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.

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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 30,000 IU/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.*

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.

Table4 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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Arteriosclerosis, Thrombosis, and Vascular Biology

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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

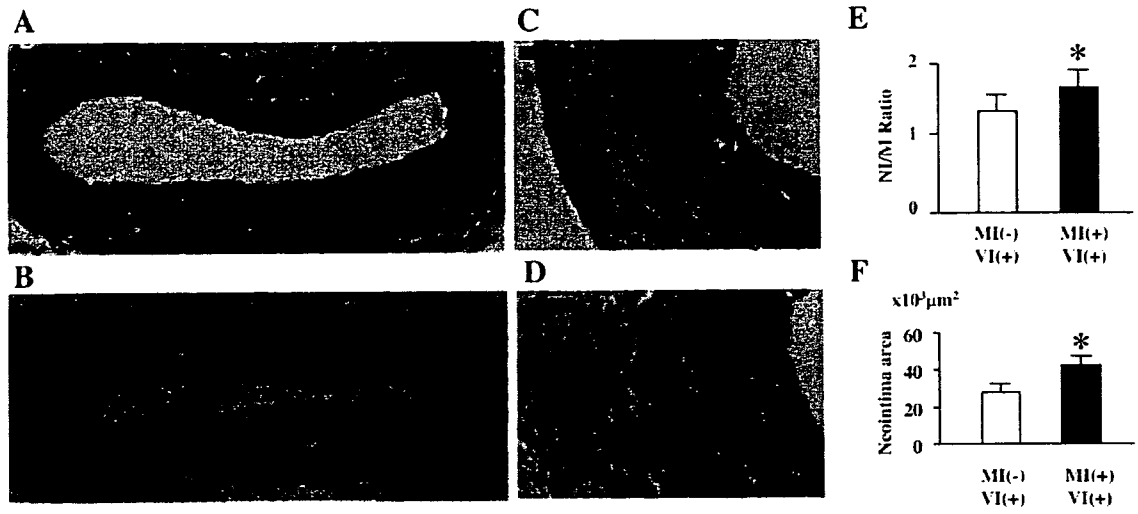

Figure 1. AMI augments neointimal hyperplasia in injured femoral arteries. A and B, Hematoxylin and eosin-stained sections of femoral artery from VI (A) and AMI+VI (B) mice (8 to 10 weeks old) harvested 4 weeks after vascular injury; scale bars represent 50 μm. C and D, α-SMA immunostained sections of 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).

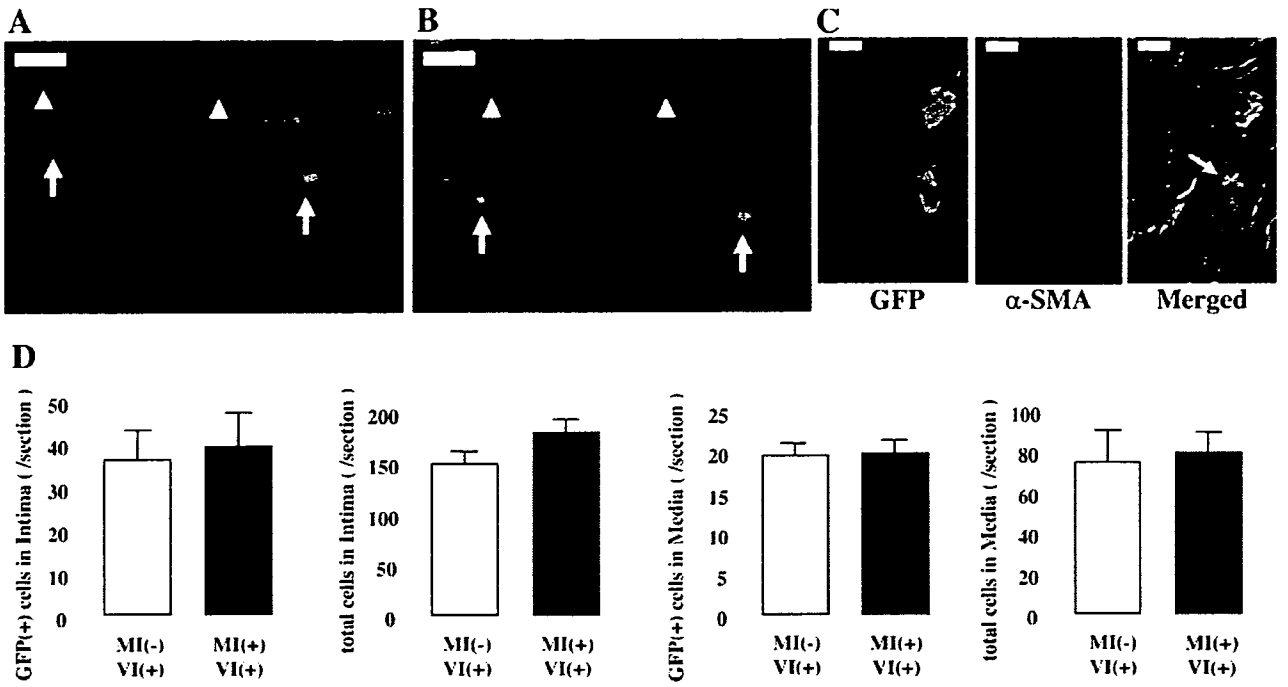

Figure 2. BM-derived GFP-positive cells within injured arteries. Vascular injuries were induced in $BMT^{GFP\rightarrow Wild}$ mice, after which the femoral arteries were fixed in 4% paraformaldehyde and embedded in plastic resin. Injured arteries from VI (A) and AMI+VI (B) mice were harvested after 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.

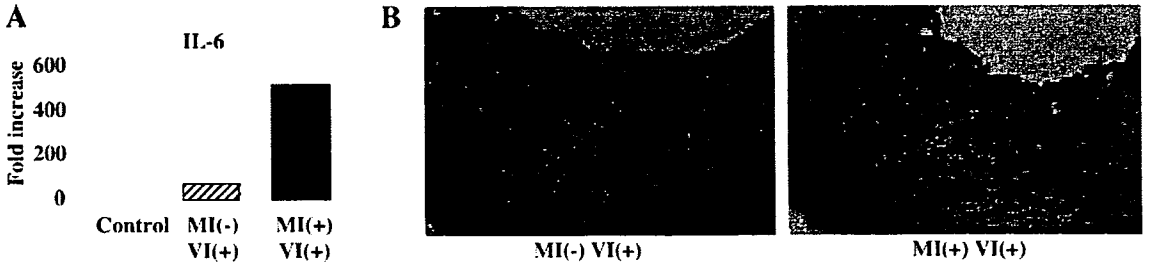

Figure 3. A, Effect of myocardial infarction on expression of the IL-6 within injured arteries: white bars, control; hatched bars, VI; black bar, AMI+VI. Tissue samples were prepared from injured and uninjured arteries 14 days after surgery (control). The result shown is representative of data obtained 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.

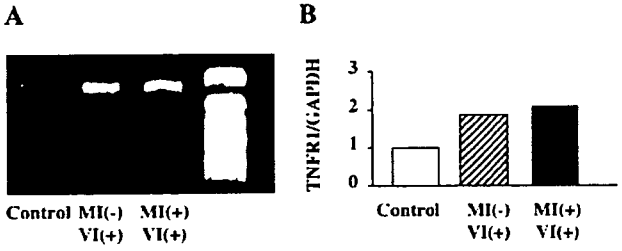

Figure 4. Effect of vascular injury on arterial expression of TNFR1. Tissue samples were prepared from injured and uninjured (control) femoral arteries 14 days after surgery: A, A representative image of RT-PCRs for TNFR1 expression. B, Quantitative real-time PCR analysis of TNFR1 expression levels obtained from 3 to 4 mice in each group.

Discussion

The main findings of the present study are that: (1) the inflammatory response to AMI augments neointimal hyperplasia at sites of injury within distant arteries; (2) BM-derived cells contribute to neointimal hyperplasia after vascular injury, but not to the AMI-related augmentation of the response; (3) cardiac synthesis of TNF-α and circulating TNF-α levels are both increased after AMI, as is expression of TNFR1 mRNA in injured arteries; (4) IL-6 is preferentially upregulated in the neointima of injured arteries after AMI; and (5) treatment with PTX, an inhibitor of TNF-α synthesis, inhibited the AMI-induced increases in plasma TNF-α and attenuated neointima formation after vascular injury. It thus appears that the inflammatory response to AMI stimulates neointimal hyperplasia at sites of vascular injury at least in part by stimulating signaling via TNF-α, TNFR1, and IL-6.

Cardiac levels of several vasoactive cytokines are elevated after AMI. For instance, levels of VEGF are increased in both infarcted hearts and the plasma. We also have observed that plasma levels of placental growth factor (PlGF), another VEGF family cytokine, are elevated within infarcted hearts as a result of its synthesis in endothelial cells within the infarcted region.[23] Earlier works by Hattori et al indicate that both VEGF and PlGF stimulate matrix metalloproteinase (MMP)-9 expression in BM stromal cells via the flt pathway, and that MMP-9 cleaves membrane bound Kit ligand into soluble Kit ligand, which in turn activates hematopoietic stem cells.[24,25] In addition, numbers of CD34-positive cells also are increased after AMI.[13] Based on these findings, we suggested that conditions directly caused by AMI stimulate neointimal hyperplasia at remote sites of vascular injury, and that BM-derived cells contribute significantly to the AMI-related augmentation of neointimal hyperplasia. In that regard, one recent report showed that BM-derived cells contribute to neointimal hyperplasia after mechanical vascular injury, especially after severe wire-induced injury.[26] In the present

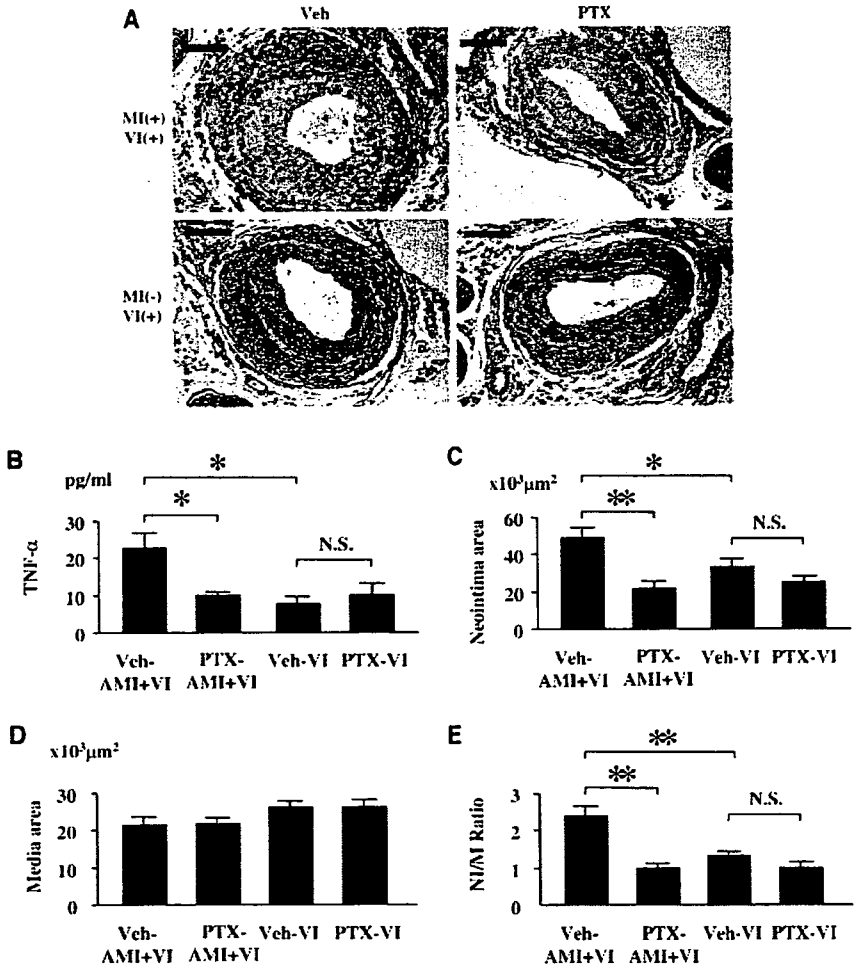

Figure 5. Inhibitory effect of PTX in AMI+VI and VI mice. A, Hematoxylin and eosin-stained sections of femoral artery from Veh-treated AMI+VI, Veh-treated VI, PTX-treated AMI+VI, and PTX-treated VI mice (8 to 10 weeks old) harvested 4 weeks after vascular injury; scale bars represent 100 μm. B, Circulating TNF-α levels in AMI+VI and VI mice continuously infused for 4 weeks with Veh or PTX. C to E, Effect of PTX treatment on morphological changes. Morphometric analysis of injured femoral arteries in AMI+VI and VI mice, with or without PTX, 4 weeks after wire-induced injury. Values are mean±SEM; *P<0.05; **P<0.01; n=6 to 10; N.S indicates not statistically significant.

study, we confirmed the presence of BM-derived cells in the neointima and media of the affected artery after wire-induced vascular injury. But as shown in Figure 2, although the number of neointimal BM-derived cells tended to be higher in mice after AMI than after sham operation, the difference was not sufficient to explain the augmented neointimal hyperplasia seen after AMI. Apparently, AMI does not stimulate recruitment of BM-derived cells into the neointima at sites of vascular injury. Pathological significance of BM-derived cells in the setting of AMI is needed to be further elucidated.

Our analysis of cDNA arrays, performed to identify key molecules responsible for the AMI-related augmentation of neointimal hyperplasia after vascular injury, revealed upregulation of MCP-1, VEGF, TGF-β, SDF-1α, and IL-1β within injured arteries, with and without AMI. However, levels of IL-6 were $\approx$7-fold higher in the injured arteries of AMI+VI mice than in those of VI mice. Studies have shown that IL-6 mRNA is expressed in the atherosclerotic lesions of apolipoprotein E knockout mice[27] and humans[28,29] and in the neointimas of injured arteries,[5] and that STAT3, which is activated by IL-6, contributes to neointima formation by promoting neointimal SMC proliferation and survival.[30] However, earlier reports mainly emphasized the importance of MCP-1, VEGF, TGF-β,SDF-1α, and IL-1β rather than IL-6 in neointima formation. The present study confirms the importance of IL-6 in neointimal hyperplasia after wire-induced injury and suggests that IL-6 plays a more important role in AMI-related neointimal hyperplasia than the other aforementioned mediators.

Bearing that in mind, a key question is, what are the signals that stimulate the preferential elevation of IL-6 expression in injured arteries after AMI? A number of earlier reports have shown that TNF-α and IL-1β are strong stimulators of IL-6 expression.[22,31] Consistent with that earlier work, we found that TNF-α expression is upregulated in infarcted hearts during the 2-week period after AMI and that plasma TNF-α levels were increased to 22.6$\pm$4.2 pg/mL, which is sufficient to stimulate IL-6 expression.[32–34] We also detected expression of TNFR1 mRNA in injured arteries, but not in healthy ones. TNF-α exerts its effects through both TNFR1 and TNFR2, but blockade of TNFR1 gene expression reportedly reduces neointimal hyperplasia after vascular injury by 2-fold, whereas blocking TNFR2 expression has no effect.[35] Thus, in the setting of AMI, it is likely that TNF-α released from the infarcted heart binds to newly upregulated-TNFR1 on the surface of cells at remote sites of arterial injury, leading to IL-6 production and, ultimately, stimulation of neointimal hyperplasia.

To prove a cause–effect relationship between expression of TNF-α in heart and IL-6 in arteries, and one between AMI and augmented neointima formation, we demonstrated that prevention of TNF-α synthesis by PTX inhibited the AMI-induced increases in plasma TNF-α and attenuated neointimal formation after vascular injury. Collectively, these results are indicative of the important role played by TNF-α in experimental postangioplasty restenosis. An earlier report showed the short-term, exogenous administration of TNF-α did not increase neointima formation after balloon injury in rabbits,[36] suggesting that prolonged and persistent elevation of circulating TNF-α is required to promote neointimal hyperplasia after vascular injury. In this study, we used PTX as an inhibitor of TNF-α synthesis instead of a neutralizing antibody or a soluble form of TNF-α receptor. Although both neutralizing antibody and soluble form of TNF-αreceptor are more specific than PTX, they are immunogenic,[37] when they are used in vivo experiments especially in the long-term experiment like the present experiment. PTX reduces the synthesis of TNF-α by blocking its transcription,[38] and has been used successfully in earlier works.[39,40] Therefore, we adopted PTX to inhibit TNF-α pathway. However, it might be possible that present findings were modulated by other molecules transcriptionally suppressed by PTX.

In the clinical point of view, primary purpose of the present study is to elucidate whether post-AMI conditions enhance the neointimal hyperplasia and restenosis after PCI, that is why we generated a mouse model of AMI plus vascular injury. However, further studies using model in larger animal such as porcine are necessary to get more directly evidence that AMI augments neointimal hyperplasia and restenosis after PCI of infarct-related artery.

In conclusion, the present findings provide experimental evidence supporting the idea that conditions directly resulting from AMI exacerbate neointimal hyperplasia after vascular injury through activation of TNF-α, TNFR1, and IL-6 network.

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Disclosures

None.

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α2 Isoform–specific activation of 5′ adenosine monophosphate–activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle

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Abstract

5′ Adenosine monophosphate–activated protein kinase (AMPK) has been implicated in exercise-induced stimulation of glucose metabolism in skeletal muscle. Although skeletal muscle expresses both the α1 and α2 isoforms of AMPK, the α2 isoform is activated predominantly in response to moderate-intensity endurance exercise in human and animal muscles. The purpose of this study was to determine whether activation of α2 AMPK plays a role in increasing the rate of glucose transport, promoting glucose transporter 4 (GLUT4) expression, and enhancing insulin sensitivity in skeletal muscle. To selectively activate the α2 isoform, we used 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), which is metabolized in muscle cells and preferentially stimulates the α2 isoform. Subcutaneous administration of 250 mg/kg AICAR activated the α2 isoform for 90 minutes, but not the α1 isoform in hind limb muscles of the C57/B6J mouse. The maximal activation of the α2 isoform was observed 30 to 60 minutes after administration of AICAR and was similar to the activation induced by a 30-minute swim in a current pool. The increase in α2 activity paralleled the phosphorylation of Thr^{172}, the essential residue for full kinase activation, and the activity of acetyl-coenzyme A carboxylase β, a known substrate of AMPK in skeletal muscle. Subcutaneous injection of AICAR rapidly increased, by 30%, the rate of 2-deoxyglucose (2DG) transport into soleus muscle; 2DG transport increased within 30 minutes and remained elevated for 4 hours after administration of AICAR. Repeated intraperitoneal injection of AICAR, 3 times a day for 4 to 7 days, increased soleus GLUT4 protein by 30% concomitant with a significant 20% increase in insulin-stimulated 2DG transport. These data suggest that moderate endurance exercise promotes glucose transport, GLUT4 expression, and insulin sensitivity in skeletal muscle at least partially via activation of the α2 isoform of AMPK.

1. Introduction

Physical exercise is a potent stimulator of glucose transport and glucose transporter 4 (GLUT4) expression in skeletal muscle. An acute bout of exercise increases the rate of glucose transport into contracting muscles by inducing translocation of GLUT4 to the cell surface via an insulin-independent mechanism (contraction-stimulated glucose transport) [1]. Acute exercise also activates expression of GLUT4 protein, and the GLUT4 protein expression is elevated with repeated bouts of acute exercise [2]. The exercise-induced increase in GLUT4 is associated with improved insulin sensitivity (ie, increased rates of insulin-stimulated GLUT4 translocation and glucose transport into

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skeletal muscle) [3]. These mechanisms of enhanced glucose transport help improve glycemic control in patients with diabetes and may help prevent nondiabetic subjects from developing glucose intolerance.

Recent studies have suggested that 5′ adenosine monophosphate–activated protein kinase (AMPK) is an important signaling intermediary leading to contraction-stimulated GLUT4 translocation and glucose transport [4-9] and GLUT4 expression [10-15] in skeletal muscle. AMPK is a heterotrimeric protein composed of a catalytic α subunit and regulatory subunits, β and γ. Although the α subunit exists in different isoforms in skeletal muscle [16], $\alpha 1$ and $\alpha 2$, the $\alpha 2$ isoform–containing AMPK is preferentially activated in response to exercise. For example, cycle ergometer exercise at 50% of maximum energy consumption ($\dot{V}O_2max$) does not change $\alpha 2$ or $\alpha 1$ activity, and exercise at 60% to 75% of $\dot{V}O_2max$ increases $\alpha 2$, but not $\alpha 1$, activity in biopsy samples of vastus lateralis muscle from healthy subjects [17-19]. Similar activation of $\alpha 2$ occurs in response to cycle ergometer exercise at 70% of $\dot{V}O_2max$ in patients with type 2 diabetes mellitus who have similar protein expression of α isoforms as healthy subjects [20]. In contrast, both isoforms are significantly activated in response to high-intensity exercise such as sprint exercise requiring power output 2- to 3-fold greater than that attained during maximal aerobic exercise [21]. In rat skeletal muscle, voluntary treadmill running exercise increases only $\alpha 2$ activity, whereas high-intensity contractions, such as electrically induced tetanic contractions, increase the activities of both isoforms in isolated rat skeletal muscle [8]. These observations in human and animal muscles suggest that regulation of the α isoforms is intensity-dependent in contracting skeletal muscle, and that the $\alpha 2$ isoform, rather than $\alpha 1$, is involved in the metabolic responses to moderate-intensity endurance exercise.

We explored the physiological relevance of the predominant $\alpha 2$ activation in skeletal muscle, focusing particularly on glucose transport, GLUT4 expression, and insulin sensitivity by selectively activating $\alpha 2$ AMPK using the AMPK-stimulating agent, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR).

2. Materials and methods

2.1. Materials

AICAR was obtained from Sigma (St Louis, MO). Phosphospecific antibody directed against AMPKα Thr^{172} was obtained from Cell Signaling Technology (Beverly, MA) and that directed against acetyl-coenzyme A carboxylase β (ACCβ) Ser^{79} from Upstate Biotechnology (Lake Placid, NY). Anti-GLUT4 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All radioactive materials were purchased from NEN Life Science Products (Boston, MA). Reagents for the protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Sigma or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

2.2. Animals

Male C57/B6 mice, aged 7 to 10 weeks, were obtained from Shimizu Breeding Laboratories (Kyoto, Japan) and fed standard laboratory chow and water ad libitum. They were housed in plastic cages in an environmentally controlled room maintained at 23°C with a 12-hour light-dark cycle. Mice were fasted for 8 to 10 hours before the experiments, except as otherwise described. Blood samples were collected from the tail vein. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan.

2.3. Administration of AICAR

For studies of a single administration of AICAR, AICAR was dissolved in saline (20 g/L) and injected subcutaneously or intraperitoneally without anesthesia at a dose of 250 mg/kg body weight. Mice were then killed by cervical dislocation at the indicated time points, and either hind limb muscles (gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus [EDL] muscles) or soleus and EDL muscles were dissected. For studies of repeated injections of AICAR, 250 mg/kg of AICAR was dissolved in saline (20 g/L) and injected into fed mice intraperitoneally 3 times a day for up to 8 days. Mice were killed by cervical dislocation 12 to 16 hours after the last injection, and the hind limb or soleus and EDL muscles were collected. The muscles were either processed fresh to measure 2-deoxyglucose (2DG) transport or frozen and stored in liquid nitrogen for later assays. Saline was injected as a control condition in the studies using the single and repeated administration of AICAR.

2.4. Swimming exercise

Mice swam in groups of 6 or less at a time at ~60% of $\dot{V}O_2max$ (5 L/min flow rate) for 30 minutes during the dark cycle as described previously [22]. A large adjustable-current pool (90 × 45 × 45 cm) filled to a depth of 38 cm [22] allowed each mouse to swim without interference with other mice. A constant current was generated by circulating water with a pump, and the flow was monitored by a water flow meter, which was used to adjust the strength of the current. The temperature of the water was maintained at 34°C with a water heater and thermostat. For studies involving a single bout of exercise, mice were killed by cervical dislocation immediately after swimming, the hind limb muscles were dissected, and the muscles were frozen and stored in liquid nitrogen. For studies involving repeated bouts of exercise, fed mice swam for 30 minutes during the dark cycle twice a day for up to 7 days. Twelve to 16 hours after the last exercise session, the mice were killed and muscle samples were dissected, frozen, and stored in liquid nitrogen.