), heparin cofactor II (HCII), plasminogen (PLG), fibrinogen, and C4b binding protein (C4BP) were also examined [10]. Factors showing reduced function were investigated by gene analyses after informed consent was obtained.

Methods of basic laboratory tests

Basic laboratory tests were performed to exclude severe liver dysfunction, disseminated intravascular coagulation, and vitamin K deficiency as causes and to evaluate antithrombotic therapy. Pooled normal plasma was prepared from 68 healthy individuals (33 men aged 22–58 years, 35 women aged 23–59 years) and used as a standard or control plasma for screening tests [10].

Anticoagulant activities of PC and PS were determined using the Staclot Protein C kit and the Staclot Protein S kit (Diagnostica Stago, Asnieres, France), respectively. Concentrations of PC, free PS, and total PS were measured by enzyme-linked immunosorbent assay (ELISA) (Asserachrom Protein C and Asserachrom (free/total) Protein S, Diagnostica Stago, Asnieres, France). A chromogenic substrate was used to assay for AT activity as heparin-dependent inhibition of bovine thrombin (heparin cofactor activity) (Chromostrate ATIII kit, Hitachi, Tokyo, Japan). Plasma concentrations of AT and C4BP were determined by liquid-phase nephelometry (N Antiserum to Human Antithrombin III kit, Dade Behring, Tokyo, Japan) and by latex agglutination (Liatest C4b-BP kit, Diagnostica Stago, Asnieres, France), respectively [10,14]. Family members of some patients (18 family members out of 49 patients) were also examined to ascertain whether similar abnormalities were present in them.

Criteria for abnormality

To define the criteria for the reduced activities and plasma concentrations, two-standard deviations (2SD) and three-standard deviations (3SD) from the mean were determined using data from healthy subjects transformed by the parametric minimal skewness method. A level within 2SD was regarded as the reference interval [15], and a level below −3SD was defined as reduced activity in this study.

Gene analyses of aberrant factors

This clinical study has been formally certified by the Institutional Review Board of the Graduate School of

Table 1a
DVT patients having reduced activity of PS, PC and AT

Type of thrombosis	Examined patients	Number of patients having reduced activities of PS and/or PC	Numbers of patients having reduced activity of		
			PS	PC	AT
Number of DVT patients	85	49[a]	40 (18 patients having reduced activities of PS and PC)	27	6
DVT alone	55	30	23	16	2
DVT with PE	23	14	13	8	2
DVT with mesenteric venous thr	1	1	1	0	0
DVT with portal vein thr	6	4	3	3	2

thr: thrombosis, AT: antithrombin, PC: protein C, PS: protein S, DVT: deep vein thrombosis, PE: pulmonary embolism.

[a] Some patients in whom reduced activity was detected were re-examined approximately 1 month after the first examination. The reduced activities of PC and PS were confirmed by repetitive measurements in appropriate intervals. Namely, 15 out of 49 patients were confirmed by duplicate measurements, 23 out of 49 patients were confirmed by triplicate, 5 out of 49 patients were confirmed by four measurements and 6 out of 49 patients were confirmed by more than five measurements.

Medical Sciences, Kyushu University. Prior to the trial, informed consent was individually obtained from all participants by the attending physicians following full explanations of the aim of the research and guarantee of privacy. Genes analyzed were AT (*AT3* exon 1–6) [16], PC (*PROC* exon 1–9) [17], PS (*PROS1* exon 1–15) [18,19], PLG (*PLG* A601T, V355F, D676N) [20–22], C4BP β-chain (*C4BPB* exon 3–8) [23], and HCII (*HC2* exon 1–5) [24].

Genomic DNA was prepared from peripheral blood leukocytes, and the exon and exon–intron boundary regions were amplified by polymerase chain reaction (PCR). The PCR products were subjected to direct sequencing using ABI 377 (Perkin Elmer Applied Biosystems, CA, USA) or analyzed after subcloning into the TA vector. PLG and protein $S_{Tokushima}$ (K155E) were analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP) using restriction enzyme fragments of PCR products. Gene analyses was performed as described previously [19].

Results

Age distribution of patients

Thrombotic factors were examined in 85 patients suffering from DVT. The age of the subjects ranged from below 10 to 79, with peak distribution (n = 52) occurring in the 20–40 year-old group.

Types of DVT patients

The subjects consisted of 55 patients suffering from DVT alone, 23 patients suffering from DVT with pulmonary embolism, 1 patient suffering from DVT with mesenteric thrombosis, and 6 patients suffering from DVT with portal vein thrombosis (Table 1a).

Summary of basic laboratory tests of healthy individuals and of DVT patients

The reference intervals and criteria for the reduced activity of each of the factors are described in "Subjects and methods." A level below −3SD was defined as reduced activity in this study (Table 1b). For healthy individuals, only 1 individual out of 126 (73 men: mean ± SD = 35.4 ± 10.1 years old; 53 female: mean ± SD = 40.5 ± 13.1 years old) had reduced PS activity and 1 out of 95 healthy individuals (48 men: mean ± SD = 32.8 ± 8.6 years old; 47 female: mean ± SD = 40.9 ± 12.7 years old) had reduced PC activity (Table 1b), while for DVT patients, reduced activity in PS and/or PC was found in 49 of the 85 patients (Table 1a). Among DVT patients, 18 had both reduced PS and PC activities. Reduced AT activity was found in 6 of the patients (Table 1a).

To eliminate the effect of warfarin on PS/PC activities, it would be ideal to measure PS and PC activities during a period when patients have ceased taking drugs for at least

Table 1b
Criteria for reduced activity of PS, PC and AT, and number of healthy individuals having the reduced activity

Items	Reference intervals (mean ± 2SD)	Criteria for the reduced activity (below mean − 3SD)	Number of healthy individuals examined	Healthy individuals having reduced activity
Protein S (PS)			126	1
	M: 73–121%	M: below 61%	(M: 73)	(M: 0)
	F: 59–128%	F: below 50%	(F: 53)	(F: 1)
Protein C (PC)	75–131%	below 61%	95	1
Antithrombin (AT)	80–120%	below 70%	95	0

M: male; F: female.

Prevalence of the reduced activity of protein S in the Japanese general population has been reported as 1–2% [36,37].

Table 2
Gene mutations of protein S, protein C, antithrombin identified in patients suffering from DVT

	Patient no.	Age at first thrombosis	Sex	Kinds of DVT	Familial inq.	Activity (%)	Antigen (%)		C4bBP (%)	Previous publication	Nucleotide changes*	Amino acid mutated*
							Free	Total				
Protein S patients examined probands: $n = 39$	PS 1	9	F	DVT	Familial inq.	<10	7	50	148	New	IVS1-1 G > C	–
	PS 2	37	M	DVT, Mesenteric v.	Familial inq.	10	24	58	100	New	IVS13-2 A > G	–
	PS 3	35	M	DVT, PE		33	25	90	142		600 G > T	G54R
	PS 4	40	F	DVT, PE	Familial inq.	34	38	63	105	New	1998 C > T	R520W
	PS 5	19	M	DVT	Familial inq.	28	34	50	130	New	2224 A > G	Y595C
	PS 6	26	F	DVT, pregnancy	Familial inq.	49	56	98	120	New	1041 G > C	E201Q
	PS 7	23	M	DVT		60	73	88	141	New	2206 C > T	T589I
	PS 8	16	M	DVT	Familial inq.	33	22	46	91	New	1789 C > A	A450D
	PS 9	32	F	DVT	Familial inq.	3	36	103	111	New	795 G > T 903 A > G	E119X *K155E* (compound heterozygotes)
	PS 10	39	F	DVT		19	22	84	161	New	1057 G > T	C206F
	PS 11	42	M	DVT, PE	Familial inq.	35	78	94	127		903 A > G	*K155E/ K155E*
	PS 12	23	M	DVT		58	84	84	108		903 A > G	*K155E*
	PS 13	63	F	DVT, Portal v.		38	49	78	80		903 A > G	*K155E*
	PS 14	34	F	DVT, pregnancy		50	75	88	96		903 A > G	*K155E*
	PS 15	22	M	DVT	Familial inq.	6	17	42	103	New	1163 T > G	C241W
	PS 16/ PC 4	13	M	DVT	Familial inq.	21	14	31	43	New	1341 G > T	E301X
	PS 17	66	F	DVT		48	53	79	103		2317 C > T	P626L
	PS 18	16	M	DVT, PE	Familial inq.	11	10	35	106		1268 C > T	R410X
	PS 19	18	M	DVT		32	35	60	84		1860 C > T	R474C
Healthy individuals examined: $n = 47$												No mutation observed
Protein C patients examined probands: $n = 23$	PC 1	25	F	DVT, portal v.		62		73			728 C > T	R169W
	PC 2	24	M	DVT		50		81			1394 G > A	G391S
	PC 3	63	M	DVT		34		31		New	1085 C > A	L288I
	PC 4/ PS 16	13	M	DVT	Familial inq.	18		31		New	IVS2-4 C > T	–
	PC 5	30	F	DVT	Familial inq.	60		90			662 C > T	R147W
	PC 6	65	M	DVT		42		68			671–673 del AAG	K150del
	PC 7	55	F	DVT		59		85			671–673 del AAG	K150del
	PC 8	30	M	DVT		39		97			221 C > T	R-1C
Healthy individuals examined: $n = 30$	PC N1					63		88			671–673 del AAG	K150del observed in an individual
Antithrombin patients examined: $n = 6$	AT 1	41	F	DVT, PE		52		46		New	203 A > T	K11X
	AT 2	64	F	DVT		51		52		New	219 C > A	P16H
Healthy individuals examined: n=24												no mutation observed

*Expression of nucleotide changes is according to the method described in Human Mutation 11, 1–3 (1998). X: stop codon.
*Numbering of nucleotide is according to NCBI reference sequence BC015801 for PS, NM_000312 for PC and NM_000488 for AT, respectively.
DVT: deep vein thrombosis, PE: pulmonary embolism, Mesenteric v.: mesenteric venous thrombosis, Portal v.: portal vein thrombosis, Familial inq.: familial inquiry, M: male, F: female.

8 days. It was impractical to achieve this goal in some patients. However, the activities of PS and PC in 12 patients who had no PS or PC gene mutation were 83 ± 21% of PS (n = 12) and 68 ± 14% of PC (n = 12), respectively, even though they had taken 2–5 mg warfarin per day for years (data not shown). Compared to these values, the means and standard deviations of PS and PC activities in the 18 patients having gene mutations in Table 1a were 24 ± 13% (n = 18) and 29 ± 15% (n = 18), respectively. These patients had also taken 2–5 mg warfarin per day for years. Thus, our criteria for the selection of patients with reduced activities could be regarded as reasonably acceptable.

Gene analyses of aberrant factors

Gene analyses were performed on factors related to the reduced activities in the affected patients. The numbers of individuals examined were 39 for PS, 23 for PC, and 6 for AT. The numbers of patients having gene mutations were 19 for *PROS1*, including 5 individuals with $PS_{Tokushima}$ (K155E) which is a known polymorphism of the PS gene (*PROS1*) in Japan [25]; 8 for *PROC*; and 2 for *AT3*, among DVT patients having reduced activities (Table 2). One patient had mutations in both *PROS1* and *PROC* genes (patient No. PS16 or PC4 in Table 2). It was noted that as much as 22% (19/85) of Japanese DVT patients showed mutations of the *PROS1* gene, and this frequency would more likely be increased if we could have analyzed all of the patients having reduced activity. The frequencies of mutations of *PROS1* and *PROC* genes in Japanese DVT patients were approximately 10 times and 3 times, respectively, higher than those of *PROS1* and *PROC* gene mutations in Caucasian DVT patients [26–28]. The frequency of the *AT3* gene mutation in Japanese DVT patients was not different from that reported in Europe [26,27]. The detected mutations are summarized in Table 2.

PS gene (PROS1) analysis

All the exons and intron–exon boundaries of the *PROS1* gene were analyzed in 39 patients who showed reduced activity. *PROS1* gene mutations were detected in 19 of 39 DVT patients (49%) (Table 2). All of these patients showed heterozygous mutations except for one who was homozygous $PS_{Tokushima}$ (K155E) (patient No. PS11 in Table 2). We also analyzed all the exons and intron–exon boundaries of the *PROS1* gene in 47 healthy subjects and could not find any mutation among the 94 chromosomes (Table 2). These results indicated that the frequency *PROS1* mutation was high in Japanese DVT patients, although there was no particular hot spot within the *PROS1* gene except $PS_{Tokushima}$ (K155E).

PS-Tokushima (K155E)

The $PS_{Tokushima}$ (K155E) is a polymorphism of *PROS1* found in the Japanese population. RFLP analysis of 304 healthy subjects (168 men aged 22–74 years, 136 women aged 23–74 years) showed the K155E mutation in 5 subjects (1.6%, 5/304) (Table 3), consistent with a previous report [25]. Total and free antigen levels of subject PS11 (Table 2), who was homozygous for $PS_{Tokushima}$ (K155E), were 78% and 94%, respectively, which are within the reference intervals, and PS activity was 35%, indicating that the specific activity of the $PS_{Tokushima}$ protein (35/94 = 0.37) is lower than that of the normal PS protein. This result is consistent with results of our expression experiments in HEK 293 cells [29].

Among 39 patients with reduced PS activity, $PS_{Tokushima}$ (K155E) was found in 5 patients with DVT. The odds ratio of $PS_{Tokushima}$ (K155E) frequency in Japanese DVT patients (5 of 85 subjects) against its frequency in healthy individuals (5 of 304 subjects) was 3.74 (95% confidence interval 1.06–13.2) (Table 3), suggesting that this polymorphism is a risk factor for DVT.

PC gene (PROC) analysis

All the exons and intron–exon boundaries of *PROC* were analyzed in 23 patients. PC mutations were detected in 8 out of 23 DVT patients and two of them were new mutations (Table 2). An R169W mutation was found not only in one DVT patient (patient No. PC1 in Table 2) but also in three patients suffering from pulmonary embolism who were not included in this study. We analyzed all the exons and intron–exon boundaries of the *PROC* gene in 30 healthy subjects and did not find the R169W mutation among the 60 alleles (data not shown), suggesting that R169W may not be a common polymorphism but may be a causative mutation in DVT or pulmonary embolism in Japanese individuals. One mutant allele, K150Del, was found in two patients with DVT (patient No. PC6, PC7 in Table 2) and in one individual allele among the 30 healthy subjects (30 year-old female) (Table 2). Whether the K150Del mutation causes reduced PC activity remains to be resolved.

Analyses of AT gene (AT3) and other genes

All the exons and intron–exon boundaries of the *AT3* gene were analyzed in 6 patients (Table 2). Abnormalities of *AT3* were detected in 2 out of 6 patients with DVT, with one having a nonsense mutation and the other a missense mutation. These mutations were not found in our examination of the exons and intron–exon boundaries of the *AT3* gene in 48 alleles of 24 healthy subjects.

We found 3 patients having low PLG activity, 5 patients having low HCII activity, and 2 patients having low C4BP concentration among 85 DVT patients, but could not find

Table 3
Frequency of $PS_{Tokushima}$ (K155E) in healthy individuals and DVT patients

	Numbers of individuals examined	$PS_{Tokushima}$	Odds ratio (95% confidence interval)
Healthy individuals	304	5	1.00
DVT patients	85	5	3.74 (1.06–13.2)

The frequency of $PS_{Tokushima}$ (K155E) in 304 healthy individuals was examined with PCR-RFLP analysis.

any mutations in *PLG*, *HC2*, or *C4BP* genes. Factor V Leiden mutation was not found in any of the individuals examined in the present study.

Family history of patients having gene mutations

In this study, gene analysis was performed also on the family members of 18 patients having gene mutations. Among them, six family members had no mutations of the aberrant factor gene, while family members of 12 patients had the same mutations as their respective probands (Table 4). Many family members having the same mutations as the probands were not suffering from DVT, except the family members of PS5 and PS16. All of the affected family members of PS5 and PS16 were suffering from DVT (Table 4). The mother of PS5, having the heterozygous *PROS1* gene mutation Y595C, was suffering from DVT. PS16 (13 year-old boy) carried mutations in both *PROS1* (E301X) and in *PROC* (IVS2-4 C > T). His father (56 year-old) and uncle (53 year-old) had the same double mutation and were also suffering from DVT (Table 4). Their onset years of DVT were 43 years old for his father and 41 years old for his uncle.

Other family members having the same mutations as the patients had lower activities than the reference intervals but were asymptomatic (Table 4). PS6 patient (26 year-old female) and her 2 month-old daughter were the exception since the PS activities of her mother and baby were 109% and 65%, respectively. In general, the PS activity of a healthy baby is lower than that of a healthy adult. The typical activity of 2 month-old healthy babies is approximately 45%. Thus, we concluded that both of the PS activities were within the reference intervals. The PS activity of the PS6 patient was reduced when she was pregnant. Her PS activity did not return to the reference interval range after she delivered her first baby.

Discussion

We have been conducting an ongoing investigation of constitutional predispositions of DVT in Japanese individuals by systematically examining the relationship between coagulation/fibrinolysis-related factors and thromboses in our research laboratory [10]. Currently, our results indicate that the frequency of Japanese DVT patients having mutated PS and/or PC genes is higher than those reported for Caucasian patients [1,26–28], with mutated PS molecules being especially higher as reported in the results section. In our study, the frequency of the mutated AT molecule in Japanese DVT patients was much less frequent than those of mutated PS and/or PC molecules. Although functional analyses of these mutated PS molecules indicated that not all of these molecules were responsible for the reduced PS activity, most of the reduced PS activity in DVT patients could be explained by PS gene abnormalities [14,19,29,30].

The VITA project in a Caucasian population reported that PS or PC heterozygous mutations were observed in 2–4% of patients with thrombosis, and enhanced the risk of phlebothrombosis [28]. The frequency of *PROS1* and

Table 4
Family members of probands and their pathological findings

	DVT patient	Mutations identified	Probands and the activity (%)	Family members and their activity (%)			
1	PS 1	IVSI-1 G > C	Proband <10	Grandmother[a] 33	Father 71	Mother[a] 22	Sister 74
2	PS 2	IVSI3-2 A > G	Proband 10	Daughter 1[a] 21	Daughter 2 66		
3	PS 4	1998 C < T	Proband 34	Father[a] 55	Sister[a] 51	Niece 136	
4	PS 5	2224 A > G	Proband 28	Father 77	Mother[b] 27		
5	PS 6	1041 G > C	Proband 49	Father 112	Mother[a] 109	Daughter[a] (2 month-old) 65	
6	PS 8	1789 C > A	Proband 33	Father 83	Wife 76	Brother 112	Daughter[a] (9 year-old) 9
7	PS 9	795 G > T 903 A > G	Proband 3	Father[a] 65 903 A > G	Mother[a] 45 795 G > T		
8	PS 11	903 A > G 903 A > G	Proband 35	Father[a] 45 903 A > G	Mother[a] 39 903 A > G		
9	PS 15	1163 T > G	Proband 6	Father[a] 34	Mother 114		
10	PS 16/PC 4	1341 G > T	Proband 21	Father[b] 8/50	Uncle[b] 23/55		
11	PS 18	1268 C < T	Proband 11	Father 129	Mother[a] 29	Sister[a] 25	Aunt 144
12	PC 5	662 C > T	Proband 60	Father 88	Mother[a] 73	Brother 77	

[a] Family members having the same mutations as probands but no symptom.
[b] Family members having the same mutations as probands and suffering from DVT.

PROC gene mutations (22% and 9%, respectively) of Japanese DVT patients was certainly higher than those of Caucasian patients, while the frequency of the *AT3* gene mutation (2%) was similar in both populations. These data suggest that dysfunction of the PS/PC anticoagulation system due to *PROS1* and *PROC* gene mutations is a major risk factor for Japanese DVT patients.

Another interesting point related to PS activity is the $PS_{Tokushima}$ (K155E) mutation, which has been shown to be a polymorphism of PS in Japanese [25,31]. The Lys155 (K155) residue is located in the second EGF-like domain which is important in intermolecular interactions, and the cofactor activity of $PS_{Tokushima}$ (K155E) to activated PC (APC) is reduced [25,32–34]. This would indicate that $PS_{Tokushima}$ (K155E) reduces APC activity and suppresses coagulation control. Indeed, one patient with homozygous $PS_{Tokushima}$ (K155E) who suffered DVT had 35% of PS activity. The odds ratio of 3.74 would suggest that this polymorphism is another risk for DVT.

Results from our gene analysis studies show that none of the family members having the same mutations as the probands were suffering from DVT, except two who had DVT. Thus, subjects having a heterozygous abnormal *PROS1* or *PROC* gene alone did not show DVT symptoms, suggesting that the heterozygous *PROS1* or *PROC* gene mutations as well as $PS_{Tokushima}$ (K155E) causes symptoms of DVT when unknown factors additionally exist in these probands.

Our results suggest that the Y595C mutation may be a causative mutation for DVT even in the heterozygous state, because the proband's mother with the heterozygous Y595C gene mutation was also suffering from DVT (PS5 in Table 4). Indeed, our expression experiment of Y595C mutation indicated that Y595C mutation caused an impaired secretion of PS and decreased APC cofactor activity [29].

Since factor V Leiden mutation shows resistance to the PS/PC system [7,35], coagulation regulation by the PS/PC system profoundly fails in individuals having factor V Leiden mutation, yielding the same phenotypes as those with reduced PS/PC anticoagulation activity in heterozygous deficient subjects. It is interesting that while in Japanese DVT patients, factor V Leiden mutation was not detected, the frequency of Japanese DVT patients having heterozygous *PROS1* or *PROC* gene mutations is equivalent to the frequency (20–50%) of Caucasian DVT patients having factor V Leiden mutation. In Caucasian thrombophilia, the coagulation regulation is disturbed most directly due to factor V Leiden mutation bypassing the PS/PC anticoagulation system, while in Japanese thrombophilia, coagulation regulation is disturbed due to weaker PS/PC activity caused by *PROS1* and *PROC* gene mutations. Phenotypically, the relative hypofunction of the PS/PC anticoagulation system is an important risk factor for DVT in Japanese as well as in Caucasian individuals.

In conclusion, our study reinforces that the genetic anomaly in the PS/PC anticoagulation system is an important risk factor for thrombophilia in the Japanese population.

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Appendix A. Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:
DDBJ, http://sakura.ddbj.nig.ac.jp/. (accession numbers: PS1 [AB083386], PS2 [AB083387], PS3 [AB083388], PS4 [AB083390], PS5 [AB083688], PS6 [AB083391], PS7 [AB083687], PS8 [AB083393], PS9 [AB083689], PS10 [AB083394], PS15 [AB087994], PS16 [AB084904], PS17 [AB084900], PS18 [AB087995], PC1 [AB083697], PC2 [AB083696], PC3 [AB083693], PC5 [AB086849], PC6–7 N1 [AB083698], PC8 [AB086851], PC9 [AB083694], AT1 [AB083706], AT2 [AB083707]).

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CLINICAL BIOCHEMISTRY

Review

Mitochondrial DNA in somatic cells: A promising target of routine clinical tests

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Abstract

Alterations of mitochondrial DNA have long been considered only from a point of view of rare genetic disorders causing neuromyopathy. Recently, alterations of mitochondrial DNA have been found in so-called common diseases such as heart failure, diabetes, and cancer; some of these alterations are inherited, and some are generated and/or accumulated in somatic cells with age. Mitochondrial DNA is more vulnerable to alteration than is nuclear DNA. For example, mitochondria produce a large amount of reactive oxygen species as an inevitable byproduct of oxidative phosphorylation. Therefore, mitochondrial DNA is under much stronger oxidative stress than is nuclear DNA. In spite of the importance, it is much less elucidated in the mitochondrial genome than in the nuclear genome how the genome is maintained. In this review, we focus on maintenance of mitochondrial DNA in somatic cells and its clinical importance.

Keywords: Mitochondria; Mitochondrial DNA; Reactive oxygen species (ROS); Aging; DNA damage; DNA repair; Nucleoid

Contents

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Introduction

Mitochondrial DNA (mtDNA) suffers much more damage than nuclear DNA. Firstly, mtDNA is under much stronger oxidative stress than nuclear DNA. mtDNA is in proximity to the ROS-generating respiratory chain. The oxidative damage of mtDNA is exemplified by the fact that 8-oxoG accumulates to a greater extent and increases more rapidly with age in mtDNA than in nuclear DNA, though the absolute amount of 8-oxoG in mtDNA had been overestimated [1]. Secondly, mtDNA suffers damage from toxic chemicals much more strongly than does nuclear DNA [2]. To accomplish aerobic ATP production, mitochondria maintain a membrane potential with the matrix side negative. This membrane potential tends to accumulate lipophilic cations inside mitochondria. Mitochondria import lipophilic cations from the cytosol and accumulate these cations up to 1000-fold [3], although mitochondria have several transport pathways to get rid of toxic foreign molecules. Some medicinal drugs and biologically toxic chemicals are lipophilic and have positive charges. And thus, for example, mtDNA is modified by alkylating agents several 10-fold more than is nuclear DNA [2]. The greater damage would naturally cause a higher mutation rate in mtDNA.

Considering that over 90% of mtDNA is occupied by a coding region while 1.5% total of the whole nuclear genome is occupied by coding regions, mitochondrial genome single nucleotide polymorphisms (mtSNPs) may be more probable to be related with phenotypes than are nuclear SNPs [4]. It is just a recent event, compared to the long history of humankind, that human lifespan rapidly increased particularly in developed countries. Hence, it is plausible to assume that many mtSNPs that could be involved in the susceptibility of elderly onset diseases have not been targets of natural selection during a long history of humankind. Recently, the relationship between mtSNPs and common diseases has been intensively studied: aging, diabetes, obesity, hypertension, cardiac infarction, neurodegeneration, and so on. A typical example is mtSNP of adenine at nucleotide position (np) 5178 (C5178A), which is related to longevity [5]. Interestingly, some haplotypes of mtDNA are preferentially found in top marathon runners and so are proposed to be associated with high performance in running. Surprisingly, it is reported that human mtDNA haplotypes are associated with intellectual ability [6]. At least in mice, there is fairly reliable experimental evidence for this [7].

Thus, subtle changes in mtDNA genetic information can exert diverse and profound influences on the lives of individuals. Alterations of mtDNA have long been considered by many clinicians only from a point of view of rare genetic disorders causing encephalomyopathy. Now, it is being recognized that somatic mutation of mtDNA occurs more frequently than expected and that the somatic mutation is physiologically and pathologically important. In this review, we focus on mtDNA in somatic cells.

General characteristics of mitochondrial DNA

For those readers who are not familiar with mtDNA, we will first briefly introduce the general characteristics of mtDNA [8]. Human mtDNA is double stranded, circular, and about 16.5 kb in length (Fig. 1). The two strands are named heavy (H)- and light (L)-strands based on their gravitational density. The mtDNA codes for only 13 proteins of the mitochondrial respiratory chain and a minimal set of 2 rRNAs and 22 tRNAs for constructing the mitochondrial translational machinery. All of the genes, however, are considered essential for proper function of aerobic ATP production by the respiratory chain. mtDNA lacks introns and so is very compact. There are two noncoding regions, the D-loop and O_L. The former, about 1 kb in length, is a main regulatory site for transcription and replication (Fig. 2). The latter, ~30 bases in length, is proposed by Clayton et al. to be an initiation site for L-strand replication [9].

There are several tens to thousands copies of mtDNA in one cell. Its number largely depends on the energy demand of a cell. Owing to the presence of multiple copies of mtDNA in one cell, mutant and wild-type mtDNAs can coexist in one cell, which is called heteroplasmy. On the other hand, it is called homoplasmy when a cell contains only one kind of mtDNA irrespective of mutant or wild type. The degree of heteroplasmy varies among cells, tissues, and organs, which allows a variety of symptoms among patients who suffer the same mtDNA alterations. One cell usually maintains its mitochondrial respiratory activity until the mutant mtDNA reaches a certain level in the cell. This phenomenon is termed a threshold effect of heteroplasmy. The threshold is about 50% for large deletion mutations or point mutations in tRNAs, while it can be up to ~80% for point mutations in protein-encoding genes. This suggests that proteins derived from wild-type mtDNA can compensate for the malfunction of mutant mtDNA-derived proteins

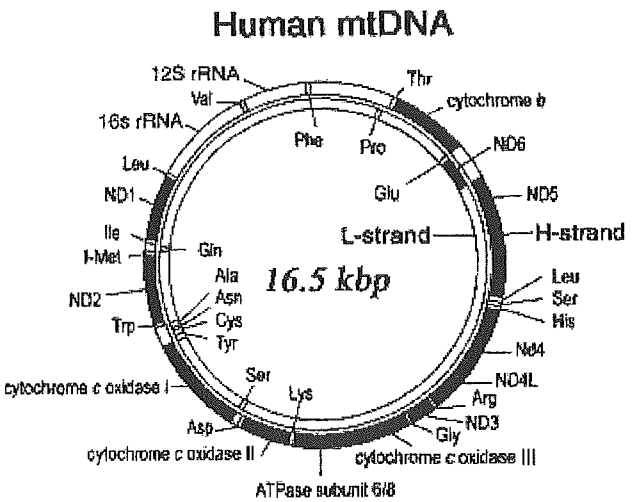

Fig. 1. Human mtDNA. Hunan mtDNA is ~16.5 kbp circular DNA. 13 proteins, 22 tRNAs, and 2 rRNAs are encoded on both strands.

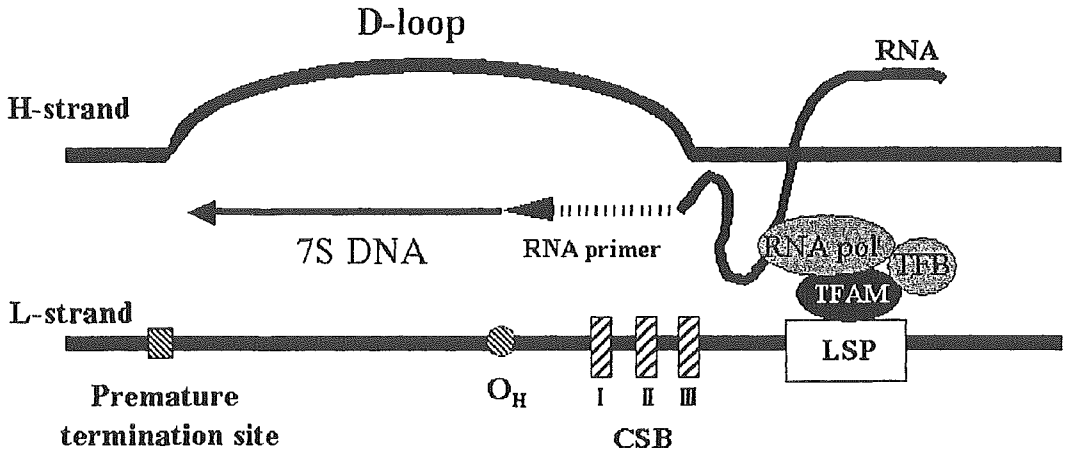

Fig. 2. Schematic description of a D-loop region. A D-loop region is a major non-coding region in human mtDNA. TFAM, mitochondrial transcription factor B (TFB), and mitochondrial RNA polymerase (RNA pol) are assembled on a light-strand promoter (LSP), and then transcription is started. The transcript makes an RNA–DNA hybrid in the conserved sequence blocks (CSB) and is considered to serve as a primer for DNA synthesis (RNA to DNA transition). The transition occurs mainly in a region ranging np 190 to 110 (O_H). Most of the DNA synthesis prematurely terminates about 700 bases downstream, which produces a characteristic triplex D-loop structure. In the strand-asymmetric model, the DNA synthesis proceeds to replication of mtDNA when the premature termination does not occur.

in a cell. In other words, mitochondria in a cell communicate with one another or mitochondria function as one unit in a cell. Consistent with this functional uniformity, dynamic fusing and fission of mitochondria are observed in a living cell.

mtDNA is exclusively transmitted maternally, although only one case is reported that paternal mtDNA has caused mitochondrial myopathy [10]. mtDNA demonstrates homoplasmy when transmitted from a mother to the next generation. mtDNA is considered to be randomly segregated into daughter cells. Here, for example, we assume that a cell contains 500 copies of type A mtDNA and 500 copies of type B mtDNA. It is an astronomically rare event that all 500 copies of type A are stochastically segregated into one daughter cell and all 500 copies of type B into the other cell after cell division. However, the copy number of mtDNA per cell is considered to dramatically decrease during one period of oocyte maturation due to cell division without mtDNA replication, which increases a chance to be homoplasmic by random segregation (so called a bottleneck effect). Thus, a heteroplasmic state of the mother normally disappears within three generations.

Conversion from heteroplasmy to homoplasmy actually can occur even in somatic cells without the bottleneck mechanism. If we assume that heteroplasmic mtDNA molecules are segregated randomly into two daughter cells after cell division and the two daughter cells equally divide again, homoplasmic cells would occupy only an extraordinarily small part of a large descendant population after numerous cell divisions. However, given that only one of the two daughter cells continues to divide, the cell could drift to homoplasmy during continuous cell division. A similar situation is seen in a stem cell that self-replicates while producing non-dividing differentiated cells.

Maintenance of mitochondrial DNA

Mitochromosome

Human mtDNA is frequently described as not being protected by histones, and so it is more vulnerable to damage than nuclear DNA. This description per se is largely true. Human mtDNA is indeed not wrapped with histones and, as such, is more vulnerable than nuclear DNA. This description, however, creates a misunderstanding that human mtDNA is naked and therefore vulnerable. In *Saccharomyces cerevisiae*, Miyakawa et al. first raised the possibility that mtDNA is compactly packaged with proteinous substances and takes on a nucleosome-like structure called a nucleoid [11]. This concept has been virtually ignored in mammalian mtDNA for a long time. Just recently, the mammalian mtDNA nucleoid has come to be firmly recognized by several lines of evidence [12–18] (Figs. 3 and 4). The molecular structure of the nucleoid is, however, little elucidated even for yeast mtDNA, much less for a mammalian one.

Mitochondrial transcription factor A (TFAM) was reported to be present about 15 molecules per one mtDNA molecule when it was purified as a transcription factor [19]. The authors and others have reported that there are about one thousand molecules of human TFAM per one mtDNA molecule, i.e. one TFAM for every 15 bases of mtDNA [12,18]. This amount well corresponds to the amount of yeast Abf2 that is a yeast homolog of TFAM. TFAM is a typical member of the high mobility group (HMG) of family proteins, many of which bind to DNA in a sequence-independent manner and exert a variety of influences including transcription activation via changing DNA-strand structure [20]. In fact, TFAM can bind to DNA irrespective of DNA sequence, though it has a higher binding affinity to the

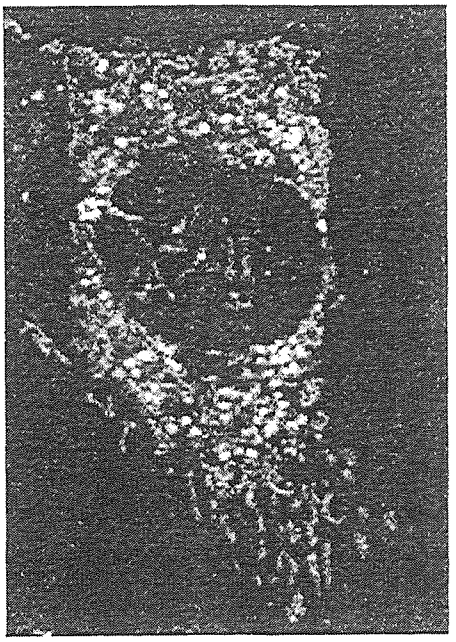

Fig. 3. Staining of mtDNA with ditercalinium chloride. Ditercalinium chloride accumulates in mitochondria and binds to mtDNA. The fluorescence of ditercalinium chloride is seen granularly in a thin tubular background. The granular signals may represent mtDNA nucleoids.

L- and H-strand promoter sequence. TFAM occupies about 20 bases on DNA. Thus, the amount of TFAM is sufficient to cover the whole region of mtDNA given that most of TFAM molecules are bound to mtDNA. We propose that TFAM also plays a histone-like role in packaging mtDNA as a main component of the nucleoid because TFAM that is devoid of transcription-activating activity can maintain mtDNA [16]. The importance of the nucleoid structure in the maintenance of mtDNA is shown in the observation that the amount of mtDNA changes almost in parallel with forced decrease and increase in TFAM. The nucleoid units may assemble to form a further higher structure as nuclear nucleosomes form chromosome. A concept of the mitochromosome will be required hereafter.

DNA repair

In 1974, Clayton et al. [21] reported that UV damage of mtDNA is not repaired, which has been verified. However, the report produced a longstanding misunderstanding that mitochondria lack DNA repair systems because DNA repair systems were not well elucidated at that time except for nucleotide excision repair systems that are responsible for pyrimidine dimers produced by UV irradiation. However, mitochondria indeed repair certain types of DNA damage. Unlike nuclear DNA, mtDNA is continuously replicated, even in terminally differentiated cells, such as nerve cells and cardiomyocytes. Hence, somatic mtDNA damage (resulting in mtDNA mutation) potentially causes more adverse effects on cellular functions than does somatic nuclear DNA damage in such terminally differentiated cells. Accordingly, DNA repair systems in mitochondria would be actually more important than those in nuclei, particularly for non-dividing cells.

As will be described below in the Somatic mutation of mitochondrial DNA section, a mitochondrial respiratory chain is a physiological source of reactive oxygen species (ROS). mtDNA is located in close proximity to the ROS-producing respiratory chain and therefore is oxidatively damaged more than nuclear DNA. A representative ROS-modified base is 8-oxoguanine that is a guanine base oxidized at its 8th position. The oxidative damage occurs not only in DNA strands but also in free nucleotides. 8-Oxo-dGTPase eliminates oxidized free dGTP and so prevents incorporation of an oxidized guanine base into DNA during replication [22]. Human 8-oxo-dGTPase, termed hMTH1, is located both in the cytosol and mitochondrial matrix [23].

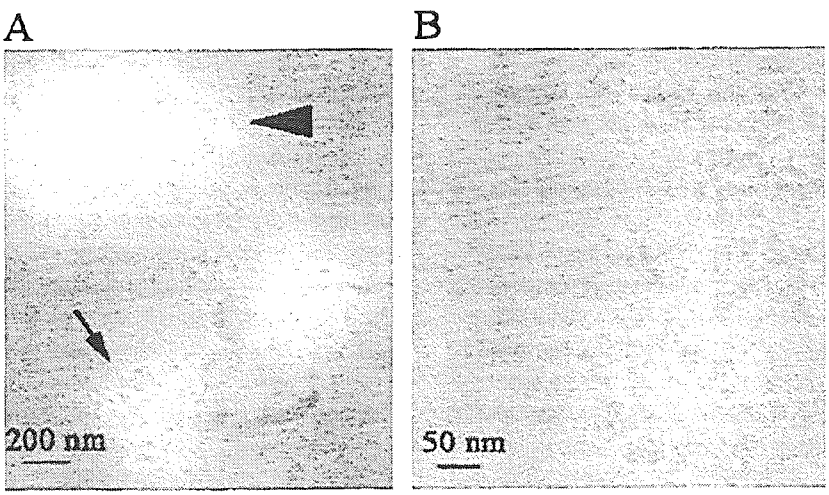

Fig. 4. Electron microscopic images of mtDNA nucleoid. After solubilization of human mitochondria with a non-ionic detergent NP-40, insoluble mtDNA nucleoid was separated by sucrose density gradient centrifugation. (A) Vesicle-like structure (arrow head) and mtDNA extruded from a lipid vesicle (arrow) are seen. (B) Beaded structure of mtDNA is shown in a more magnified field.

hMTH1 is a first mitochondrial DNA-repair-related enzyme whose gene, activity, and protein are clearly identified. All other base excision repair enzymes required for repair of the oxidized guanine, such as OGG1 [24] and MYH [25], are also in mammalian mitochondria. Thus, mitochondria are fully equipped with a repair system for the oxidized base. There are mainly four DNA repair systems in nuclei: base excision repair, nucleotide excision repair, mismatch repair, and recombination repair. There is very little evidence that the latter three systems work in mitochondria [26]. Considering that base excision repair is mainly responsible for repair of oxidative damage, it may be reasonable that mitochondria retain only the base excision system. Interestingly, over-expression of MTH [27] or OGG1 [28] in mitochondria makes the cells resistant to oxidative stress, suggesting importance of the DNA repair system for oxidative damage.

DNA replication

It has long been believed that mammalian mtDNA is replicated in quite a different way from that of nuclear DNA [9]. This classical theory of mtDNA replication is now called a strand-asymmetric model. According to this model, a transcript starting from a light-strand promoter (LSP) serves as a primer for initiation of the H-strand synthesis at the O_H region in the D-loop region. DNA synthesis proceeds, displacing the parental H-strand to form a triplex structure (Fig. 2). A large part of the DNA synthesis prematurely terminates when it goes forward about 700 bases, resulting in the formation of a so called D-loop. The ~700 bases nascent H-strand in the D-loop is called 7S DNA or a D-loop strand. If the H-strand synthesis does not prematurely terminate, the DNA synthesis is considered to enter a complete replication cycle. The displacement of the parental H-strand as single-stranded DNA continues until the synthesis reaches O_L which is located about 10 kb downstream. When the O_L is once exposed on a single-stranded H-strand, the O_L region takes on a special stem-loop structure that initiates synthesis of an RNA primer for L-strand DNA synthesis. Thus, in this model, the lagging L-strand synthesis is delayed, and so the synthesis of both strands proceeds asymmetrically. This model is quite unique and is not seen in the replication of mammalian nuclear DNA or bacterial DNA.

Very recently, Holt and Jacobs have proposed a new model of mtDNA replication, i.e. a strand-symmetric model [29,30]. They analyzed replication intermediates of mtDNA by two-dimensional agarose gel electrophoresis. They did not find any intermediates harboring long single-stranded DNA that should be formed in the strand-asymmetric model. Instead, they have found typical Y-fork intermediates that are formed in a usual nuclear DNA replication system. Based on these observations, they concluded that lagging strand synthesis occurs simultaneously and symmetrically with leading strand synthesis, as generally seen in DNA replication. In addition, they propose that H-strand synthesis initiates from a broad region ranging about 5 kb but not from the replication origin of the H-strand, O_H.

Their two-dimensional gel results are very clear. However, Okazaki fragments that should be formed for lagging strand synthesis in the symmetric model are not identified yet. One expected feature in the asymmetric model is that 7S DNAs have the same free 5′ ends as those of the replicative nascent H-strands. This prediction is experimentally confirmed [31]. Furthermore, frequency of the premature termination is well inversely related to the rate of mtDNA replication [32–34], which is feasibly explained by the asymmetric model but not sufficiently by the symmetric model. At present, the two theories are not yet reconciled with each other [35,36].

Somatic mutation of mitochondrial DNA [26,37,38]

As described above, many longstanding concepts on mtDNA are currently challenged, and new images of mtDNA are being constructed. Such a situation is also true for mutation of mtDNA. Recently, using denaturant gradient gel electrophoresis, very low level mutations in mtDNA have been detected, and the mutation rate in human mtDNA indeed has been shown to be several hundred-fold higher than nuclear gene mutation rates [39]. Furthermore, Turnbull et al. measured cytochrome *c* oxidase (complex IV) activity-negative cryptic cells of colon epithelia, which turned out to be due to mtDNA mutation [58]. The mutation rate of mtDNA was estimated to be about 100-fold higher than that of nuclear DNA based on the incidence of the cytochrome *c* oxidase activity-negative cryptic cells. Point mutations in the control/D-loop region of human mtDNA accumulate in an age-dependent manner [41], and age-related large rearrangements of mtDNA have also been reported [42,43]. DNA polymerase gamma, which is the only DNA polymerase identified in mammalian mitochondria and which is supposed to be responsible for mtDNA replication, has 3′–5′ exonuclease activity for proofreading [44]. DNA polymerase gamma synthesizes DNA as faithfully as DNA polymerase for nuclear DNA replication, suggesting that the higher mutation rates in mtDNA are due to stronger damage and/or weaker repair activities.

Somatically occurring variant mtDNA molecules could be amplified depending on conditions and potentially leading to cellular dysfunction with age. For example, stem cells in the colon crypt, which sustain the turnover of colon epithelium cells by self-replication, accumulate mtDNA mutations with age. Many of the mutations are homoplasmic, and some of them indeed make the cells rho (-), i.e. aerobic ATP synthesis incompetent [40]. In transgenic mice expressing DNA polymerase gamma devoid of the proofreading exonuclease activity [45], about a 10-fold higher mutation rate of mtDNA is observed than that of wild type.

Interestingly, these transgenic mice showed premature aging phenotypes such as alopecia and kyphosis. The lifespan was about 3-fold shorter. These two reports indicate two important facts. First, mtDNA mutation in normal somatic cells can accumulate to a homoplasmic level with age. Second, accumulation of somatic mtDNA mutation can cause aging phenotypes.

Oxidative stress on the heart

Adenine nucleotide translocase 1 (ANT1) is expressed in skeletal and cardiac muscles [46]. The *Ant1*-deficient mice developed mild cardiomyopathy. ANT is responsible for importing ADP into the mitochondria and exporting ATP out of the mitochondria. Thus, ANT provides substrates for ATP synthase. Without phosphorylation of ADP at the ATP synthase, electrons do not normally flow through the respiratory chain. Hence, the inactivation of ANT would also cause the block of the electron flow, and the block could enhance the production of O_2^-. SOD2, which is superoxide dismutase locating in the mitochondrial matrix and is normally induced by oxidative stress, was strikingly increased in the *Ant1* mutant mice. Interestingly, mtDNA rearrangements markedly accumulated in *Ant1* mutant mice but not in *Sod2*-deficient mice [47]. Consistent with this, heterozygous missense mutations have been identified in the human nuclear gene encoding ANT1 in several families with autosomal dominant progressive external ophthalmoplegia (adPEO), which is a rare human disease which presents large-scale mtDNA deletions [48]. O_2^- is converted to H_2O_2 by SOD2, and then H_2O_2 is converted to hydroxyl radicals via Fenton chemistry. Therefore, it is anticipated that hydroxyl radicals increase more in the *Ant1* mutant mice and O_2^- increases in the *Sod2*-deficient mice. This suggests that hydroxyl radicals but not O_2^- anions play a main role in mtDNA damage by ROS.

A few percent of O_2 consumed in mitochondria is normally converted to ROS. To assess whether an increase in O_2 consumption per se results in oxidative stress, two kinds of cardiac working overload models are employed: hyper-electric pacing and post-infarction hypertrophy.

Dog hearts were forcefully beaten at 240 beats/min using an electric pacemaker. This forced pacemaking causes heart failure in 4 weeks. The level of lipid peroxides (as a level of thiobarbituric acid-reactive substances) increased in these failing hearts, suggesting that working overload causes oxidative stress [49]. Complex I activity, but not complex II activity, was decreased in the failing hearts. As expected, the mitochondria prepared from the failing hearts showed an increase in NADH-dependent, but not succinate-dependent, O_2^- production. Thus, complex I but not complex II is damaged in this working overload-induced heart failure model.

When partial infarction of a mouse heart is made by occlusion of one coronary artery, the remaining non-ischemic portion must work more to maintain pumping function. The overload finally leads to heart failure with dilatated cardiomyopathy in 4 weeks. This model may also assess work overload. The lipid peroxidation level of the hearts was higher in this post-infarction model than in controls, indicating an increase in oxidative stress in the post-infarction heart [50]. Remarkably, the amount of mtDNA in the hearts decreased to about 50% of the control. In parallel, a decrease in the amount of mRNAs encoded by mtDNA was also seen. The activities of the complexes I, III, and IV, all of which contain the subunits encoded by mtDNA, decreased. However, the activity of complex II, which is not encoded by mtDNA, remained almost at a normal level. These results suggest that a primary target of ROS in post-infarction hearts is mtDNA, not proteins of the respiratory chain [50].

Results in this model of heart failure are reminiscent of the *Ant1*-deficient mice. If O_2^- anions were responsible for the oxidative damage as in *Sod2*-deficient mice, the iron-sulfur clusters, which are contained in complex II, would be first impaired. Hence, hydroxyl radicals are supposed to be responsible in this model of heart failure. The post-infarction hearts indeed clearly showed an increased level of hydroxyl radicals [50], and this increase in hydroxyl radicals could explain the primary damage of mtDNA. Interestingly, the expression of MTH1 was upregulated in the post-infarction hearts, presumably serving to prevent oxidative mtDNA damage [51]. We propose that mtDNA can be a direct and primary target of ROS under conditions where SOD2 is present or upregulated.

Mitochondrial dysfunction in neurodegenerative diseases

Parkinson's disease

Parkinson's disease is one of the most widespread age-associated neurodegenerative diseases with motor abnormalities. This disease is caused by a reduction in dopamine in nerve cells in the substantia nigra. Mitochondrial dysfunction in nigral neurons is supposed to be involved in its etiology and progression of the symptoms. Particularly, a decline of complex I activity is well-recognized [52]. The impaired activity of complex I could enhance ROS production. In fact, nigral cells of Parkinson's disease patients are intensely stained with anti-4-hydroxynonenal antibodies [53]. 4-Hydroxynonenal is a degradation product of lipid peroxides and is frequently used as a marker of lipid peroxidation. Hence, it is suggested that in Parkinson's disease the brain is under increased oxidative stress. More mtDNA molecules with a common deletion (4977-bp deletion with break points at np 8470 and 13,447) are found in Parkinson's disease than in age-matched controls [54], suggesting that mtDNA is also more strongly damaged in this disease. We observed that nigral cells show staining

in the cytoplasm when exposed to anti-8-oxoG antibodies [55] but that staining is not observed in cortex cells. These results suggest that the oxidative stress and damage are more intense in nigral cells in Parkinson's disease patients. The cytoplasmic staining of 8-oxoG also suggests that mtDNA, but not nuclear DNA, is principally damaged. hMTH1, human 8-oxo-dGTPase, is upregulated in the nigral cells of patients with Parkinson's disease. In addition, the upregulation of hMTH1, which is located both in the cytosol and mitochondria, is mainly observed in mitochondria [55]. Considering that mtDNA suffers the most oxidative damage, the upregulation of hMTH1 in mitochondria is reasonable in prevention of the 8-oxoG-induced mtDNA mutation. Because the upregulation of hMTH1 or accumulation of 8-oxoG is not observed in multiple systemic atrophy, a disease state with similar depletion of nigral cells, the mitochondrial oxidative stress is not likely to be a result of the depletion of the nigral cells but is instead likely a causative event specific to Parkinson's disease. Thus, mitochondrial oxidative stress may play an important role as one of the etiologies in Parkinson's disease.

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized clinically by dementia and histologically by amyloid-β (Aβ) deposition in the brain. Mitochondrial dysfunction has been proposed as an underlying mechanism of AD pathogenesis [56]. The different mitochondrial enzymes are reduced in activity. Among them, a selective decline in cytochrome *c* oxidase activity in particular has been biochemically shown in AD patient brains [57,58]. When cybrid cells are made by fusing AD patient platelets and mtDNA-less (rho^0) cells, the mitochondrial malfunction of AD patients can be transmitted to the cybrid cells in many cases ([59] and references therein), suggesting that mitochondrial malfunction originated at least in part from mtDNA of AD patients. Somatic mtDNA rearrangement mutations have been observed in AD patient brains. For example, a common 5 kb deletion of mtDNA is observed to be about 15-fold elevated in AD patient brains [60]. It is reported by several groups that the mutation at np 4336 is associated with AD [61,62]. Conversely, the European mtDNA lineages with J and Uk haplotypes are protective of AD [63,64]. Recently, it has been reported that AD brains frequently harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication [65]. Thus, somatic mtDNA mutations as well as mtSNPs of germ-line cells are involved in the pathogenesis of AD.

Mitochondrial respiratory chain dysfunction would naturally increase ROS production. Consistent with this scenario, the cybrids harboring mtDNA of AD patients produce more ROS than do the control cybrids [66]. In fact, increased oxidative damage has been observed in the brains of late-onset sporadic AD patients [67]. The increased ROS production could enhance the Aβ production from amyloid precursor protein. In turn, Aβ is known to decrease the activity of the mitochondrial respiratory chain and induce oxidative stress in neuronal cells [56]. Interestingly, this Aβ toxicity per se is enhanced in the AD cybrids [68]. The alterations of mtDNA in AD patients appear to play multiple roles in the progression of AD: increase in ROS, increase in Aβ formation, and increase in Aβ toxicity.

Diabetes mellitus

It is well understood that normal mitochondrial function is essential for insulin secretion in pancreatic beta-cells [69]. For example, when insulin-secreting cells are depleted of mtDNA, they lose the ability to secrete insulin upon glucose stimulation [70]. mtDNA molecules harboring mutations tend to accumulate in non-dividing cells, such as nerve cells and skeletal/cardiac muscles. Diabetes mellitus often accompanies congenital encephalomyopathies with mtDNA defects [71], suggesting that pancreatic beta-cells are also prone to accumulate mtDNA molecules with mutations. An A to G mutation of mtDNA at nucleotide position 3243 (A3243G mutation) is responsible for about 80% of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), one of the most common forms of mitochondrial encephalomyopathy. Interestingly, 1–2% of diabetic patients who do not have symptoms of encephalomyopathy also carry this mutation [72,73]. Because hundreds to thousands of copies of mtDNA molecules are present in one cell, wild-type and mutant mtDNA molecules can co-exist. As previously explained, this is termed heteroplasmy. The degree of heteroplasmy varies among cells and tissues. Defects in mitochondrial respiration do not become apparent until the mutant mtDNA molecules occupy a large part of the total mtDNA population. Those diabetic patients with the A3243G mutation are likely to be a subtype of MELAS patients, where the mutation accumulates mainly in the pancreatic beta-cells. Thus, 1–2% of all diabetic mellitus cases would be explained by mitochondrial A3243G mutation.

The A3243G mutation is examined most often by using peripheral leukocytes, although recently urinary cells have been reported to carry higher heteroplasmy of A3243G [74]. The mutant heteroplasmy in leukocytes is known to be much lower than that in muscles in mitochondrial disease. Therefore, there is a possibility that the presence of the mutation may be missed upon analysis due to the low heteroplasmy in leukocytes and thus underestimate the prevalence of mitochondrial diabetes mellitus. We have developed a sensitive method by which 0.01% of heteroplasmy can be detected [75]. However, even after applying this method, the A3243G mutation was detected in only ~2% of randomly selected patients with diabetes mellitus. Hence, patients with mitochondrial diabetes mellitus with

the A3243G mutation may not exceed 2% of all diabetes mellitus patients in Japan. Even so, this is the biggest single etiological entity identified in diabetes mellitus. Based on high sensitive detection of the A3243G mutation by our and other groups [75,76], the A3243G mutation seems to exist at a very low level (below 0.01% heteroplasmy) in apparently healthy subjects. It may be an important question why some people accumulate the mutation at a high level while others do not.

Over 200 point mutations have been found so far in mitochondrial diseases. Assuming that all these mutations are potentially a cause for diabetes mellitus, some researchers propose that up to 20% of diabetes mellitus patients may be mtDNA-related. Based on maternal inheritance of diabetes, one epidemiological study supports the idea that 20–30% of diabetes could be mtDNA-related [77].

Mitochondrial DNA in cancer

Mitochondrial aberrations such as altered expression and mutations in mtDNA-encoded products have been identified in a variety of human tumors (see [78] for a review). Recently, it has come to be known that mtDNA mutations are very often found in primary tumors but not in surrounding normal tissues. The mutations are homoplasmic in nature, range over almost entire regions of mtDNA, and include point, deletion, and insertion mutations. This homoplasmic nature of mutated mtDNA raises the possibility that some mutations are involved in tumorigenesis itself by affecting the energy metabolisms and/or ROS production. However, many of the identified mutations are silent in their translation products. These mutations may instead simply reflect the clonal expansion of cells that converted to homoplasmy after incidental mtDNA mutations. Many cancer cells might have a nature of cancer stem cells. Statistical calculations indicate that the observed homoplasmic mitochondrial mutations in cancer cells can be explained without assuming any advantage of mtDNA replication or cell survival [79]. The mutated mtDNA found in cancer is 100-fold more abundant than mutated nuclear p53 DNA [80], indicating that the mitochondrial mutations could be feasible as a molecular marker for detection of cancer.

The effect of drugs on mitochondrial DNA

AZT, an anti-HIV drug, is well known to cause myopathy due to mtDNA depletion by inhibition of mtDNA replication [81]. Many cancer chemotherapeutic agents also act on mtDNA. DNA-binding chemotherapeutics can inhibit processes such as DNA replication and/or transcription and

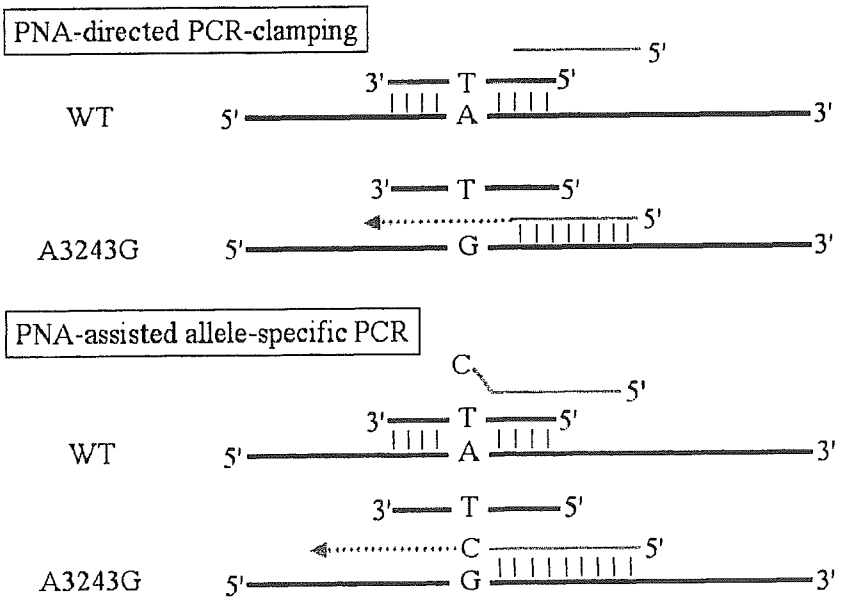

Fig. 5. Schemes for PNA-directed PCR-clamping and PNA-assisted allele-specific PCR. The L-strand of mtDNA around np 3243 is shown as a black bold line. The 3243 site is only shown by a nucleotide. Green and blue lines are a DNA primer and peptide nucleic acid (PNA), respectively. A broken red arrow denotes newly synthesized DNA. The PNA is designed to completely match to a wt mtDNA region including np 3243 at its middle. In PNA-directed PCR-clamping (upper panel), an antisense primer DNA is designed to partly overlap the PNA-binding region but not to reach np 3243. PNA normally binds to a DNA strand more strongly than does normal DNA, and so the PNA expels the 3′ side of the primer from wt mtDNA and inhibits the amplification of wt mtDNA. In general, one nucleotide mismatch makes the binding of PNA much weaker. Therefore, the primer instead of the PNA binds to 3243 mutant mtDNA, leading to the amplification of the mutant mtDNA. In PNA-assisted allele-specific PCR (lower panel), an antisense primer is designed to end at np 3243 and to match to the 3243 mutant. Due to this 3′ mismatch, the amplification of wild-type mtDNA is largely suppressed. In addition, because the PNA expels the 3′ side of the primer, the amplification of wild-type mtDNA is further inhibited. The PNA is more efficiently detached from the 3243 mutant mtDNA in PNA-assisted allele-specific PCR than in PNA-directed PCR-clamping because the primer overlaps the PNA region longer in the former than in the latter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thus act as anticancer drugs. Generally, these agents bind to the minor grooves of DNA and intercalate between bases. As examples, actinomycin D and doxorubicin are clinically important DNA intercalators. Mitochondrial DNA is an important target of side effects of these anticancer drugs. One reason is that the mitochondrial respiratory chain can reducibly activate redox-cycling agents and in turn the activated agents produce ROS. Doxorubicin is a member of antracyclines which consist of quinoloid xenobiotics. Unfortunately, this reagent can produce a characteristic form of cardiac injury involving mitochondrial abnormalities during treatment. It is known that doxorubicin is reduced to a semiquinone radical form by complex I of the mitochondrial respiratory chain, producing a large amount of ROS [82], which can cause oxidative mtDNA damage. In addition, many anticancer drugs will target mitochondria as a side effect because mitochondria accumulate lipophilic cations, as already described. Ditercalinium was originally designed to bis-intercalate into DNA with high affinity and indeed displays strong cytotoxicity on experimental tumor cells. However, it has turned out after a clinical trial that irreversible hepatotoxicity is a dose-limiting side-effect [83]. This reagent specifically accumulates in mitochondria[83] and depletes mtDNA [84]. When mouse cells are incubated with ditercalinium, mitochondria but not nuclei are stained (Fig. 3). Importantly, granularly stained materials, which may represent mtDNA nucleoids, are observed in mitochondrial tubular backgrounds [85], suggesting that ditercalinium accumulates in mitochondria and strongly binds to mtDNA. In the end, this drug was abandoned as a clinical anticancer drug.

Concluding remarks

Until recently, clinical interests in alterations of mtDNA were confined to relatively rare inborn mitochondrial encephalomyopathies, and so the clinical examination of mtDNA within disease was rather confined. It has now been recognized that mitochondrial oxidative stress and related oxidative mtDNA damage are implicated in wide and common pathological states including aging, cancer, infection, and even medicinal therapies. Sequencing of mtDNA is widely used for identification of individuals in a forensic field. The mtDNA sequencing could also be clinically used for assessment of individual risk factors in common diseases. The quantification of mtDNA can be a routine test for monitoring side effects of anti-HIV and anticancer drugs. Detection of oxidative damage of mtDNA is theoretically a very sensitive way for monitoring in vivo oxidative stress. Recently, we have reported one routinely available method for quantitative detection of the A3243G mutation [86] (Fig. 5). Efforts for developing new feasible and reliable methods are further required to allow mtDNA examinations to become routine clinical tests undertaken in many laboratories.

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Overexpression of Mitochondrial Transcription Factor A Ameliorates Mitochondrial Deficiencies and Cardiac Failure After Myocardial Infarction

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Background—Mitochondrial DNA (mtDNA) copy number is decreased not only in mtDNA-mutation diseases but also in a wide variety of acquired degenerative and ischemic diseases. Mitochondrial transcription factor A (TFAM) is essential for mtDNA transcription and replication. Myocardial mtDNA copy number and TFAM expression both decreased in cardiac failure. However, the functional significance of TFAM has not been established in this disease state.

Methods and Results—We have now addressed this question by creating transgenic (Tg) mice that overexpress human *TFAM* gene and examined whether TFAM could protect the heart from mtDNA deficiencies and attenuate left ventricular (LV) remodeling and failure after myocardial infarction (MI) created by ligating the left coronary artery. *TFAM* overexpression could ameliorate the decrease in mtDNA copy number and mitochondrial complex enzyme activities in post-MI hearts. Survival rate during 4 weeks of MI was significantly higher in Tg-MI than in wild-type (WT) littermates (WT-MI), although infarct size was comparable. LV cavity dilatation and dysfunction were significantly attenuated in Tg-MI. LV end-diastolic pressure was increased in WT-MI, and it was also reduced in Tg-MI. Improvement of LV function in Tg-MI was accompanied by a decrease in myocyte hypertrophy, apoptosis, and interstitial fibrosis as well as oxidative stress in the noninfarcted LV.

Conclusions—Overexpression of *TFAM* inhibited LV remodeling after MI. TFAM may provide a novel therapeutic strategy of cardiac failure. (***Circulation.* 2005;112:683-690.**)

Key Words: free radicals ■ genes ■ heart failure ■ myocardial infarction ■ remodeling

Myocardial infarction (MI) leads to complex structural alterations (remodeling) involving both the infarcted and noninfarcted left ventricular (LV) myocardium. Early remodeling is LV cavity dilatation occurring during the early phase of MI, which is likely due to wall thinning of the infarct region. During the first several days, LV enlargement follows, and thereafter a progressive dilatation of the noninfarcted LV associated with myocyte hypertrophy and interstitial fibrosis occurs over weeks. These progressive changes in LV geometry contribute to the development of depressed cardiac function, clinical heart failure, and increased mortality. Accordingly, it is of critical importance to explore the mechanisms and to develop therapeutic strategies that will effectively inhibit this deleterious process.

Mitochondria have their own genomic system, mitochondrial DNA (mtDNA), a closed-circular double-stranded DNA molecule. MtDNA contains 2 promoters, the light-strand and heavy-strand promoters (LSP and HSP, respectively), from which transcripts are produced and then processed to yield the individual mRNAs encoding 13 subunits of the oxidative phosphorylation system, ribosomal and transfer RNAs.[1,2] Transcription from the LSP also produces RNA primer, which is necessary for initiating mtDNA replication. Mitochondrial function is controlled by the mtDNA as well as factors that regulate mtDNA transcription and/or replication.[3] This raises the possibility that mitochondrial gene replication and thus the mitochondrial DNA copy number and/or mitochondrial gene transcription are impaired in heart failure. Indeed, heart failure is frequently associated with qualitative and quantitative defects in mtDNA.[4–7] Recently, we demonstrated that the decline in mitochondrial function and mtDNA copy number plays a major role in the development of heart failure that occurs after MI.[8,9]

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Mitochondrial transcription factor A (TFAM) is a nucleus-encoded protein that binds upstream of the LSP and HSP of mtDNA and promotes transcription of mtDNA. It also plays an important role in regulating mtDNA copy number.[10] In fact, disruption of the *Tfam* gene in mice causes depletion of mtDNA, loss of mitochondrial transcripts, loss of mtDNA-encoded polypeptides, and severe respiratory chain deficiency.[11] Moreover, targeted disruption of *Tfam* in cardiac myocytes induced deletion of mtDNA and dilated cardiomyopathy.[12,13] These lines of evidence obtained from knockout mice have established a critical role for TFAM in regulation of mtDNA copy number and mitochondrial function as well as maintenance of the physiological function of the heart in vivo. In addition, a reduction in TFAM expression has been demonstrated in several forms of cardiac failure.[7,9,14,15] Therefore, an increase in *TFAM* expression may exert beneficial effects on cardiac remodeling after MI. However, it has not yet been analyzed whether an increase in *TFAM* expression can ameliorate mitochondrial dysfunction in heart failure and whether this protein may have therapeutic potential. To address these questions, we created transgenic (Tg) mice containing human *TFAM* gene. Accordingly, human *TFAM* Tg mice and their wild-type (WT) littermates were randomized to have either a large transmural MI induced by coronary artery ligation or sham operation.

Methods

Generation of Tg Mice

Human *TFAM* cDNA was inserted into the unique *Eco*RI site between the CAG (modified chicken β-actin promoter with CMV-IE enhancer) promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector[16] and used to generate Tg mice (Figure 1A). The pronuclei of fertilized eggs from hyperovulated C57BL/6 mice were microinjected with this DNA construct. The presence of the *TFAM* transgene was confirmed by polymerase chain reaction (PCR) before the experiments. Four independent founder lines were identified and mated to C57BL/6 WT mice to generate pure C57BL/6 genetic background WT and Tg offspring. Heterozygous Tg mice were used at 10 to 13 weeks of age. The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society.

Western Blotting

The protein levels human TFAM and mouse Tfam were analyzed in cardiac tissue homogenates by Western blot analysis with a polyclonal antiserum against human TFAM and mouse Tfam, respectively. In brief, the LV tissues were homogenized with the lysis buffer (1% SDS, 1.0 mmol/L sodium orthovanadate, 10 mmol/L Tris; pH 7.4). After centrifugation, equal amounts of protein (5 μg protein per lane), estimated by the Bradford method with the use of a protein assay (Bio-Rad), were electrophoresed on a 12.5% SDS–polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane (Millipore). After blocking with 5% nonfat milk in PBS containing 0.05% Tween-20 at 4°C overnight, the membrane was incubated with the first antibody and then with the peroxidase-linked second antibody (Amersham Pharmacia). Chemiluminescence was detected with an ECL Western blot detection kit (Amersham Pharmacia) according to the manufacturer's recommendation.

Immunohistochemistry

Frozen sections of cardiac tissues were incubated in the presence of 100 nmol/L Mitotracker Red CMXRos (Molecular Probes) at 37°C for 20 minutes. We did not repeat freezing-thawing to avoid the loss of mitochondrial integrity. After they were washed with PBS (10 mmol/L sodium phosphate, pH 7.4, and 150 mmol/L NaCl), the sections were fixed with 3.7% formaldehyde for 5 minutes. After they were washed, the fixed sections were incubated with 100-fold diluted anti-TFAM affinity purified antibodies (10 μg/mL) in PBS at 4°C overnight. Fluorescence images were taken with a confocal laser scanning microscope (Bio-Rad MRC 1000) with laser beams of 488 and 568 nm for excitation.

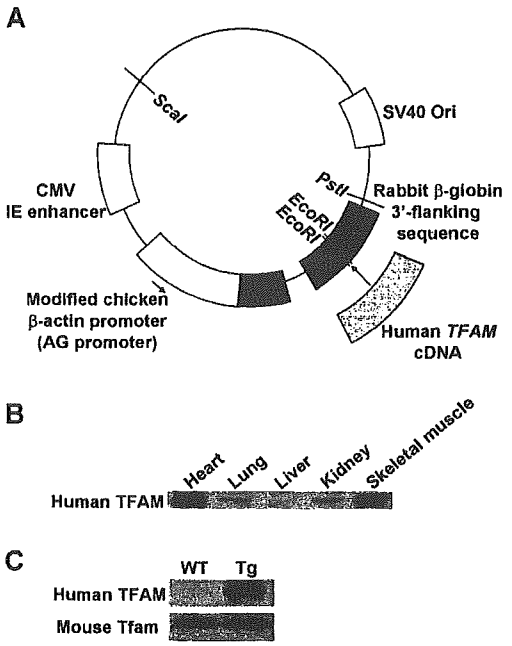

Figure 1. Characterization of human *TFAM* Tg mice. A, Diagram of the human *TFAM* transgenic construct. Plasmid was constructed by inserting a human *TFAM* cDNA (0.74 kb) into the unique *Eco*RI site between the CAG promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. Tg mice harboring human *TFAM* cDNA were identified by PCR with genomic DNA prepared from tail biopsies. CMV indicates cytomegalovirus; IE, immediate early; SV40, simian virus; and Ori, origin of DNA replication. B, Western blot analysis of human TFAM protein in various tissues from Tg mice. Total protein extracts from heart, lung, liver, kidney, and skeletal muscle were probed with a polyclonal antiserum against human TFAM. The antibody recognized TFAM as a single band of 24 kDa. C, Western blot analysis of human TFAM and mouse Tfam protein levels in the heart from Tg and WT mice.

Creation of MI

We created MI in mice by ligating the left coronary artery. Sham operation without coronary artery ligation was also performed.[9] Tail clips were applied, and a PCR protocol was performed to confirm the genotype by a group of investigators. Next, MI was induced in these mice by another subset of investigators, who were not informed of the genotyping results. This assignment procedure was performed with numeric codes to identify the animals.

Survival

To perform the survival analysis, cages were inspected for deceased animals during the study period of 4 weeks. All deceased mice were examined for the presence of MI as well as pleural effusion and cardiac rupture.

We performed the subsequent molecular (mtDNA copy number and mtRNA), biochemical (mitochondrial enzyme activity and apoptosis), and histopathological (myocyte cross-sectional area, collagen volume fraction, and mitochondrial ultrastructure) analysis by using the LV from sham-operated mice and the noninfarcted LV from MI mice.