TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r-square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means ± S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t-test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was established at $p<0.05$. Linkage disequilibrium was calculated using SNPAlyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*−832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5'UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5'UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5'UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaaggtg [g/t] acggtggcgaca	
1754C>T		Exon(3'UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3'UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3'UTR)		99.6	0.4	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3'UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3'UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3'UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3'flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3'flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r-square=0.84, group B r-square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women (n=1215)	Men (n=1032)	p
Age, years ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's t-test or X^2 analysis.

mutations were missense mutations (R385K; n=2, A455V; n=53 heterozygous, n=14 homozygous, D468Y; n=2). Four mutations within the TM promoter region and the 5′-untranslated region (5′-UTR) (−832C>A, −754G>C, −265C>A, −58G>C) were rare. Twenty-five patients were heterozygous carriers for the −202G>A mutation within the promoter region, which was reported as a −33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r-square>0.8) with 2005G>A in the 3′-UTR. No patients were carriers for previously reported mutations in the lectin-like domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain (n=53 heterozygous, n=14 homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r-square>0.9) with the 2729A>C mutation within the 3′-UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (−202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects. As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p=0.02$ and odds ratio = 2.98, 95% confidence interval = 0.21–7.33; $p=0.02$, respectively) (Table 6). This mutation was in linkage disequilibrium (r-square > 0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3′-UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β-estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2, or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3'-UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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To the editor:

Protein S–K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients

Deep vein thrombosis (DVT) is a multifactorial disease caused by interactions between acquired risk factors and coagulation abnormalities.[1] In whites, the factor V–Leiden and the prothrombin-20210G>A are widely recognized as genetic risk factors for DVT. However, these 2 mutations are not present in Japanese populations, and little is known about the genetic risk factors for DVT in these populations. In this study, we evaluated the genetic contributions of 5 polymorphisms in Japanese DVT patients. The plasminogen-A620T mutation, formerly referred to as plasminogen-Tochigi, and the protein S–K196E mutation, formerly referred to as protein S–Tokushima, exhibited decreased activities of plasminogen and protein S despite normal antigen levels.[2-4] The ADAMTS13-P475S mutation exhibited low von Willebrand factor–cleaving activity in vitro.[5] The factor XII–4C>T substitution in the 5′-untranslated region, formerly referred to as 46C>T, showed decreased plasma levels of both antigen and activity.[6] The plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism is related to in vitro differences in transcription activity.[7] We genotyped subjects for these 5 polymorphisms and compared their genotypic frequencies between 161 DVT patients and 3655 population-based controls. The protocol for this study was approved by the ethical review committee, and only those subjects who provided written informed consent for genetic analyses were included in this study. All participants of this study were Japanese. The controls were from a general population randomly selected from the residents of Suita City located in the second largest urban area in Japan (the Suita Study).[8] One hundred sixty-one DVT patients, 78 men and 83 women, were registered by the Study Group of Research on Measures for Intractable Diseases, working under the auspices of the Ministry of Health, Labor, and Welfare of Japan. Six centers (Tochigi, Tokyo, Nagoya, Kyoto, and 2 in Osaka) participated in this study. The patients' mean age was 49.5 years (range, 12-87 years) and their mean body mass index was 23.6 ± 3.3. Thirteen percent of patients had a family history of thrombosis, and 16% of the patients had recurrent thrombosis.

Of all the polymorphisms tested, only the frequency of protein S–K196E was statistically different between the 2 groups ($\chi^2 = 38.3$, $P < .001$) (Table 1). No other frequency differences were statistically significant. Two DVT patients were homozygous for the protein S–196E allele; however, no homozygotes were identified in the control group. One patient with the 196EE genotype first developed DVT following surgery at age 47, while the other patient developed DVT during pregnancy at age 32.

The mutant protein S with the E allele has already been intensively studied as protein S–Tokushima.[11] The protein S mutant showed the reduced activated protein C cofactor activity compared with wild-type protein S, suggesting a direct link between the protein S–K196E mutation and the development of DVT. By the genotyping of the general population, the protein S–196E allele frequency was estimated as 0.009. Thus, a substantial portion of the Japanese population harbors this mutant allele and is at higher risk for DVT.

Table 1. Numbers and genotypic frequencies of protein S–K196E mutation in the DVT and control groups

Genotypes	General population, no. (%)	DVT group, no. (%)
Additive model*		
KK	3585 (98.2)	146 (90.7)
KE	66 (1.8)	13 (8.1)
EE	0 (0.0)	2 (1.2)
Total	3651 (100.0)	161 (100.0)
Dominant model†		
KK	3585 (98.2)	146 (90.7)
KE + EE	66 (1.8)	15 (9.3)
Total	3651 (100.0)	161 (100.0)

DNA genotyping was performed by the TaqMan allele discrimination method.[9] We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide + 1, and the initial Met residue is denoted as amino acid + 1, resulting in the renaming of several mutant alleles.[10] Comparisons between the DVT cases and the controls were analyzed using a χ^2 test with the genotypes as independent variables (indicated by *P* and OR) or using multiple logistic analyses with the genotypes as independent variables and age and sex as covariates (indicated by *P*′ and OR′).

*For comparison of general population to DVT group, *P* was not determined.

†For comparison of general population to DVT group, $P < .001$; OR = 5.58 (3.11-10.01); $P' < .001$; OR′ = 4.72 (2.39-9.31).

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Risks and Pregnancy Outcome in Women With Prosthetic Mechanical Heart Valve Replacement

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Background Pregnancy after mechanical heart valve replacement is highly risky for both mother and child because of the aggravation of maternal heart function and adverse effects of anticoagulation therapy. In Japan, however, the risks and pregnancy outcomes in women with prosthetic mechanical heart valve replacement remain to be elucidated.

Methods and Results In the present study 16 pregnancies in 12 women with prosthetic mechanical heart valve replacement were identified between 1983 and 2005. At 6–13 weeks of gestational age, warfarin, an anticoagulant agent, was changed to heparin and administration was continuously adjusted according to the activated partial thromboplastin time level up to the time of delivery. Major maternal complications and pregnancy outcomes were retrospectively investigated. The valve replaced was mitral (n=7), tricuspid (n=7), and aortic (n=2). Eight (50%) of 16 had cesarean live births. One case was delivered at full term, and 7 cases were delivered preterm (26–36 weeks) because of maternal indications. Two babies died in the neonatal period. Therapeutic abortion was performed in 3 cases, 4 cases ended in early miscarriage, and 1 case ended in intrauterine fetal death (30 weeks). Three mothers developed valve (mitral, tricuspid, aortic) thrombosis. There was 1 maternal death from heart failure.

Conclusions Pregnancy after mechanical heart valve replacement requires strict control of coagulation. Special attention should be paid to the occurrence of complications during anticoagulation therapy. (*Circ J* 2007; **71:** 211–213)

Key Words: Fetal outcome; Heparin; Maternal complication; Mechanical heart valve replacement; Pregnancy outcome; Warfarin

Although the recent decrease in the prevalence of rheumatic heart disease in young women has decreased the prevalence of mechanical heart valve replacement, and developments in cardiac surgery have replaced mechanical heart valves with biological heart valves, these same advances in cardiac surgery have enabled women with mechanical heart valves after surgery for complex cardiovascular anomalies to survive long term. For such women, long-term management of coagulation is essential.

Pregnancy after mechanical heart valve replacement has the potential risks of maternal heart failure, arrhythmia, infectious endocarditis, and maternal death with advancing gestational age! For such cases, obstetricians have sometimes chosen therapeutic abortion or premature birth to save the mother's life. In addition, anticoagulation therapy using warfarin throughout the pregnancy carries risks of inducing congenital fetal anomaly, abortion, and early neonatal death, as well as maternal hemorrhage? However, inadequate anticoagulation therapy may induce thromboembolism. Thus, pregnancy in women with prosthetic mechanical heart valve replacement remains problematic and troublesome even now. In Japan, however, only limited data are available with regard to the impact of mechanical heart valve replacement and anticoagulation therapy on pregnancy outcome and risks.

Methods

We retrospectively identified 16 pregnancies in 12 women with prosthetic mechanical heart valve replacement managed between 1983 and 2005 at the Department of Perinatology, National Cardiovascular Center, Osaka, Japan. Warfarin, an anticoagulation agent, was changed to heparin around 6–13 weeks of gestational age, and administration of heparin was continued to parturition. The dose of heparin was regulated from 20,000 to 30,000IU/day, according to the circulating activated partial thromboplastin time (APTT) levels. APTT in patients was maintained 2–3-fold higher than in controls. Except for those who strongly desired to

Table 1 Fetal Outcomes for the Enrolled Subjects (n=16*)

Live birth	*8*
Term	*1*
Preterm	*7*
Spontaneous abortion	*4*
Therapeutic abortion	*3*
Intrauterine fetal death	*1*

**Sixteen pregnancies in 12 women.*

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Table 2 Details of the Live Births

Case no.	Valve site	Mode of delivery	Birth weight (g)	Fetal outcome	Change to heparin (weeks of gestation)	Heparin delivery	Warfarin	Complications
1	Mitral	38W·CS	2,458	Alive	6	SC	–	–
2	Mitral	33W·elective CS	1,730	Alive	8	SC	–	Valve thrombosis
3	Mitral (marfan syndrome)	33W·CS	1,968	Alive	6	SC	–	Dissection of carotid artery
4	Tricuspid (functional MV)	33W·CS	1,620	Alive	13	SC	–	Subchorionic hematoma
5	Tricuspid (functional MV)	27W·CS	1,063	Alive (hydrocephalus)	4–15, 26	DIV	+	Fetal hydrocephalus
6	Tricuspid (functional MV)	36W·elective CS	2,104	Alive	5	DIV	–	–
7	Aortic	26W·CS	797	Dead (IVH)	5	DIV	–	ICH
8	Tricuspid	27W·CS	1,063	Dead (pulmonary hemorrhage·IVH)	6	DIV/SC/DIV	–	ICH, Valve thrombosis

W, weeks; CS, cesarean section; SC, subcutaneous; MV, mitral valve; DIV, drip infusion; IVH, intra ventricular hemorrage; ICH, intracranial hemorrhage. Cases 5 and 6 were the same mother; cases 4, 13, 14, and 15 were the same mother.

Table 3 Details of Fetal Loss

Case no.	Valve site	Change to heparin (weeks)	Abortion or IUFD (weeks)	Outcome
9	Mitral	5	7	SA
10	Mitral	9	9	SA
11	Mitral	10	10	SA
12	Mitral	10	10	SA
13	Tricuspid (functional MV)	9	9	Therapeutic abortion
14	Tricuspid (functional MV)	10	19	Subchorionic hematoma/Therapeutic abortion
15	Tricuspid (functional MV)	5	15	Subchorionic hematoma/Therapeutic abortion
16	Aortic	27	30	IUFD during extracorporeal circulation

IUFD, intrauterine fetal death; SA, spontaneous abortion. Other abbreviation see in Table 1. Cases 4, 13, 14, and 15 were the same mother.

Table 4 Comparison of Complications Between Women Receiving Subcutaneous and Intravenous (Drip Infusion) Heparin

Anticoagulation regimen	Valve thrombosis	Bleeding			Maternal death
		Subchorionic hematoma	ICH	Perinatal bleeding	
Heparin (SC) (n=7)	2	3	0	2	0
Heparin (DIV) (n=4)	0	0	2	2	0

Abbreviations see in Table 2.

remain as outpatients, patients was hospitalized in principle throughout pregnancy. For the patient who hoped to be managed at home, heparin was administered by subcutaneous injection, and APTT for 6 h afterward was controlled to 2–3-fold the normal value. Continuous intravenous infusion of heparin was given to all cases before labor, and administration of heparin was stopped at delivery. Six to 12 h after parturition, heparin was administered again and then changed to warfarin after the decrease in postpartum uterine bleeding. Pregnancy outcomes, including maternal complications and fetal outcomes, were retrospectively investigated. Valvular thrombosis, subchorionic hematoma, maternal intracranial hemorrhage, and perinatal bleeding were compared between the women receiving subcutaneous injections and those receiving drip infusions.

Results

Table 1 shows the fetal outcomes for 16 pregnancies in 12 women with mechanical heart valve replacement: there were 8 live births (50%) and 8 cases of fetal loss.

Table 2 shows the details of the 8 live births. The gestational ages of these infants ranged from 26 to 38 weeks. One case (case 1) was delivered at full term, and 7 cases (cases 2–8) were delivered preterm because of maternal indications. All cases were delivered by cesarean section. Two neonates died (cases 7, 8). Two patients (cases 2, 8) who had received subcutaneous heparin therapy developed valvular (mitral, tricuspid) thrombosis, and thrombolytic therapy was performed during pregnancy. Patient 5 who had received heparin from 4 to 15 weeks and then warfarin from 16 to 26 weeks, underwent termination because of fetal hydrocephalus associated with intracranial hemorrhage.

Table 3 shows the details of the women with fetal loss. Four pregnancies (cases 9–12) resulted in spontaneous abortion at an early stage. Therapeutic abortion was performed in 3 cases (cases 13–15). In case 13, abortion was performed at the patient's request. Case 14 ended in thera-

peutic abortion because of intrauterine infection associated with the enlarged subchorionic hematoma. In case 15, aggravation of maternal anemia from massive subchorionic hemorrhage resulted in therapeutic abortion. Case 16 was a very severe case. The patient did not receive anticoagulation therapy at her own request 26 weeks of gestational age, and she then developed aortic valve thrombosis. At 30 weeks, the aortic valve was surgically replaced, but intrauterine fetal death occurred during intraoperative extracorporeal circulation and the mother also died of heart failure 3 days after cardiac surgery.

Table 4 is a comparison of complications in the patients receiving subcutaneous and those receiving intravenous (drip infusion) heparin. Valvular thrombosis formation occurred more frequently in the subcutaneous group, but the risk of bleeding was comparable between the 2 groups. Mean gestational age at the change to heparin in the cases of early miscarriage was 8.5 weeks (5–10 weeks), compared with 6.6 weeks (5–13 weeks) in the cases of continuing pregnancy. It appears that in cases of early miscarriage the change to heparin was often delayed. There was no heparin-induced thrombocytopenia.

Complications occurred in 3 patients with prosthetic mechanical heart valve replacement (cases 2, 8, 16) because of valve thrombi formation, in 4 cases (cases 3–6) because of bleeding after surgery, and in 3 cases (cases 4, 14, 15) because of subchorionic hematoma.

Discussion

Currently, biological heart valve replacement is being performed in young women because it does not require anticoagulation therapy[3] but biological valves require replacement after 10–15[4] whereas mechanical valves do not, although they require long-term anticoagulation therapy to minimize the high risk of associated thromboembolic complications. Pregnancy is a physiologic hypercoagulable state that further increases the risk of functional deterioration of the valve. There are several reports of the risks and pregnancy outcomes in women with mechanical heart valve replacement in Western countries[1-3,6,7] but only limited data are available in Japan.

Although warfarin is an easily administered and controllable drug, switching to heparin in the early weeks of gestational age is recommended because warfarin carries a potential risk of teratogenicity and higher rate of fetal loss[6,7] We have conventionally performed anticoagulation with subcutaneous heparin administration, but it can sometimes be difficult to control maternal coagulability with this technique because of the drug's narrow therapeutic range. In fact, in the present study, 2 patients developed valve thrombosis during subcutaneous heparin therapy, but this was not observed in women receiving intravenous heparin. Intravenous drip infusion of heparin may be superior to conventional subcutaneous administration.

The rate of healthy babies born to these mothers was 37.5% (6/16), which appears to be lower than that reported by Nassar et al[2] It is possible that the anticoagulation therapy we administered was inappropriate. Two mothers in the intravenous heparin therapy group developed intracranial hemorrhage, but in both cases the APTT was extended approximately 2-fold, so it is unlikely that drip infusion of heparin contributed to the maternal intracranial hemorrhage. Recently, in Japan, there have been several reports that lower doses of warfarin are recommended for non-pregnant Japanese patients with atrial fibrillation or prosthetic heart valves, in order to maintain anticoagulability and reduce side-effects[8-11]

Vitale et al demonstrated dose-dependent fetal complications of warfarin in pregnant women with mechanical heart valves and they concluded that a dose less than 5 mg of warfarin does not have a major impact on the fetus[12] However, it remains unclear whether warfarin at less than 5 mg/day would be appropriate for pregnant Japanese women because of racial differences in lifestyle, food intake, drug metabolism, body size, and bleeding tendency. Thus, further data for Japanese women should be gathered.

In the present study the rate of healthy babies born to women with mechanical heart valve replacement was lower than that reported from Western countries. Anticoagulation therapy using heparin might be inappropriate, so it is necessary and warranted to determine the appropriate dose and route of heparin therapy for pregnant Japanese women with prosthetic mechanical heart valve replacement.

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【症候への産科プライマリケア　4】

胸痛・背部痛

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はじめに：妊娠と症候との関連性

妊娠中に胸痛や背部痛を主症状して発症する疾患には，生命を脅かす疾患が含まれている．例えば，平成８年度厚生省心身障害研究「妊産婦死亡の防止に関する研究」（主任研究者：武田佳彦）による[1]，平成3～4年の2年間にわが国で死亡した320例の妊産婦死亡の原因のなかで，肺塞栓症，羊水塞栓症の直接産科的死亡24例，心血管系疾患と呼吸器疾患の間接産科的死亡10例が胸背部痛を主症状とした．このような，緊急性を有する疾患を疑い，可能性のある場合には，高次施設へ紹介することが必要である．

しかし，ほとんどの胸痛を訴える妊産婦は，生命の危険がないものである．Klinkmanら[2]は，胸痛を主訴に救急外来を受診した399例の原因を解析したMIRNET研究を行った．その結果，不安定狭心症と心筋梗塞など，緊急性を持つ患者は全体の1.5％とわずかであり，そのほかは生命の危険がないものであった．そのうち，肋軟骨炎を含む筋・骨格系疾患が最も多く36％，その次に逆流性食道炎が13.4％，安定狭心症は10.5％であった．その他，心因性8％，肺疾患5％，その他不明16％と続いた．この研究は，妊婦を扱ったものではないため，妊産婦のみを対象とした場合，その頻度は変わってくるであろう．例えば，妊娠に多い逆流性食道炎などによる胸痛の比率が増加してくることが考えられる．しかし，妊産婦といえども，胸背部痛を訴える患者を，「マイナートラブル」と片付けずに，常に心筋梗塞，肺塞栓，大動脈解離および緊張性気胸などの生命を脅かす，緊急性のある疾患を除外する態度が必要である．

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プライマリケアとしての鑑別診断

胸痛を起こす疾患，臨床所見，鑑別に必要な所見を表1に[3,4]に示す．心筋梗塞，肺塞栓，大動脈解離および緊張性気胸は，緊急に診断し治療しないと生命にかかわり，killer fourと呼ばれる．

1．心筋梗塞

虚血性心疾患は妊娠可能年齢の女性に発症することは少なく，妊娠中に遭遇することは稀である．しかし，近年の高齢妊娠の増加や冠動脈障害をきたした川崎病既往患者が成人に達することを考えると，今後合併妊娠の増加も考えられる．また分娩時，児娩出後に使用する麦角アルカロイドは末梢血管の収縮や冠動脈の攣縮を起こすことがあるので，使用に際しては心電図のST-T変化に注意しながら慎重に投与する必要がある．

2．肺塞栓症，羊水塞栓症

突然発症する前胸部全体の漠然とした痛みで程度はあまり強くないことが多い．しかし，強い呼吸困難を伴うことが特徴であり，チアノーゼ，咳，ときに血痰もみられることがある．妊娠中，産褥期に発症しやすく，特に切迫早産による長期臥床後や帝王切開後の初回歩行時などに注意が必要で

表1　胸痛を起こす疾患[3,4]

	疾患	臨床所見	鑑別に有用な所見
1. 心臓	1. 狭心症	前胸部の圧迫感，絞扼感，下顎，心窩部，肩，左腕への関連痛	労作時，寒冷，興奮などが誘引となっている 持続時間＜2～10分
	2. 安静または不安定狭心症	狭心症より程度が強い	持続時間＜20分
	3. 急性心筋梗塞	狭心症より程度が強い	突然発症し，持続時間は通常＞30分 息切れ，虚脱感，悪心・嘔吐，冷汗をしばしば伴う
	4. 心膜炎	鋭い痛み，体位で変化する	心膜摩擦音
2. 大血管	1. 大動脈解離	前胸部，背部，腰部の突然の激痛	耐え難い疼痛が，大動脈の走行に沿って移動する 高血圧・マルファン症候群などの基礎疾患の存在
	2. 肺塞栓	突然起こる呼吸困難と疼痛	呼吸困難，多呼吸，頻脈および右心不全を伴うことあり
	3. 肺高血圧	前胸部の圧迫感，運動にて増悪	呼吸困難およびそのほかの肺高血圧症の症状
3. 肺	1. 胸膜炎 and/or 肺炎	胸膜痛，罹患部に一致することが多い	側方から正中部にかけ，呼吸困難を伴う
	2. 気管・気管支炎	正中部の灼熱感・違和感	正中部で，咳を伴う
	3. 自然気胸	突然起こる一側の疼痛 呼吸困難を伴う	突然発症，呼吸困難
4. 消化管	1. 逆流性食道炎	前胸部の灼熱感と不快感 10～60分間の持続が多い	多い食餌量や食後に横になったときに増悪 制酸剤にて軽快
	2. 胃潰瘍	持続する前胸部の灼熱感	制酸剤や食餌をとることで軽快
	3. 胆嚢の疾患	持続する心窩部や右季肋部痛	自発的および食後に起こる
	4. 膵炎	持続する強い心窩部や前胸部痛	アルコール，高トリグリセリド血症，薬物などのリスク
5. 筋骨格系	1. 肋軟骨炎	突然起こる一過性の疼痛	罹患部の圧迫によって疼痛，同部に炎症所見
	2. 頸部椎間板ヘルニア	突然起こる一過性の疼痛	一定の首の位置で，疼痛が誘発される
6. 感染症	帯状疱疹	デルマトームに一致した持続性の焼けるような痛み	皮疹，デルマトーム
7. 神経症	パニック障害	運動や労作と関係のない，前胸部の絞扼感，疼痛 呼吸困難を伴うことあり 30分以上持続することあり	別の神経症を有することあり

（文献3，4を引用・改変）

ある．また，周産期に特異的な肺塞栓症として羊水塞栓が挙げられる．羊水塞栓症の症状は，肺血栓塞栓症に類似するが，血液凝固異常が強い特徴がある．

3．大動脈解離

大動脈解離の最も重要な素因は高血圧であるが，妊娠中の若年女性ではMarfan症候群などのコラーゲン線維の先天異常や大動脈弁輪拡大，大動脈二尖弁，稀にTurner症候群，大動脈縮窄症，コカイン中毒などが原因となることもある．われわれは，妊娠中の大動脈解離例をこれまで23年間に5例経験したが，3例が未診断のMarfan症候群であった[5]．妊娠前に指摘されていなくても，特徴的な体型などから積極的に疑うことも必要である．

胸痛の特徴は，鋭い裂くような胸背部痛であり，大動脈の走行に沿って移動することがある．来院時には高血圧であることが多いが，逆にバイタルサインが不安定でプレショックとなっている場合もある．

4．自然気胸

長身，痩せ型，扁平胸郭の体型の人に多い．突然出現する比較的鋭い痛みであり，ほとんど左右どちらかの片側性である．胸痛はむしろ安静時に多い．しばしば咳，呼吸困難，頻脈を伴う．患側における呼吸音の減弱をみる．胸痛はむしろ安静時に多い．呼吸状態が落ち着かず，進行性に増悪するのは緊張性気胸の可能性がある．

以上のkiller fourの疾患以外の，胸背部痛を訴える主な疾患を概述する．

5．狭心症

胸痛は心筋梗塞に類似するが，痛みは心筋梗塞より軽く，より漠然としていることが多い．再現性を持って，一定の労作で狭心症が誘発されれば労作性狭心症と考えられる．持続時間は通常15分以内で，安静や硝酸薬の舌下により軽快する．冠動脈攣縮による異型狭心症では発作は夜間就寝時，特に夜中から明け方に多いのが特徴である．

6．心外膜炎・心筋炎

発熱や上気道炎を伴い，亜急性に胸骨後部の漠然とした痛みが出現する．胸痛は体位や呼吸で変化し，臥位になったときに痛みが増悪する．

7．僧帽弁逸脱症

若年女性に多い心疾患であり，妊娠に合併することがある．痩せた背の高い患者が労作とはあまり関係のない胸痛，動悸，胸部違和感をときどき訴える場合に本症を疑う．聴診上，mid systolic clickからそれに続くlate systolic murmurが典型的であり，心エコー検査にて診断を確定する．

8．胸膜炎

発熱や咳を伴い，亜急性に出現する．多くは側胸部に片側性の痛みを生じる．痛みは呼吸や体位で変化することが特徴的である．

9．逆流性食道炎

前胸部の灼熱感と不快感が10～60分間，持続することが多い．妊娠により増悪する傾向にある．

妊娠に配慮した検査の進め方

急性の胸背部痛を訴える患者に対しては，バイタルサインのチェックと並行して静脈路を確保し，下記の検査を進めていく．

1．心電図

12誘導心電図は，心筋梗塞や狭心症などの急性冠症候群（acute coronary syndrome：ACS）において，胸痛患者が来院したとき，第一に素早く行う検査として位置づけられている[1]．心筋梗塞では典型的にはST上昇，異常Q波がみられるが，正常心電図所見であっても心筋梗塞や狭心症を否定することにはならず，疑わしい場合は，心筋障害血清マーカーなどのほかの検査や，繰り返し心電図検査を行う必要がある．狭心症ではST低下が認められる．心膜炎と心筋炎では広範囲の誘導でST上昇がみられる．肺塞栓症では，洞性頻脈とⅠ誘導における深いS波とⅢ誘導におけるq波，また右側胸部誘導の陰性T波や右脚ブロックが認められることが多い．

2．血液検査

心筋障害マーカーとして，CK-MB，心筋特異的トロポニンTおよびI（cTnT,cTnI），心臓型脂肪酸結合蛋白（H-FABP）が頻用される．心筋炎でもこれらの心筋障害マーカーが上昇を認めるが，変化が緩やかであることが特徴である．また，胸

膜炎，心膜炎，心筋炎など炎症性疾患では白血球数の増加やCRP上昇など炎症所見がみられる．肺塞栓では，動脈血ガス分析で著明な低酸素血症と低二酸化炭素血症が同時に認められる．非妊娠時の肺塞栓の診断に有用である血清Dダイマーは，妊娠中には正常でも上昇傾向にあり，陽性であっても診断には使用できない．

3．心エコー

心筋梗塞においては，梗塞部位の壁異常運動（アシナジー）が早期から認められ，心室瘤や乳頭筋断裂などの合併症も診断できる．心筋炎では，全般的な壁異常運動に加えて，心筋間質の浮腫により発症初期に一過性の壁肥厚を認めることが多い．心膜炎や大動脈解離の一部では，心嚢液の貯留がみられる．大動脈解離では大動脈径の拡大やflapが観察されることがある．臨床症状により，大動脈解離が疑われるときには，経食道エコーは，特に上行性大動脈が解離するStanfordA型の診断にきわめて有用である．肺塞栓において，右室の拡大，心室中隔の左室への圧排，三尖弁および肺動脈弁閉鎖不全といった右心負荷の所見が診断に有用である．

4．胸部X線写真

縦隔影の拡大は，大動脈解離や突発性食道破裂で，胸水貯留は突発性食道破裂や胸膜炎で認められる．突発性食道破裂では気胸も合併することがある．自然気胸では虚脱肺の上端が鎖骨より下であればⅡ度以上であり，胸腔穿刺の適応となる．重症の肺塞栓では血管影が減弱することがある．心拡大がある場合，心膜炎による心嚢液の貯留や心筋梗塞，心筋炎による心不全などが考えられる．

5．胸部CT

大動脈解離の診断には造影CTがきわめて有用である．また，肺塞栓の診断においても，近年CT装置の発達は，従来の肺換気・血流シンチグラムの必要性をなくすぐらいに精度が上がっており，妊娠中といえども，第一選択とすべき場合がある．ヘリカルCTの導入により3次元再構成なども可能となっている．そのほか，突発性食道破裂，心膜炎による心嚢液の貯留，自然気胸におけるブラの検索にも有用である．

他科専門医への紹介の必要性とタイミング

先に挙げた心筋梗塞，大動脈解離，緊張性気胸，および肺塞栓の4つの疾患をはじめとした生命にかかわる疾患は，専門施設における集中治療が必要である．問診，身体所見から，これらの疾患が疑われる場合は，自施設で可能な検査を進めつつ，速やかに母子ともに管理可能な施設へ転送することが必要である．

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Inflammatory Response to Acute Myocardial Infarction Augments Neointimal Hyperplasia After Vascular Injury in a Remote Artery

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Objective—Percutaneous coronary intervention (PCI) is currently the most widely accepted treatment for acute myocardial infarction (AMI). It remains unclear, however, whether post-AMI conditions might exacerbate neointimal hyperplasia and restenosis following PCI. Given that both a medial smooth muscle cell lineage and a bone marrow (BM)-derived hematopoietic stem cell lineage are now thought to contribute to neointima formation, the primary aims of the present study were to determine whether AMI augments neointimal hyperplasia at sites of arterial injury, and whether BM-derived cells contribute to that process.

Methods and Results—We simultaneously generated models of AMI and arterial injury in the same mice, some of which had received BM transplantation. We found that AMI augments neointimal hyperplasia at sites of femoral artery injury by ≈35% ($P<0.05$), but that while BM-derived cells contributed to neointimal hyperplasia, they did not contribute to the AMI-related augmentation. Expression of interleukin (IL)-6 mRNA was ≈7-fold higher in the neointimas of mice subjected to both AMI and arterial injury than in those of mice subjected to arterial injury alone. In addition, we observed increased synthesis of tumor necrosis factor (TNF)-α within infarcted hearts and TNF-α receptor type 1 (TNFR1) within injured arteries. Chronic treatment with pentoxifylline, which mainly inhibits TNF-α synthesis, reduced levels of circulating TNF-α and attenuated neointimal hyperplasia after AMI.

Conclusions—Conditions after AMI could exacerbate postangioplasty restenosis, not by increasing mobilization of BM-derived cells, but by stimulating signaling via TNF-α, TNFR1 and IL-6. (***Arterioscler Thromb Vasc Biol.* 2006; 26:2083-2089.**)

Key Words: bone marrow ■ inflammation ■ myocardial infarction ■ restenosis ■ smooth muscle cell

Both the occurrence and eventual healing of acute myocardial infarction (AMI) evoke inflammatory processes that lead to clinical components of instability, as evidenced by the high rate of subsequent coronary artery events, including recurrent MI and in-stent restenosis after percutaneous coronary intervention (PCI).[1–3] It is well known from both experimental and clinical observations that local upregulation of the expression of proinflammatory cytokines in activated smooth muscle cells (SMCs) contributes significantly to restenosis after balloon angioplasty and stent implantation.[4,5] In the setting of AMI, moreover, various proinflammatory cytokines and growth factors, including TNF-α, IL-1β, IL-6 and vascular endothelial growth factor (VEGF), are expressed in both infarcted and noninfarcted regions of the heart, and their plasma levels are elevated for ≈2 weeks after AMI,[6–10] raising the possibility that they, too, contribute to neointimal hyperplasia after PCI.

Recent findings suggest that 2 lineages of neointimal SMCs are involved in vascular remodeling after injury: a medial SMC lineage whose activation is triggered by various proinflammatory cytokines (the classical scenario), and a newly identified bone marrow (BM)-derived hematopoietic stem cell lineage.[11,12] It now appears that hematopoietic stem cells and endothelial progenitor cells are released from BM into the peripheral circulation during the early phase of AMI.[13,14] Thus, the mechanism for AMI-related vascular remodeling is apparently more complex than was recognized before the emergence of these new findings.

Within that context, the first aim of the present study was to determine whether AMI is, itself, capable of promoting neointimal hyperplasia at distant sites of arterial injury, such as would be caused by PCI. If so, the second aim of this study was to determine whether BM-derived cells contribute to that process. To accomplish these aims, we simultaneously gen-

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erated experimental models of AMI and femoral arterial injury in the same mice, some of which had previously received BM transplantation (BMT) from green fluorescence protein (GFP) mice. Here we show that the inflammatory response to AMI augments neointimal hyperplasia in the injured femoral artery, but whereas BM-derived cells contribute to that neointima formation, they do not significantly contribute to the AMI-related augmentation of the response. Moreover, we demonstrate that the TNF-α synthesis inhibitor pentoxifylline (PTX)[15–17] reduces levels of circulating TNF-α and attenuates neointimal hyperplasia after AMI. Apparently, cross-talk between the heart and injured artery via signaling pathways mediated by inflammatory cytokines, especially TNF-α, TNF receptor type 1 (TNFR1) and IL-6, are involved in this process.

Materials and Methods

Animals

C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (GFP mice) were a generous gift from Dr Masaru Okabe (Osaka University, Osaka, Japan).[18] All experimental procedures were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University and National Cardiovascular Center.

Bone Marrow Reconstitution

Bone marrow reconstitution (BMT) was performed as described previously.[11] One day after exposing 8-week-old male wild-type mice to a lethal dose (9.0 Gy) of X-irradiation, they received a tail vein injection of unfractionated BM cells (1×10^6) that had been harvested from the femora and tibias of GFP mice and suspended in 0.2 mL of phosphate-buffered saline. Eight weeks after BMT, peripheral leukocytes had been reconstituted to >90% of control, as determined by flow cytometry.

AMI

AMI was induced in mice as described previously.[19,20] The precise methods are described online only. Please see http://atvb.ahajournals.org.

Vascular Injury

Vascular injury (VI) was induced as described previously.[21] Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and the femoral artery was exposed. A straight spring wire (0.38 mm in diameter, No. C-SF-15 to 15; COOK, Bloomington, Ind) was then inserted into the femoral artery, left in place for 1 minute to denude and dilate the artery, then removed.

Measurement of Neointimal Hyperplasia

For morphometric studies, femoral arteries were harvested 4 weeks after injury, and digitalized images of these vessels were obtained and analyzed using image analysis software (Version 3.2; Soft Imaging System, Munster, Germany). The lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were defined, and the intimal (tissue between lumen and IEL) and medial (tissue between IEL and EEL) areas were recorded. Neointima/media area (NI/M) ratios were also calculated.

Immunohistochemistry and Immunofluorescent Staining

Methods for immunohistochemistry and immunofluorescent staining were performed standard methods, and their details were described online only. Please see http://atvb.ahajournals.org.

cDNA Array Analysis

Total RNA was isolated from pooled arteries (n=6 for each group) using a QIAGEN RNeasy Minikit (QIAGEN Inc, Valencia, Calif). Murine U74A version 2 GeneChips were purchased from Affymetrix (Santa Clara, Calif) and hybridization was carried out according to the manufacturer's instructions.

Measurement of Proinflammatory Cytokine mRNA

RNA was isolated from pooled arteries (n=6 to 8 for each group) using a QIAGEN RNeasy Minikit (QIAGEN Inc, Valencia, Calif) and then amplified using a MessageAmp™ Kit (Ambion, Austin, Tex), which enables amplification of very small amounts of RNA. RNA also was isolated from hearts using TRIzol Reagent (Invitrogen, Carlsbad, Calif), after which cDNA was generated using both RNA samples and an Invitrogen SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, Calif). Real-time polymerase chain reaction (PCR) was then performed in an ABI-Prism 7700 (Applied Biosystems, Foster City, Calif) using Taqman Universal PCR MasterMix (Applied Biosystems). The oligonucleotide probes and primers for IL-6, MCP-1, VEGF, transforming growth factor (TGF)-β,stromal cell-derived factor (SDF)-1α, IL-1β, and TNF-α were purchased from Applied Biosystems.

Measurements of Plasma TNF-α Levels

Plasma TNF-α levels were measured using a mouse TNF-α enzyme-linked immunosorbent assay kit (eBioscience, San Diego, Calif) according to the manufacturer instructions. The minimum detectable concentration of TNF-α was 8 pg/mL.

Experimental Protocols

Depending on the experiment, mice were placed into one of four groups: the AMI+VI group were subjected to both AMI and femoral arterial injury; the VI group was subjected to a sham operation and femoral arterial injury; and the AMI and sham-operated groups received only AMI or the sham operation, respectively. Mice that did not receive BM cells were used to compare neointimal hyperplasia and mRNA expression among the groups. Two weeks after AMI, femoral arteries were carefully excised from 6 to 8 mice in each group and pooled for analysis of mRNA expression. Data from 2 independent experiments were averaged. Four weeks after AMI, femoral arteries were excised from 10 mice in each group to measure neointimal hyperplasia. Again, 2 series of these experiments were performed. In addition, to detect BM-derived cells within the neointima, we performed similar experiments using 6 mice that had received BM cells in each group.

In some mice in AMI+VI and VI groups, PTX (30 mg/kg per day) or vehicle (Veh) (phosphate-buffered saline) was infused intraperitoneally using an osmotic minipump (Alzet, Cupertino, Calif) for 4 weeks after AMI or sham operation. At the end of the 4-week treatment period, the mice were euthanized and peripheral blood was collected to measure circulating TNF-α levels, and the injured and sham-operated femoral arteries were collected to assess the neointimal hyperplasia.

Statistical Analysis

All results are expressed as means±SEM. Differences between groups were evaluated for statistical significance using Student *t* test. Values of $P<0.05$ were considered significant.

Results

Myocardial Infarction Augments Neointimal Hyperplasia in Injured Arteries

Four weeks after the surgery, neointima formation was observed in mice in both the AMI+VI and VI groups (Figure 1A and 1B). As can be seen in Figure 1, however, the neointimal hyperplasia was substantially more prominent in

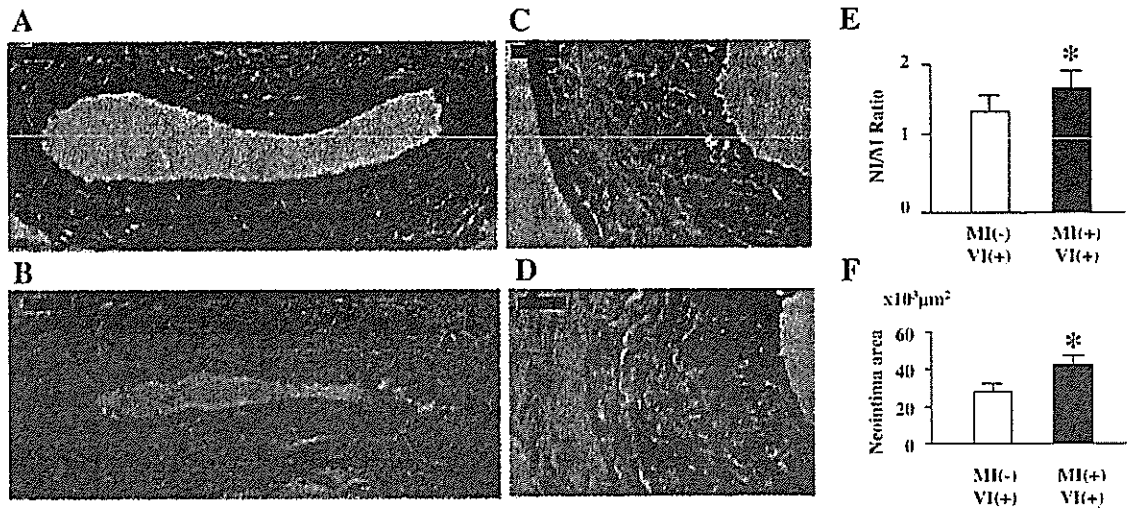

Figure 1. AMI augments neointimal hyperplasia in injured femoral arteries. A and B, Hematoxylin and eosin-stained sections of femoral artery from VI (A) and AMI+VI (B) mice (8 to 10 weeks old) harvested 4 weeks after vascular injury; scale bars represent 50 μm. C and D, α-SMA immunostained sections of femoral artery from VI (C) and AMI+VI (D) mice; scale bars represent 25 μm. Arrowheads indicate the internal elastic lamina. E,F, NI/M ratios (E) and neointimal area (F) in the injured femoral arteries of VI (open bars) and AMI+VI (solid bars) mice. Bars are means±SEM of 8 mice per group;*$P<0.05$ vs the VI group.

the AMI+VI group than in the VI group. Immunohistochemical staining revealed that the neointimas in both groups were mainly composed of α-SMA-positive SMCs (Figure 1C and 1D), suggesting that the inflammatory response to AMI increases SMC numbers within the neointimas of distant injured arteries. When we measured the neointimal and medial areas using computerized morphometry, we found that the NI/M ratios and neointimal areas were significantly greater in the AMI+VI group than in the VI group (Figure 1E and 1F).

BM-Derived Cells Contribute to Neointima Formation but Not to the AMI-Related Augmentation

To determine the extent to which BM-derived cells contribute to the AMI-related augmentation of neointimal hyperplasia in injured arteries, we next performed a set of experiments using mice that had received BM cells from GFP mice. Four weeks after the vascular injury, we observed that GFP-positive cells had accumulated in the neointimas and medias of the injured arteries (Figure 2A and 2B) in both AMI+VI and VI mice. Moreover, immunofluorescent staining showed that some of the GFP-positive cells expressed α-SMA (Figure 2C), suggesting they had differentiated into cells similar to SMCs. The numbers of GFP-positive cells did not significantly differ in the neointimas or medias of mice in the AMI+VI and VI groups (Figure 2D), though they tended to be larger in AMI+VI mice than in VI mice.

It thus appears that BM-derived cells do indeed contribute to vascular remodeling after injury, but they are not responsible for the AMI-related augmentation of the response. It also appears that the inflammatory response to AMI did not promote significant mobilization of progenitor cells with the potential to differentiate into SMCs.

Expression of Proinflammatory Cytokines in Injured Femoral Arteries

Given the absence of a significant contribution by BM-derived cells to the augmented neointimal hyperplasia seen in injured arteries after AMI, we next sought to identify any molecules that might trigger migration and proliferation of medial SMCs by analyzing the expression profiles of various mRNAs using cDNA arrays. Among a number of upregulated molecules, levels of IL-6, MCP-1, VEGF, TGF-β, SDF-1α, and IL-1βmRNA were markedly higher in the injured arteries of AMI+VI mice than in those of sham-operated mice. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed ≈7-fold increase in IL-6 mRNA expression in the AMI+VI group, as compared with the VI group, and ≈500-fold increase, as compared with the sham-operated group (Figure 3A). Levels of MCP-1, VEGF, TGF-β, SDF-1α and IL-1β mRNA were similar in both the AMI+VI and VI groups and higher than in the sham-operated group (supplemental Figure IA to IE, available online at http://atvb.ahajournals.org.). In addition, immunohistochemical analysis showed clear upregulation of IL-6 protein that paralleled the upregulation of mRNA expression in the neointimal region (Figure 3B).

Cardiac Expression of TNF-α After AMI

Because TNF-α reportedly stimulates IL-6 expression,[22] we next used quantitative RT-PCR to examine expression of TNF-α mRNA in infarcted hearts in an effort to determine the reason why IL-6 mRNA was preferentially upregulated in injured arteries following AMI. As shown in supplemental Figure IIA, expression of TNF-α mRNA was significantly increased in infarcted hearts 1, 3, 7, and 28 days after AMI, as compared with sham-operated hearts. Moreover, immunohistochemical analysis showed clear upregulation of TNF-α protein that paralleled the upregulation of mRNA expression in the infarcted hearts (supplemental Figure IIB and IIC).

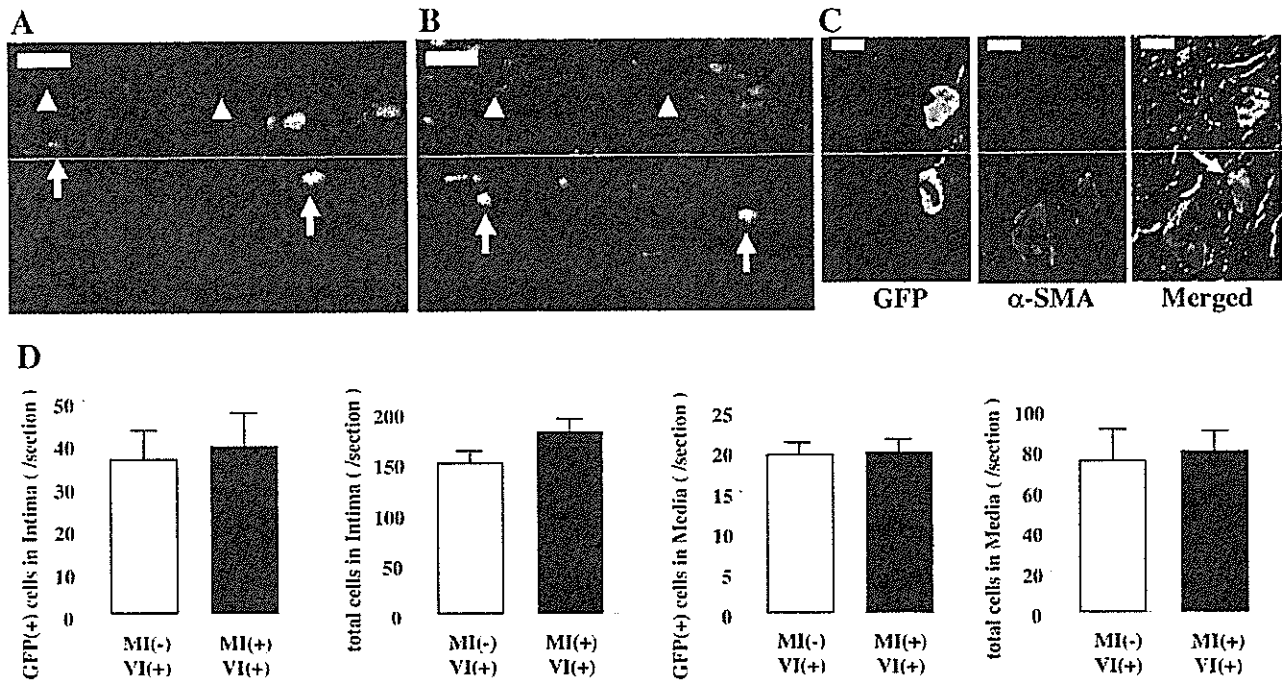

Figure 2. BM-derived GFP-positive cells within injured arteries. Vascular injuries were induced in $BMT^{GFP\rightarrow Wild}$ mice, after which the femoral arteries were fixed in 4% paraformaldehyde and embedded in plastic resin. Injured arteries from VI (A) and AMI+VI (B) mice were harvested after 4 weeks and observed under a confocal microscope. Arrowheads indicate the internal elastic lamina; arrows indicate GFP-positive cells; scale bars represent 25 μm. C, Immunofluorescent staining with Cy3-conjugated anti-α-SMA antibody (red) within injured arteries. The arrow indicates a GFP-positive SMC; scale bars represent 5 μm. D, Numbers of GFP-positive cells and total cell numbers within the injured arteries of VI (open bars) and AMI+VI (solid bars) mice. Bars are means±SEM for 5 mice per group.

Femoral Arterial Expression of TNFR1 After Vascular Injury

Given the increased cardiac expression of TNF-α and circulating of TNF-α levels after AMI, we tested the possibility that TNF-α acts via locally expressed TNFR1 to upregulate expression of IL-6 within injured arteries. Consistent with that idea, quantitative RT-PCR analysis revealed that the level of TNFR1 mRNA expression was significantly higher in injured femoral arteries from both AMI+VI and VI mice than in those from sham-operated or AMI mice (Figure 4).

Effect of Blockade of TNF-α Production on Vascular Remodeling

Finally, to confirm that the relationship between the increase in plasma TNF-α levels and the augmentation in neointima formation in remote injured arteries was causative, we tested the effects of PTX, an inhibitor of TNF-α synthesis. We found that plasma TNF-α levels were significantly higher in vehicle (Veh)-treated AMI+VI mice than in Veh-treated VI mice, but that TNF-α levels in AMI+VI mice were significantly diminished by PTX to a level similar to that seen in Veh-treated VI mice (Figure 5B). In addition, morphometric analysis revealed that neointimal areas and NI/M ratios in Veh-treated AMI+VI mice were significantly greater than in Veh-treated VI mice and that PTX significantly reduced neointimal areas and NI/M ratios (Figure 5A, 5C, and 5E). However, PTX treatment did not significantly affect neointimal areas or NI/M ratios in VI mice. Thus, prevention of the AMI-induced increase in plasma TNF-α levels by PTX attenuated neointimal hyperplasia in a remote artery after AMI.

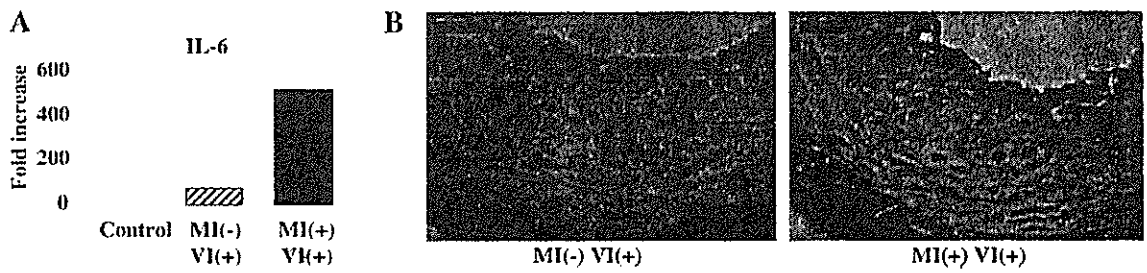

Figure 3. A, Effect of myocardial infarction on expression of the IL-6 within injured arteries: white bars, control; hatched bars, VI; black bar, AMI+VI. Tissue samples were prepared from injured and uninjured arteries 14 days after surgery (control). The result shown is representative of data obtained from 3 to 4 mice per group. IL-6 signal intensities were normalized to that of GAPDH; bars depict the fold increase relative to uninjured arteries (control). B, Immunohistochemical staining of IL6 within injured femoral arteries from VI and AMI+VI mice harvested 4 weeks after surgery. Scale bars represent 25 μm.