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Protein S and protein C gene mutations in Japanese deep vein thrombosis patients

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Abstract

Objectives: Coagulation factor V Leiden has not been detected in Japanese patients suffering from thrombosis. Hitherto, the constitutional background of Japanese thrombotic patients has never been systematically examined. We have performed a systematic investigation to determine pathogenesis for deep vein thrombosis in a Japanese population.

Design and methods: Routine coagulation and fibrinolysis tests were performed to determine the activities of protein S, protein C, antithrombin, plasminogen and fibrinogen. Gene analysis was performed in thrombotic patients having low activities of these factors.

Results: Our study indicates that the frequency (19/85 = 0.22) of mutations of protein S gene in the Japanese patients was 5–10 times higher than that of mutations of protein S gene in Caucasian patients, and the frequency (8/85 = 0.09) of mutations of protein C gene was almost three times higher than that of Caucasian patients. The frequency of antithrombin gene mutation was similar in both populations.

Conclusion: Our study reinforces that the genetic anomaly in the protein S/protein C anticoagulation system is an important risk factor for thrombophilia in the Japanese population.
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Keywords: Deep vein thrombosis; Protein S gene mutation; Protein C gene mutation; Factor V Leiden; Japanese thrombophilia

Introduction

Thrombophilia is defined as an increased tendency to thrombosis, and can be either inherited or acquired. Thrombus formation is regulated by the anticoagulatory and fibrinolytic systems on the vascular endothelial cells. Importance of thrombotic factors as the cause of thrombosis has been clarified [1] and studies have indicated that abnormalities of the anticoagulation system and fibrinolytic factors contribute to thrombosis [2,3]. Venous thrombosis is the most common clinical manifestation of such thrombophilia. The prevalence of inherited anomalies of anticoagulation factors such as antithrombin, protein S (PS) and protein C (PC) in patients with venous thrombosis has been reported to be approximately 5–10% [4–6]. In European and American Caucasian patients, factor V Leiden (R506Q), a polymorphism of coagulation factor V, is a major risk factor for venous thrombosis [7,8]. Although the constitutional background of Japanese thrombotic patients has not been well examined, factor V Leiden (R506Q) is not detected in Japanese patients suffering from thromboses [9]. Since 1994, at Kyushu University Hospital, we have been investigating constitutional predispositions of patients suffering from not only venous thrombosis but also arterial and small vessel thromboses in the Japanese population [10], indicating the important role of PS in the pathogenesis of thromboses in Japanese population. Recently, two studies from Taiwan have reported that reduced activities of the PS/PC anticoagulation system are the most important risk factors for thrombophilia in the Taiwanese–Chinese population [11,12].

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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	27	6
			(18 patients having reduced activities of PS and PC)		
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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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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Online-only Data Supplements I and II can be found at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.104.524835/DC1.

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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.

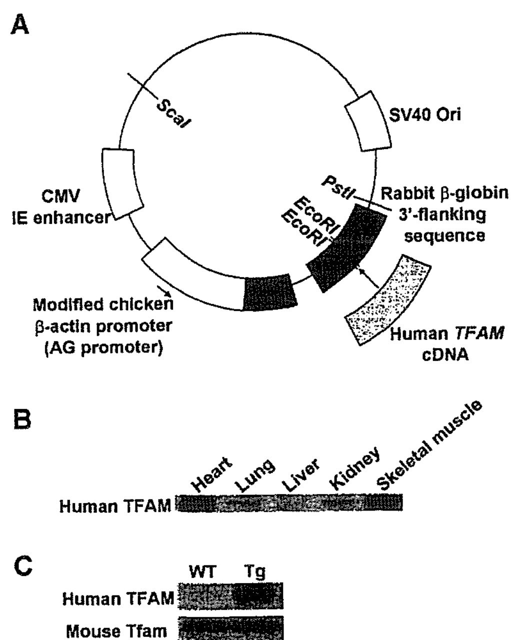

Figure 1. Characterization of human *TFAM* Tg mice. A, Diagram of the human *TFAM* transgenic construct. Plasmid was constructed by inserting a human *TFAM* cDNA (0.74 kb) into the unique *Eco*RI site between the CAG promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression 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.

Southern Blot Analysis

DNA was extracted from cardiac tissues, and a Southern blot analysis was performed to measure the mtDNA copy number as described earlier.[9] Primers for the mtDNA probe corresponded to nucleotides 2424 to 3605 of the mouse mitochondrial genome, and those for the nuclear-encoded mouse 18S rRNA probe corresponded to nucleotides 435 to 1951 of the human 18S rRNA genome. The mtDNA levels were normalized to the abundance of the 18S rRNA gene run on the same gel.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from frozen LV by the guanidinium method, and a Northern hybridization analysis was performed according to methods described previously.[9] Probes for mtRNA analysis were prepared by amplification of nucleotides 1209 to 2606 (probe 1), nucleotides 3351 to 7570 (probe 2), nucleotides 8861 to 14549 (probe 3), and nucleotides 14729 to 15837 (probe 4) of mtDNA from mouse genomic DNA.

Mitochondrial Enzyme Activity

The specific activity of complex I, complex II, complex III, and complex IV was measured in the myocardial tissues according to methods described previously.[9] The specific enzymatic activity of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I) was measured by a reduction of the ubiquinone analogue decylubiquinone. For the activity of succinate-ubiquinone oxidoreductase (complex II), the reduction of 2,6-dichlorophenolindophenol, when coupled to complex II–catalyzed reduction of decylubiquinone, was measured. For the specific activity of ubiquinol/cytochrome *c* oxidoreductase (complex III), the reduction of cytochrome *c* catalyzed by complex III in the presence of reduced decylubiquinone was monitored. The specific activity of cytochrome *c* oxidase (complex IV) was measured by following the oxidation of reduced cytochrome *c*, which had been prepared in the presence of dithionite. All enzymatic activities were expressed as nanomoles per minute per milligram protein.

Echocardiographic and Hemodynamic Measurements

After 4 weeks of surgery, echocardiographic studies were performed under light anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% wt/vol, 8 μL/g IP) and spontaneous respiration. A 2D parasternal short-axis view of the LV was obtained at the level of the papillary muscles. In general, the best views were obtained with the transducer lightly applied to the mid upper left anterior chest wall. The transducer was then gently moved cephalad or caudad and angulated until desirable images were obtained. After it was ensured that the imaging was on axis (based on roundness of the LV cavity), 2D targeted M-mode tracings were recorded at a paper speed of 50 mm/s. Our previous study has shown that the intraobserver and interobserver variabilities of our echocardiographic measurements for LV dimensions were small, and measurements made in the same animals on separate days were highly reproducible.[17] Then, under the same anesthesia with Avertin, a 1.4F micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the LV to measure pressures.[17]

Infarct Size

To measure the infarct size after 28 days of MI, the heart was excised, and the LVs were cut from apex to base into 3 transverse sections. Five-micrometer sections were cut and stained with Masson's trichrome. Infarct length was measured along the endocardial and epicardial surfaces from each of the cardiac sections, and the values from all specimens were summed. Infarct size (in percentage) was calculated as total infarct circumference divided by total cardiac circumference.[17]

In addition, to measure infarct size after 24 hours when most animals are still alive, a separate group of animals including WT-MI (n=6) and Tg-MI (n=6) was created. After 24 hours of coronary artery ligation, Evans blue dye (1%) was perfused into the aorta and coronary arteries, and tissue sections were weighed and then incubated with a 1.5% triphenyltetrazolium chloride solution. The infarct area (pale), the area at risk (not blue), and the total LV area from each section were measured.[18] In our preliminary study, we confirmed excellent reliability of infarct size measurements, in which a morphometric methodology similar to that used in this study was used. The intraobserver and interobserver variabilities between 2 measurements divided by these means, expressed as a percentage, were <5%.

Histopathology

After in vivo hemodynamic studies, the heart was excised and dissected into the right and left ventricles, including the septum. Five-micrometer sections were cut and stained with Masson's trichrome. Myocyte cross-sectional area and collagen volume fraction were determined by the quantitative morphometry of LV tissue sections.[17]

For assessment of mitochondrial ultrastructure by electron microscopy, LV tissues were fixed in a mixture of 1% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4 for 2 hours at room temperature. After they were washed in 0.1 mol/L phosphate buffer containing 0.25 mol/L sucrose, they were postfixed with 1% osmium tetroxide for 1 hour at room temperature. The tissues were then block-stained with 1% uranyl acetate in 50% methanol for 2 hours, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were double stained with uranyl acetate and lead citrate and then were observed under an electron microscope (Hitachi H7000). For quantitative morphometric analysis, the number and size of the mitochondria were examined according to methods described previously.[9] The number of mitochondria and the cross-sectional area (size) of each mitochondrion were measured within a sampling region of 100 square sarcomeres (sm^2). Eighteen regions were selected at random for each specimen, and for all regions the averages of mitochondrial number and cross-sectional area were calculated.

Apoptosis

To detect apoptosis, LV tissue sections were stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining. The number of TUNEL-positive cardiac myocyte nuclei was counted, and the data were normalized per 10^5 total nuclei identified by hematoxylin-positive staining in the same sections. We further examined whether apoptosis is present by the more sensitive ligation-mediated PCR fragmentation assays (Maxim Biotech Inc).[18]

Statistical Analysis

Data are expressed as mean±SEM. Survival analysis was performed by the Kaplan-Meier method, and between-group difference in survival was tested by the log-rank test. Between-group comparison of means was performed by 1-way ANOVA, followed by *t* tests. The Bonferroni correction was done for multiple comparisons of means. $P<0.05$ was considered statistically significant.

Results

Characterization of Human TFAM Tg Mice

Human *TFAM* cDNA was used to generate Tg mice (Figure 1A). Four lines of Tg mice were confirmed by PCR. These lines were viable and fertile, and there were no detectable differences in cardiac size and structure between Tg and WT mice either macroscopically or microscopically.

We analyzed TFAM protein levels in various tissues by Western blot analysis using anti-human TFAM antibody. We found a robust expression of human TFAM protein in the heart and skeletal muscle, but it was barely detected in the lung, liver, and kidney (Figure 1B). Among 4 established lines of Tg mice, 1 line that expressed the highest level of the human TFAM protein in the heart was used for further

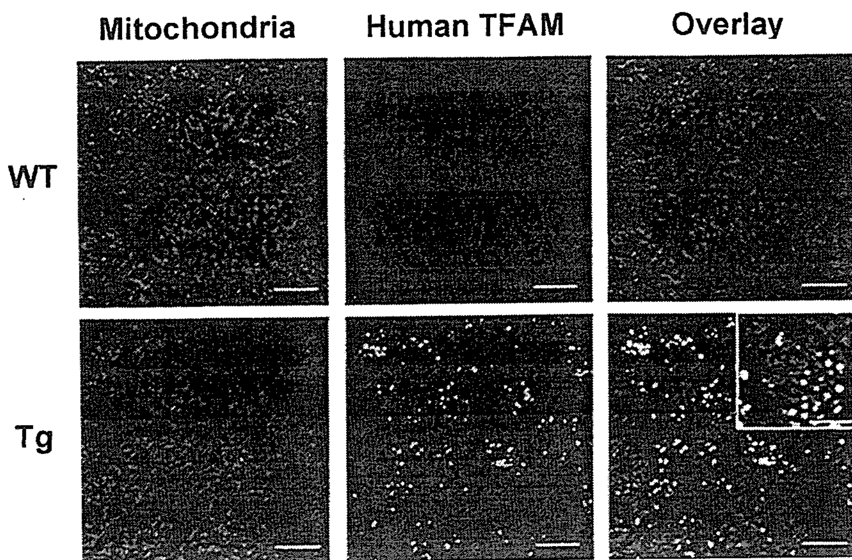

Figure 2. Myocardial tissue sections from WT (top) and Tg (bottom) mice were double-stained with MitoTracker dye (red) and a human TFAM specific antibody (green). Immunoreactivity for human TFAM was observed in the cytoplasm of cardiac myocytes. Merged images show that TFAM was colocalized with the mitochondria (yellow). Bar=20 μm. Inset shows merged images with higher magnification; bar=10 μm.

experiments. The endogenous expression level of the mouse Tfam protein was not modified or downregulated by the overexpression of human *TFAM* gene (Figure 1C). Immunohistochemical studies showed homogeneous human TFAM distribution in cardiac myocytes and colocalized with the mouse mitochondria (Figure 2). Human TFAM staining showed a relatively spotty staining pattern. With higher magnification, its expression appeared not to be restricted to a specific site of mitochondria (Figure 2, inset). These results suggest that the human TFAM exerts an expression pattern similar to that observed for the endogenous mouse Tfam and may function in the mouse heart.

MtDNA Copy Number and Mitochondrial Enzymes

We created MI in male Tg mice (Tg-MI) and nontransgenic wild-type littermates (WT-MI). Sham operation without coronary artery ligation was also performed in WT (WT-sham) and Tg (Tg-sham) mice. After 4 weeks of surgery, we measured mtDNA copy number, expressed as the ratio of mtDNA to nuclear DNA (18S rRNA), in the myocardial tissue by a Southern blot analysis. In parallel to an increase in TFAM protein, mtDNA copy number increased in the heart from Tg animals compared with WT controls (Figure 3A). In WT-MI animals, mtDNA copy number in the noninfarcted LV showed a 41% decrease ($P<0.01$) compared with sham mice, which was significantly prevented and preserved at a normal level in Tg-MI mice (Figure 3A).

To determine the effects of mtDNA copy number alterations on mtRNA, mtRNA transcript levels were measured by Northern blot analysis. As previously reported,[9] mtRNA transcript levels, including ND1+ND2, ND4, ND4L, ND5, cytochrome *b*, COI, COII, and COIII transcripts as well as 16S rRNA, were lower in WT-MI than those in WT-sham. However, overexpression of human *TFAM* did not increase, and even decreased, these mRNA levels in Tg-sham as well as in Tg-MI (online-only Data Supplement I). These results indicate that the regulation of mtRNA transcripts is dissociated from that of mtDNA copy number.

We next measured the respiratory chain enzyme activities. Despite the significant increase in mtDNA copy number in the heart from Tg, complex I, complex II, complex III, and complex IV demonstrated no significant changes in the enzymatic activity in comparison with WT controls (Figure 3B). Consistent with mtDNA copy number, the enzymatic activities of complex I, complex III, and complex IV were significantly lower in the noninfarcted LV from WT-MI than those from WT-sham. Most importantly, there was no such decrease observed in Tg-MI (Figure 3B). The enzymatic activity of complex II, exclusively encoded by nuclear DNA, was not altered in either group. These results indicate that mtDNA and mitochondrial enzymatic activities are downregulated in the hearts after MI, and human *TFAM* gene overexpression efficiently counteracts these mitochondrial deficiencies.

The overall number of mitochondria and the overall average size of the mitochondria demonstrated no significant changes in Tg-sham in comparison with WT controls. In contrast, the mitochondrial number was significantly increased and their size was decreased in WT-MI, both of which were attenuated in Tg-MI (online-only Data Supplement II).

Survival

The survival analysis was performed in 4 groups of mice during the study period of 4 weeks; WT-sham (n=20), WT-MI (n=21), Tg-sham (n=29), and Tg-MI (n=29). There were no deaths in sham-operated groups. The survival rate was significantly higher in Tg-MI compared with WT-MI (100% versus 66%; $P<0.01$; Figure 4A).

Infarct Size

We determined the infarct size by morphometric analysis in the surviving mice 28 days after MI, and it was comparable between WT-MI and Tg-MI (Figure 4B). To further confirm that overexpression of *TFAM* gene did not alter the infarct size, both area at risk and infarct area were measured in a separate group of mice 24 hours after coronary artery ligation.

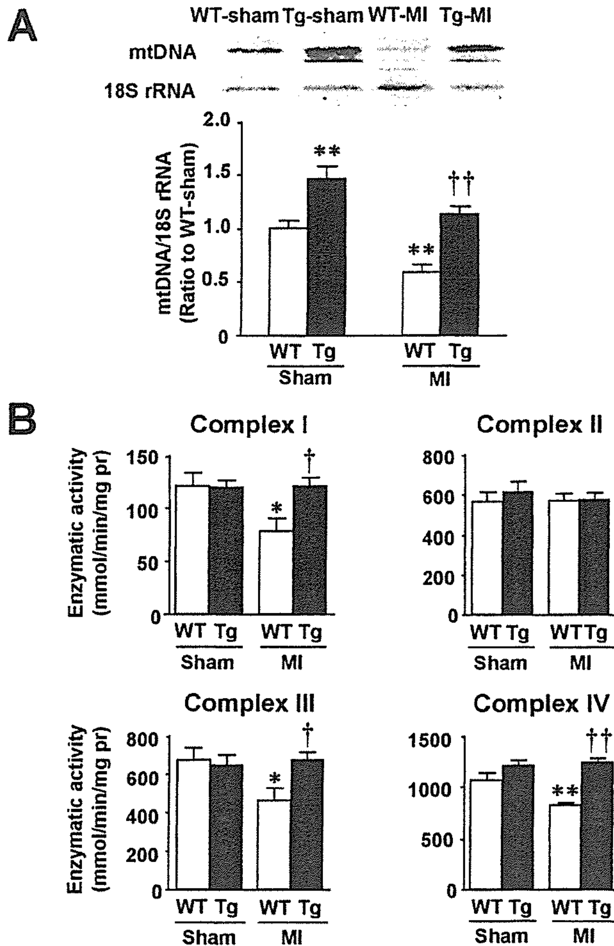

Figure 3. mtDNA and mitochondrial function. A, top, Southern blot analysis of mtDNA copy number in total DNA extracts from the heart from WT-sham, Tg-sham, WT-MI, and Tg-MI mice. Top bands show signals from the mtDNA fragment, and bottom bands show signals from the nuclear DNA fragment containing the 18S rRNA gene. A, bottom, Summary data for a Southern blot analysis of mtDNA copy number in 4 groups of animals (n=8 for each). Data were obtained by a densitometric quantification of the Southern blots such as those shown in A. B, Enzymatic activity of respiratory chain complex I, complex II, complex III, and complex IV in isolated mitochondria from 4 groups of animals (n=6 for each). Each assay was done in triplicate. Values are mean±SEM. *P<0.05, **P<0.01 for difference from WT-sham values. †P<0.05, ††P<0.01 for difference from WT-MI values. pr indicates protein.

The infarct size (infarct/risk area) was also comparable between WT-MI and Tg-MI mice (84.5±0.4% for n=6 versus 83.2±1.1% for n=6; P=NS; Figure 4C).

Cardiac Function and Structure

The echocardiographic studies of surviving mice at 4 weeks showed that cardiac diameters were significantly increased in WT-MI over the values in WT-sham or Tg-sham. Tg-MI showed less cavity dilatation and improved contractile function compared with WT-MI (Figure 5).

There was no significant difference in heart rate and aortic blood pressure among 4 groups of mice (Table). LV end-diastolic pressure increased in WT-MI and was significantly attenuated in Tg-MI. Coinciding with increased LV end-diastolic pressure, lung weight/body weight increased in WT-MI and was also attenuated in Tg-MI (Table). The prevalence of pleural effusion, a clinical sign of heart failure, was significantly lower in Tg-MI than that in WT-MI (Table).

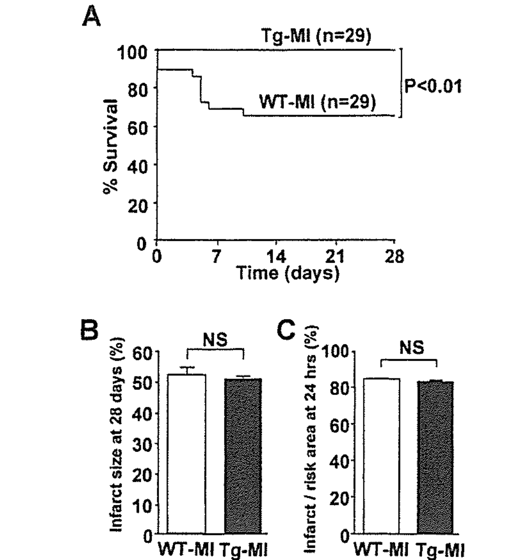

Figure 4. Survival and infarct size. A, Kaplan-Meier survival analysis. Percentages of surviving WT-MI (n=29) and Tg-MI (n=29) mice were plotted. Between-group difference was tested by the log-rank test. B, Infarct size values from WT-MI (n=6) and Tg-MI (n=6) mice in surviving mice 28 days after MI. C, Infarct size (infarct/risk area) values from WT-MI (n=6) and Tg-MI (n=6) mice 24 hours after MI. Values are mean±SEM.

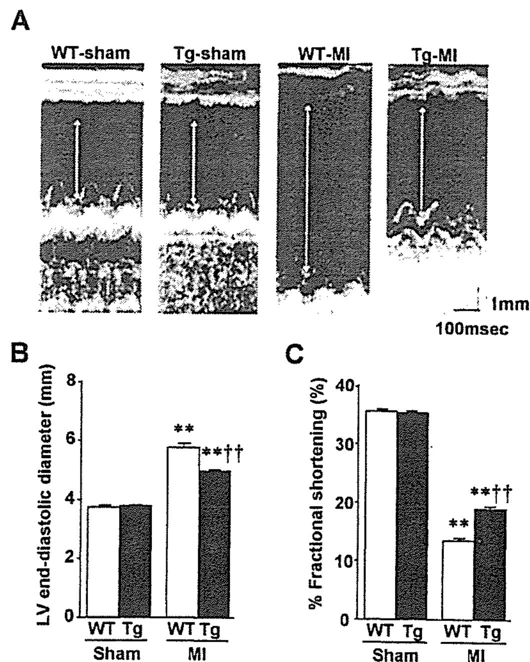

Figure 5. A, Representative M-mode echocardiograms obtained from WT-sham, Tg-sham, WT-MI, and Tg-MI mice. Arrows indicate LV end-diastolic diameter. B, C, Summary data for echocardiographic measurements in 4 groups of animals (n=6 for each). LV end-diastolic diameter (B) and percent fractional shortening (C) are shown. Values are mean±SEM. **P<0.01 for difference from WT-sham values; ††P<0.01 for difference from WT-MI values.

Characteristics of Animal Models

	WT-Sham (n=20)	Tg-Sham (n=21)	WT-MI (n=19)	Tg-MI (n=29)
Hemodynamic data				
Heart rate, bpm	469±6	471±4	479±11	477±4
Mean aortic pressure, mm Hg	77±4	76±2	73±1	74±1
LV EDP, mm Hg	0.7±0.5	0.7±0.4	13.1±2.0**	4.3±0.8*‡
Organ weight data				
Body weight, g	27.3±0.4	26.7±0.4	26.2±0.5	26.0±0.2
LV weight/body weight, mg/g	3.14±0.07	3.23±0.05	3.88±0.24**	3.69±0.09**
RV weight/body weight, mg/g	0.95±0.05	0.98±0.04	1.39±0.12**	1.12±0.05†
Lung weight/body weight, mg/g	5.3±0.1	5.3±0.1	8.3±0.6**	6.4±0.3‡
Pleural effusion, %	0	0	63**	31**†

Values are mean±SEM. EDP indicates end-diastolic pressure; RV, right ventricular.
**P<0.01 vs WT-sham; †P<0.05, ‡P<0.01 vs WT-MI.

Cross-sectional area of cardiac myocytes, an index of cellular hypertrophy, increased in the noninfarcted LV from WT-MI and was significantly attenuated in Tg-MI (Figure 6A). Collagen volume fraction, an index of myocardial interstitial fibrosis, also increased in the noninfarcted LV from WT-MI and was significantly smaller in Tg-MI (Figure 6B). These results indicate that TFAM efficiently counteracts structural and functional deterioration in post-MI hearts.

Apoptosis

To detect apoptosis, myocardial tissue sections were stained with TUNEL staining. TUNEL-positive nuclei were rarely seen in control mice, whereas their number increased in the noninfarcted LV from WT-MI and was significantly decreased in Tg-MI (Figure 7A). In addition, DNA ladder appeared faint in the noninfarcted LV from Tg-MI compared with that from WT-MI, suggesting the attenuation of apoptosis by *TFAM* overexpression (Figure 7B).

Discussion

The present study provides the first direct evidence that the overexpression of *TFAM* can prevent the decline in mtDNA as well as mitochondrial respiratory defects in post-MI hearts. TFAM significantly attenuated cardiac chamber dilatation and dysfunction as well as histopathological changes such as myocyte hypertrophy, interstitial fibrosis, and apoptosis. The apparent beneficial effects of *TFAM* overexpression were not due to its MI size–sparing effect, but they occurred secondary to more adaptive remodeling. All of these beneficial effects could contribute to the improved survival in Tg mice after MI.

Previous studies have suggested an intimate link between mtDNA damage, increased lipid peroxidation, and a decrease in mitochondrial electron transport complex enzyme activities.[4] A growing body of evidence suggests that mtDNA deficiencies and mitochondrial dysfunction play a major role in the development and progression of cardiac failure. A recent study from our laboratory demonstrated a decline in TFAM and mtDNA copy number in a murine heart failure model after MI.[9] These studies imply a relationship between TFAM, mtDNA copy

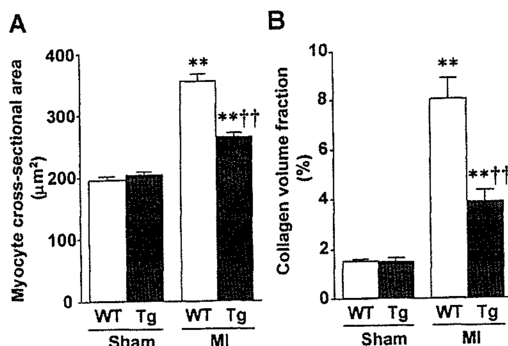

Figure 6. Summary data for histopathological analysis of LV tissue sections in 4 groups of animals (n=6 for each). Myocyte cross-sectional area (A) and collagen volume fraction (B) are shown. Values are mean±SEM. **P<0.01 for difference from WT-sham values; ††P<0.01 for difference from WT-MI values.

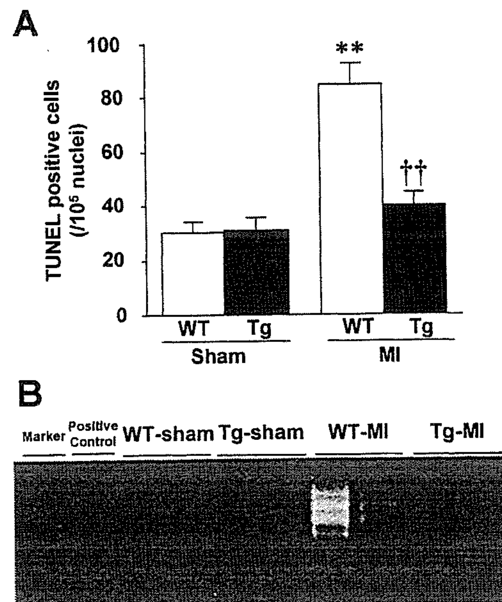

Figure 7. A, Number of TUNEL-positive myocytes in noninfarcted area of LV from 4 groups of animals (n=8 each). Values are mean±SEM. **P<0.01 for difference from WT-sham values. ††P<0.01 for difference from WT-MI values. B, DNA ladder indicative of apoptosis in genomic DNA from LV.

number, and mitochondrial function because the magnitude of the mtDNA defects is parallel to quantitative deficiencies in electron transport function. We thus proposed a direct relationship between TFAM content and electron transport chain activity during the post-MI remodeling process, ignoring the possibility of direct ischemic damage to the electron transport chain complexes. The downregulation of *TFAM* gene expression and a concurrent decrease in mitochondrial genes have been also shown in heart failure induced by aortic banding.[15] In addition, mtDNA depletion has been reported in mitochondrial myopathy and respiratory defects.[19–21] On the basis of these studies, mtDNA defects are considered to be involved not only in the pathogenesis of the diseases caused by inherited defects of mtDNA but also in those secondary to ischemia or mechanical overload.

TFAM not only regulates mtDNA transcription and replication[22] but also maintains mtDNA copy number. In fact, *Tfam* knockout mice, which had a 50% reduction in their transcript and protein levels, exerted a 34% reduction in the mtDNA copy number, 22% reduction in the mitochondrial transcript levels, and partial reduction in the cytochrome *c* oxidase levels in the heart.[11] Moreover, cardiac-specific disruption in the *Tfam* gene in mice exhibited dilated cardiomyopathy in association with a reduced amount of mtDNA and mitochondrial transcripts.[13] The transfection of antisense plasmids in culture, designed to reduce the expression of *TFAM*, effectively decreased the levels of mitochondrially encoded transcripts.[23] On the contrary, the forced overexpression of *TFAM* could produce the opposite effect.[24] Consistent with the present results (Figure 3A, 3B), a recent study by Ekstrand et al[25] demonstrated that the overexpression of human *TFAM* in the mouse increased mtDNA copy number. These lines of evidence imply the primary importance of TFAM as a regulatory mechanism of mtDNA copy number. TFAM has been shown to directly interact with mtDNA to form nucleoids.[26,27] Therefore, increased TFAM may increase the steady-state levels of mtDNA by directly binding and stabilizing mtDNA in Tg-sham mice. Our study also showed that overexpression of human *TFAM* did not increase the respiratory chain complex enzyme activities in Tg-sham mice (Figure 3C), suggesting that the regulation of mtDNA copy number is dissociated from that of electron transport function.[25] Furthermore, our proposed association between TFAM, mtDNA copy number, and electron transport chain activity may be weakened by our data that *TFAM* overexpression did not affect mtRNA levels (online-only Data Supplement I). There may be complex regulatory mechanisms responsible for the association of TFAM, mtDNA, and mitochondrial function, and further studies are clearly needed to solve this issue.

The results obtained from human *TFAM* Tg-sham mice differ from those from the inducible, cardiac-specific overexpression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) transgene in adult mice, which leads to a modest increase in mitochondrial number and development of reversible cardiomyopathy.[28] PGC-1α is the transcriptional coactivator and acts upstream of TFAM and also has the capacity to increase mtDNA levels as well as mitochondrial mass in cultured cells and in Tg mice.[29,30] The reason for the discrepant results between PGC-1 and TFAM transgene overexpression remains unsolved in this study, which, however, may be related to the complex regulatory mechanisms of mitochondrial biogenesis and function by PGC-1 and its downstream factors, including nuclear respiratory factors 1 and 2 and TFAM.[31,32] This may also be due to the difference in the timing of transgene overexpression. Moreover, even though the present study demonstrated the beneficial effects of *TFAM* overexpression on post-MI LV remodeling, it could not determine whether it must occur before the ischemic insult or only during the post-MI phase.

The present study clearly demonstrated that *TFAM* overexpression could ameliorate the decline in mtDNA copy number and preserve it at a normal level in hearts from Tg-MI mice (Figure 3A). *TFAM* overexpression might increase the steady-state levels of mtDNA by directly stabilizing mtDNA. Consistent with alterations in mtDNA, the decrease in oxidative capacities seen in MI was also prevented (Figure 3B). Moreover, our studies establish an important role of TFAM in myocardial protection against remodeling and failure (Figures 4A and 5). The beneficial effects of *TFAM* overexpression shown in the present study were not due to its MI size–sparing effect because infarct size was comparable between WT-MI and Tg-MI mice (Figure 4B, 4C). Furthermore, its effects were not due to the effects on hemodynamics because blood pressure and heart rate were not altered (Table).

Several factors may be attributable to the protective effects conferred by TFAM against myocardial remodeling and failure. First, *TFAM* overexpression prevented the decrease in mtDNA copy number (Figure 3A) and mitochondrial electron transport function (Figure 3B), which may contribute to the decrease in myocardial oxidative stress. The decreased oxidative stress could contribute to the amelioration of cardiac hypertrophy, apoptosis, and interstitial fibrosis.[18] Second, *TFAM* overexpression may induce mitochondrial biogenesis, which, however, is thought to be unlikely because the number and size of the mitochondria assessed by electron microscopy were not altered in Tg-sham mice (online-only Data Supplement II). Importantly, the beneficial effects of *TFAM* overexpression on LV remodeling and failure occurred with the attenuation of increased mitochondrial number seen in MI. Furthermore, an increase in mitochondrial number itself did not necessarily exert beneficial effects in MI.

Several pathogenic mtDNA base substitution mutations, such as missense mutations and mtDNA rearrangement mutations (deletions and insertions), have been identified in patients with mitochondrial diseases.[4] An accumulation of the deleted forms of mtDNA in the myocardium frequently results in either cardiac hypertrophy, conduction block, or heart failure.[33] Furthermore, there is now a consensus view that mutations in mtDNA and abnormalities in mitochondrial function are associated with common forms of cardiac diseases, such as ischemic heart disease[34] and dilated cardiomyopathy.[35] In these conditions, however, the strict causal relationship between abnormalities in mtDNA and cardiac dysfunction has yet to be fully elucidated.

The present study supports our earlier conclusions that the deficiencies of mtDNA contribute to cardiac failure.[9] Furthermore, it confirms that the defects in TFAM are critically involved in mitochondrial dysfunction as well as maladaptive cardiac remodeling and failure. More importantly, the increased

TFAM expression could ameliorate the pathophysiological processes seen in heart failure. MtDNA decline and mitochondrial defects are now well recognized in a variety of diseases such as neurodegenerative diseases, diabetes mellitus, cancer, and even aging. Therefore, with further knowledge on the mechanisms of TFAM for maintenance of mtDNA copy number and mitochondrial function, it may eventually be possible to develop novel strategies for the treatment of such diseases based on the manipulation of TFAM.

Acknowledgments

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Relation of Serum Total Cholesterol and Other Factors to Risk of Cerebral Infarction in Japanese Men With Hypercholesterolemia

— The Kyushu Lipid Intervention Study —

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Background Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT), and few studies have examined the risk factors in the elderly.
Methods and Results Clinical and behavioral risk factors for cerebral infarction were examined in 4,349 Japanese men aged 45–74 years with a serum total cholesterol (TC) concentration of 220 mg/dl or greater who participated in the Kyushu Lipid Intervention Study. A total of 81 men developed definite cerebral infarction in a 5-year follow-up period. The Cox proportional hazards model was used with serum TC at baseline and during the follow-up, serum high-density lipoprotein-cholesterol (HDL-C), HT, diabetes mellitus (DM), and other factors as covariates. Serum TC during the follow-up, not at baseline, was positively associated with cerebral infarction, showing a stronger association in the elderly (≥65 years old) than in the middle-aged (<65 years old). Statin use was related to a moderate decrease in the risk of cerebral infarction when follow-up TC was not considered, but the decrease was almost nullified after adjustment for follow-up TC. A low concentration of serum HDL-C, diabetes mellitus, hypertension, and angina pectoris were each related to an increased risk. No clear association was observed for body mass index, smoking or alcohol use.
Conclusions Lowering cholesterol is important in the prevention of cerebral infarction in men with moderate hypercholesterolemia. A low concentration of HDL-C, DM, and HT are independent predictors of cerebral infarction. (*Circ J* 2005; **69:** 1–6)

Key Words: Cerebral infarction; Diabetes mellitus; High-density lipoprotein-cholesterol; Hypercholesterolemia; Japanese men

Stroke is a leading cause of death and disability in industrialized countries and of the 2 major types of stroke, cerebral infarction predominates, although hemorrhagic stroke remains common in Asian populations.[1] Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT).[2] Findings regarding the relation between serum total cholesterol (TC) or low-density lipoprotein-cholesterol (LDL-C) and cerebral infarction are inconsistent in observational studies,[3–16] whereas cholesterol-lowering trials have shown a decrease in the risk of cerebral infarction among patients assigned to statin treatment.[17,18] The role of serum high-density lipoprotein-cholesterol (HDL-C) is receiving particular interest in the epidemiology of cerebral infarction. Low concentrations of serum HDL-C have been fairly consistently associated with an increased risk of cerebral infarction.[4–9,14,19] Several[5,7,8,12,15,19] but not all[3,6] prospective studies reported that diabetes mellitus (DM) was associated with an increased risk of cerebral infarction. In the study reported here, we examined the relation of serum TC and HDL-C and other factors to the risk of cerebral infarction using data from the Kyushu Lipid Intervention Study (KLIS), a primary prevention trial of coronary heart disease (CHD) events and cerebral infarction in Japanese men with moderately elevated concentrations of serum TC.[20–23] Furthermore, because few studies have investigated the risk factors for cerebral infarction in elderly persons,[6,15,19] we examined the association with these factors in middle-aged and elderly men separately.

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Methods

Details of the study design, patient characteristics at baseline, and primary results of the KLIS have been described previously.[20–23] In brief, a total of 5,640 men aged 45–74 years with serum TC concentration of 220 mg/dl or greater were enrolled by 902 physicians in Kyushu District during the period between May 1990 and September 1993.

Table 1 Characteristics of the Study Subjects by Statin Use

	Mean (SD) or proportion		p-value for difference*
	Statin (–)	Statin (+)	
No. of subjects	1,637	2,712	–
Age (years)	58.1 (8.2)	58.0 (7.9)	0.78
Baseline total cholesterol (mg/dl)	244 (17.9)	258 (25.8)	<0.0001
Follow-up total cholesterol (mg/dl)	225 (25.1)	219 (26.7)	<0.0001
Baseline HDL-cholesterol (mg/dl)	50 (12.0)	49 (12.0)	0.05
Body mass index (kg/m^2)	23.9 (2.8)	24.2 (2.7)	0.008
Angina pectoris (%)	8.2	10.5	0.01
Hypertension (%)	42.6	44.5	0.24
Diabetes mellitus (%)	24.9	22.8	0.11
Prior use of hypolipidemics (%)	7.6	14.3	<0.0001
Current smoking (%)	40.0	37.5	0.10
Daily alcohol use (%)†	41.7	39.6	0.16

Values are mean (SD) unless otherwise specified.
**Comparison of means was based on t-test, and the chi-square test was used for proportions.*
†Drinking alcohol 5 days per week or more frequently.

All the patients gave consent to participate in the study. Ineligible for the study were a history of myocardial infarction, coronary bypass surgery, coronary angioplasty, cerebral hemorrhage, or cerebral infarction; serum HDL-C concentration of 80 mg/dl or greater; and a life-limiting morbid condition such as severe renal or hepatic disease. Each physician was instructed to randomly allocate patients to either pravastatin treatment or conventional treatment as specified in a sealed envelope, but participating physicians did not necessarily follow that instruction.[20] The patients were followed up until the end of 1997 for the occurrence of coronary events and cerebral infarction. The study was approved by the ethical committee of the principal investigator's affiliated institution.

Subjects

The present analysis included 4,349 of the 5,640 enrolled men; 1,291 were excluded for the following reasons: (1) withdrawal of consent (n=147), (2) no institutional contract (n=616; this category represented the lack of a written agreement between a participating hospital or clinic and a sponsoring pharmaceutical company, which became necessary in the course of the study because of the introduction of a new regulation for clinical trials in Japan), (3) found to be ineligible during follow-up (n=97), and (4) missing data (n=431).

Laboratory and Clinical Data

Serum concentrations of TC, HDL-C, triglycerides (TG), and other clinical and biochemical variables were determined at baseline and subsequently in the follow-up. Laboratory measurements were done at different laboratories, but each physician was requested to use the same laboratory throughout the study period. Average serum TC concentrations during the follow-up were determined on the basis of periodical follow-up measurements at 3 months, 6 months, and every year thereafter; the number of measurements ranged from 1 to 10 with a median of 6. Serum LDL-C was not used in the present study because the Friedewald method[24] was not applicable for 7% of the men who even at baseline had serum TG concentrations of 400 mg/dl or greater (n=282) or who had missing data (n=5). HDL-C was not measured in 46 men during the follow-up.

Hypertension was defined as present if a patient had systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or if was under medication for HT. Subjects were defined as having DM if they had either fasting plasma glucose ≥140 mg/dl or hemoglobin A1c ≥6.5% or if they were under medication for DM. The presence of angina pectoris (AP) and prior use of hypolipidemic drugs were based on the report of the study physicians. Statin use was defined if any statin drugs were prescribed during the follow-up period. Height and body weight were recorded, and body mass index (kg/m^2) was calculated as an index of obesity. Current habits of smoking and alcohol drinking were ascertained, together with the number of cigarettes smoked per day and frequency of alcohol drinking per week.

Endpoints

Cerebral infarction was the secondary endpoint and coronary events were the primary endpoint. Definite cerebral infarction was diagnosed when typical symptoms and signs were accompanied with diagnostic findings on brain imaging or cerebral angiography. Diagnosis based only on clinical signs and symptoms was regarded as a suspected case.[20] These endpoints were determined by the Endpoint and Adverse Effect Committee on the basis of periodical reports from the study physicians and, if necessary, by supplementary inquiry. From January to May 1998, an ad hoc survey was carried out to ascertain the occurrence of coronary events and cerebral infarction up to the end of 1997. Vital status was unknown for 36 men, and cerebral infarction and coronary events were not ascertained for 97 men. A total of 81 definite cases and 10 suspected cases of cerebral infarction were identified in an average observation period of 5.05 years. One definite case and 2 suspected cases of cerebral infarction occurred subsequent to a coronary event. None developed coronary events after cerebral infarction. Only definite cases of cerebral infarction were used in the present study, and the follow-up period continued until the event of a definite cerebral infarction regardless of coronary events.

Statistical Analysis

The Cox proportional hazards model was used to examine the relation of clinical and behavioral factors to the risk of cerebral infarction. The principal model included indicator variables for age (5-year class), baseline serum TC (<240, 240–259, ≥260 mg/dl), follow-up TC (<220, 220–239, ≥240 mg/dl), serum HDL-C (<40, 40–59, ≥ 60 mg/dl), BMI (<22.5, 22.5–24.9, ≥25.0 kg/m^2), AP, HT, DM, prior

use of lipid-lowering drugs, current smoking (0, 1–19, ≥20 cigarettes per day), and alcohol use (0, 1–4, ≥5 days per week). The association with statin use was examined using the model with and without follow-up TC. Adjusted relative risk and 95% confidence intervals (CI) were obtained from a regression coefficient and standard error for the corresponding indicator variable. Statistical significance of the interaction was assessed by the likelihood ratio test. Statistical significance was declared when the 95%CI did not include unity or when the two-sided p-value was less than 0.05. Statistical computations were done with the SAS software version 8.2 (SAS Institute, Inc, Carry, NC, USA).

Results

The mean age of the study subjects was 58.0 years, and the mean concentrations of baseline and follow-up serum TC were 253 mg/dl and 221 mg/dl, respectively. The characteristics of the study subjects are summarized by statin use in Table 1. Serum TC concentrations were higher at baseline and lower during the follow-up among men taking statins than in those not taking the medication. The differences were highly significant. The number of cerebral infarctions was 47 (1.7%) in men taking statins and 34 (2.1%) in those who were not.

Although baseline TC was not associated with the risk of cerebral infarction, the follow-up TC concentration was strongly, positively associated with cerebral infarction (Table 2). A nearly 4-fold increase in the risk was observed for men with a follow-up serum TC ≥240 mg/dl compared with those with a concentration <220 mg/dl. High concentrations of serum HDL-C (≥60 mg/dl) was associated with a lower risk of cerebral infarction, although the decrease was not statistically significant (p=0.08). Diabetes mellitus was associated with a statistically significant increase in the risk of cerebral infarction. Men with AP or HT also showed a moderate increase in the risk, and the increased risk associated with HT was statistically significant. No clear association was observed for BMI. A statistically nonsignificant decrease in the risk was observed for each of the intermediate categories of smoking and alcohol use.

Statin use was associated with a statistically non-significant, moderate decrease in the risk of cerebral infarction without adjustment for follow-up TC; adjusted relative risk for statin use was 0.73 (95%CI 0.46–1.16) when follow-up TC was replaced with statin use in the model used for Table 2. When both statin use and follow-up TC were included in the model, the adjusted relative risk for statin use was 0.91 (95%CI 0.57–1.47), and adjusted relative risks for the follow-up TC concentrations of <220, 220–239, and ≥240 mg/dl were 1.00 (referent), 1.69 (95%CI 0.97–2.97), and 3.76 (95%CI 2.15–6.56), respectively. Further, the association between follow-up TC and cerebral infarction was examined by statin use. In that analysis, follow-up TC was used as continuous variable to avoid unstable estimation because of the smaller number of cases. Adjusted relative risks for an increase of 10 mg/dl in TC were 1.31 (95%CI 1.14–1.51) in non-statin users and 1.11 (95%CI 0.99–1.25) in statin users. The increased risk associated with follow-up TC seemed to be attenuated in the latter group, but the interaction was not statistically significant (p=0.16).

When separate analyses were done for men aged less than 65 years and those aged 65 years or older (Table 3), the increased risk of cerebral infarction associated with elevated concentrations of serum TC during the follow-up was slightly more evident in the elderly men than in the middle-aged men. A decreased risk associated with high concentrations of HDL-C was more apparent in the middle-aged men, but neither of the decreases in risk for the 2 age groups was statistically significant. Diabetes mellitus and AP were each associated with a statistically significant increase in the risk of cerebral infarction in the elderly only. Hypertension was statistically non-significantly associated with an increased risk in both middle-aged and elderly men. As regards alcohol use, a statistically significant decrease in the risk was observed for the category of 1–4 days per week in elderly men only.

Table 2 Adjusted Relative Risks of Cerebral Infarction According to Selected Risk Factors

Variable	*No. of men*	*No. of cases*	*Adjusted RR (95%CI)**
Baseline TC (mg/dl)			
<240	*1,463*	*21*	*1.00 (referent)*
240–259	*1,538*	*38*	*1.42 (0.83–2.46)*
≥260	*1,348*	*22*	*0.78 (0.42–1.46)*
Follow-up TC (mg/dl)			
<220	*2,097*	*29*	*1.00 (referent)*
220–239	*1,338*	*23*	*1.72 (0.99–3.00)*
≥240	*914*	*29*	*3.86 (2.23–6.62)*
HDL-cholesterol (mg/dl)			
<40	*970*	*22*	*1.00 (referent)*
40–59	*2,485*	*49*	*0.94 (0.56–1.57)*
≥60	*894*	*10*	*0.50 (0.23–1.09)*
Body mass index (kg/m²)			
<22.5	*1,197*	*22*	*1.00 (referent)*
22.5–24.9	*1,609*	*31*	*1.15 (0.66–2.01)*
≥25.0	*1,543*	*28*	*1.16 (0.65–2.07)*
Angina pectoris			
None	*3,930*	*66*	*1.00 (referent)*
(+)	*419*	*15*	*1.74 (0.98–3.08)*
Diabetes mellitus			
None	*3,325*	*54*	*1.00 (referent)*
(+)	*1,024*	*27*	*1.81 (1.13–2.89)*
Hypertension			
None	*2,445*	*30*	*1.00 (referent)*
(+)	*1,904*	*51*	*1.65 (1.04–2.63)*
Cigarettes per day			
0	*2,679*	*60*	*1.00 (referent)*
1–19	*474*	*6*	*0.54 (0.23–1.26)*
≥20	*1,196*	*15*	*0.82 (0.46–1.46)*
Alcohol use (days/week)			
0	*1,683*	*44*	*1.00 (referent)*
1–4	*910*	*10*	*0.50 (0.25–1.01)*
≥5	*1,756*	*27*	*0.85 (0.52–1.41)*

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on the Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, and listed variables.*

Discussion

Methodological problems need to be clarified before interpreting the present findings. Because the patients were non-randomly allocated to pravastatin treatment, cardiovascular risk factors were generally more prevalent among statin users, as shown in Table 1. Although statistical adjustment was done for these factors, residual confounding effects possibly remained. Statin use was strongly associated with lower concentrations of follow-up TC, and it may be difficult to conclude which is more importantly related to the risk of cerebral infarction on statistical grounds. All the patients were treated for hypercholesterolemia regard-

Table 3 Adjusted Relative Risk of Cerebral Infarction According to Selected Risk Factors in Men Aged Less Than 65 Years and Older Men

Variable	*<65 years (n=3,115)*		*≥65 years (n=1,070)*	
	No. of cases	*Adjusted RR (95%CI)**	*No. of cases*	*Adjusted RR (95% CI)**
Baseline TC (mg/dl)				
<240	*7*	*1.00 (referent)*	*14*	*1.00 (referent)*
240–259	*16*	*1.91 (0.78–4.70)*	*22*	*1.15 (0.57–2.31)*
≥260	*15*	*1.65 (0.64–4.26)*	*7*	*0.38 (0.15–0.98)*
Follow-up TC (mg/dl)				
<220	*13*	*1.00 (referent)*	*16*	*1.00 (referent)*
220–239	*12*	*1.40 (0.63–3.12)*	*11*	*1.91 (0.87–4.19)*
≥240	*13*	*2.45 (1.07–5.60)*	*16*	*5.26 (2.53–10.95)*
HDL-cholesterol (mg/dl)				
<40	*12*	*1.00 (referent)*	*10*	*1.00 (referent)*
40–59	*23*	*0.70 (0.35–1.43)*	*26*	*1.33 (0.63–2.82)*
≥60	*3*	*0.33 (0.09–1.18)*	*7*	*0.70 (0.25–1.94)*
Body mass index (kg/m²)				
<22.5	*7*	*1.00 (referent)*	*15*	*1.00 (referent)*
22.5–24.9	*14*	*1.26 (0.51–3.15)*	*17*	*1.20 (0.59–2.46)*
≥25.0	*11*	*1.45 (0.59–3.56)*	*11*	*1.01 (0.45–2.27)*
Angina pectoris				
None	*34*	*1.00 (referent)*	*32*	*1.00 (referent)*
(+)	*4*	*0.99 (0.35–2.82)*	*11*	*2.70 (1.32–5.53)*
Diabetes mellitus				
None	*26*	*1.00 (referent)*	*28*	*1.00 (referent)*
(+)	*12*	*1.39 (0.70–2.77)*	*15*	*2.42 (1.25–4.68)*
Hypertension				
None	*16*	*1.00 (referent)*	*14*	*1.00 (referent)*
(+)	*22*	*1.50 (0.77–2.90)*	*29*	*1.89 (0.98–3.67)*
Cigarettes per day				
0	*27*	*1.00 (referent)*	*33*	*1.00 (referent)*
1–19	*2*	*0.45 (0.11–1.92)*	*4*	*0.59 (0.21–1.72)*
≥20	*9*	*0.83 (0.38–1.81)*	*6*	*0.83 (0.33–2.04)*
Alcohol use (days/week)				
0	*17*	*1.00 (referent)*	*27*	*1.00 (referent)*
1–4	*7*	*0.79 (0.32–1.92)*	*3*	*0.28 (0.08–0.94)*
≥5	*14*	*0.83 (0.40–1.73)*	*13*	*0.94 (0.47–1.90)*

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on the Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, and listed variables.*

less of statin use, and cardiovascular risk factors ascertained at baseline may have changed in varying degrees. The relation of clinical risk factors at baseline to the risk of cerebral infarction may have been attenuated because comorbid conditions such as HT and DM were probably well treated during the follow-up.[25] Finally, results from the subgroup analysis should be interpreted cautiously. In the analysis by statin use or by age group, the estimated relative risks were more subject to random fluctuation because of the smaller number of cases.

The present study demonstrated an evident, positive association between the serum TC concentration in the follow-up period, but not at baseline, and cerebral infarction. Statin use was associated with a moderate, statistically nonsignificant decrease in the risk of cerebral infarction when follow-up TC was not taken into consideration. The magnitude of the decrease in the risk associated with statin use was the same as reported for pravastatin use previously in the KLIS.[21] However, the decreased risk associated with statin use was almost nullified after adjustment for follow-up TC. The positive association with follow-up TC remained after adjustment for statin use. These findings indicate that lowering cholesterol itself is important in the prevention of cerebral infarction among men with moderate hypercholesterolemia.

It remains a matter of controversy whether the reduced risk of stroke or ischemic stroke associated with use of statins can be ascribed to the cholesterol-lowering effect of statins or to other properties.[26,27] Statins are known to ameliorate endothelial dysfunction, stabilize atherosclerotic plaques, and modify inflammatory responses and thrombus formation.[28] The observation that statin treatment confers a reduced risk of ischemic stroke among hypertensive patients with average or below-average cholesterol concentrations suggests a role of the nonlipid-lowering effects of statins.[29] On the other hand, a meta-analysis of randomized controlled trials indicated that the beneficial effect on stroke incidence was seen only when the final cholesterol concentration was <232 mg/dl (6.0 mmol/L), suggesting the importance of lowering cholesterol.[30] Another meta-analysis of 7 prospective observational studies showed a statistically significant decrease of 15% in the risk of thromboembolic stroke for a 1.0 mmol/L decrease in LDL-C.[31] In this regard, the interaction between statin use and follow-up TC is of particular interest. In the present study, the positive relation between follow-up TC and cerebral infarction seemed weaker among statin users. The findings may be interpreted as suggestive of a protective effect of statins other than their cholesterol-lowering effect, but it is also possible that uncontrolled risk factors other than elevated concentrations of serum TC may be major determinants of the risk of cerebral infarction among statin users because they are generally at a higher risk of cardiovascular diseases on entry. A larger study is needed to clarify the relation between