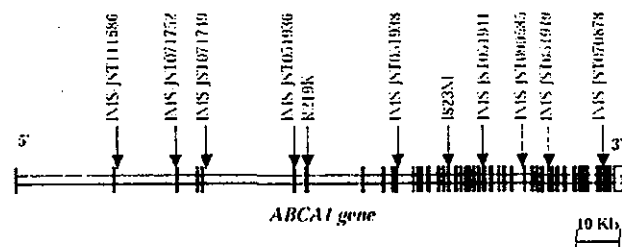


(Wang et al. 2000; Clee et al. 2001; Lutucuta et al. 2001; Harada et al. 2003). However, few of these findings have been replicated, and there are inconsistencies among previous association studies. Accordingly, the associations between *ABCA1* variants and HDL-C are still controversial (Singaraja et al. 2003). One possible reason for these differences may be that the sample sizes in these studies were relatively small and lacked statistical power. Thus, to evaluate the effect of polymorphisms in *ABCA1* on the HDL-C level, we conducted an association study using a large cohort (the Suita population, $n=1,880$), representing the general population in Japan.

Subjects

The myocardial infarction group The selection criteria and design of the myocardial infarction (MI) group have been described

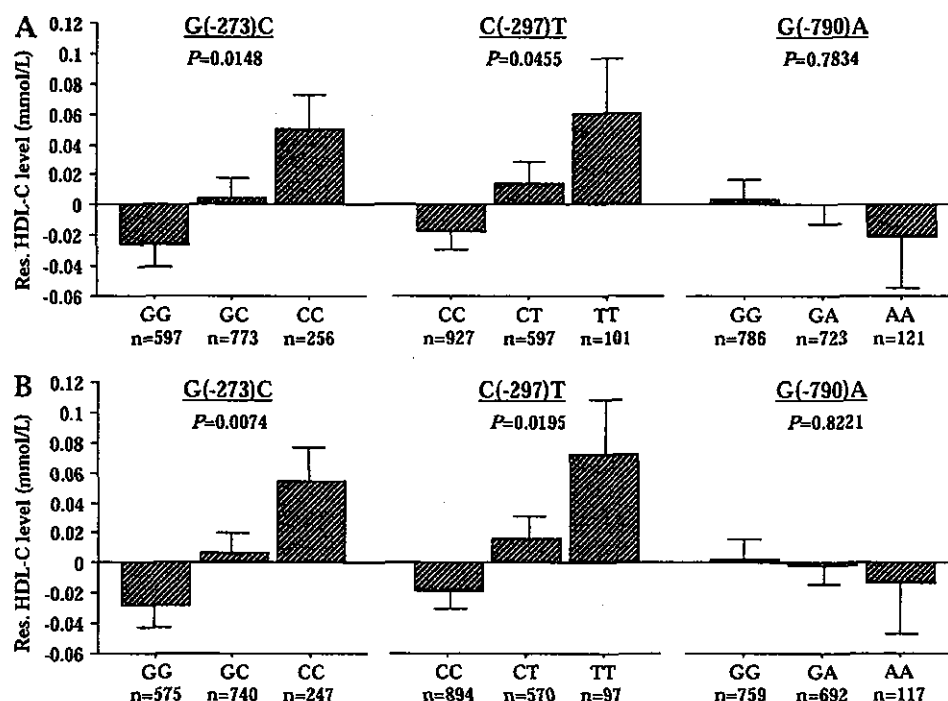


previously (Takagi et al. 2002). This group consisted of 706 patients with MI (598 men and 108 women, aged 61.3 ± 0.4 years) who were enrolled in the Division of Cardiology at National Cardiovascular Center between May 2001 and April 2003. In the present study, we investigated only males ($n = 598$).

DNA studies

The preliminary study revealed that *JST-IMS005607* had the greatest effect on the HDL-C level among seven SNPs on the *ApoA1* region, including the promoter region (up to -3Kb).

Fig. 3A, B Residual HDL cholesterol levels among the *ABCA1* G(-273)C, C(-297)T, and G(-790)A genotypes. A Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, and alcohol consumption. B Residual HDL cholesterol levels adjusted for sex, age, body-mass index, smoking, alcohol consumption, *ApoE* genotype, and *ApoA1* genotype (*JST-IMS005603*)



Thus, we selected *JST-IMS005607* for adjusting HDL-C. The genotyping of *ApoE* was performed according to a previous report (Katsuya et al. 2002). *ApoE* polymorphisms were categorized into three genotypes: E2 ($\epsilon 2/\epsilon 2 + \epsilon 2/\epsilon 3 + \epsilon 2/\epsilon 4$ subjects), E3 ($\epsilon 3/\epsilon 3$ subjects), E4 ($\epsilon 3/\epsilon 4 + \epsilon 4/\epsilon 4$ subjects) (Lefevre et al. 1997). All polymorphisms were determined by the TaqMan System.

Statistical analysis

Values are expressed as mean \pm standard error of the mean (SEM). For triglyceride values, a logarithmic transformation was applied for the statistical test, but untransformed values are shown in the Tables 1 and 2. All statistical analyses were performed with the JMP statistical package (SAS Institute). Values of $P < 0.05$ were considered to indicate statistical significance. Multiple linear regression and multiple logistic analyses were performed with other covariates. The residual HDL-C level was calculated by adjusting for sex, age, and body-mass index (BMI), smoking (cigarettes/day) and consumption of alcohol (ethanol, ml/week). For analyses of the effects of the *ABCA1* genotype (in the Suita population), the residual HDL-C level was calculated by adjusting not only for the above five factors, but also for the *ApoA1* (*JST-IMS005603*), and the *ApoE* (E2, E3, and E4) genotypes. Differences in numerical data among the groups were evaluated by one-way analysis of variance (ANOVA). Hardy-Weinberg equilibrium was calculated by a chi-square test (Table 3). To measure linkage disequilibrium (LD) between SNPs, D' and r^2 values were analyzed using the SNPalyze statistical package (Dynacom).

Results

Polymorphisms of the 5'-flanking region and exon 1 of the *ABCA1* gene

We found 14 polymorphisms in the promoter region, 1 polymorphism in exon 1 (5'-untranslated region), and 2 polymorphisms in intron 1 (Fig. 1).

LD was evaluated by calculating r^2 values (Table 1). We regarded $r^2 > 0.5$ as tight linkage. The minor allele frequency of the T(-1423)C and G52A polymorphisms was low (4% each), and these SNPs were neglected in further analyses. The frequencies of T(10), T(9), and T(8) were 4, 92, and 4%, respectively, in the (-980)T(10)/T(9)/T(8) polymorphism, and this polymorphism was also neglected because this is not suitable for TaqMan genotyping. Accordingly, we selected three polymorphisms, G(-790)A, C(-297)T, and G(-273)C, for the following association study.

Association study of *ApoA1* and *ApoE*

To observe the effect of *ABCA1* polymorphisms on the HDL-C level more clearly, the HDL-C level should be adjusted by various well-known influential factors.

The *ApoA1* *IMS-JST005603* polymorphism was associated with the levels of HDL-C and triglyceride [HDL-C: TT 1.54 ± 0.001 mmol/l, TC 1.59 ± 0.02 , CC 1.68 ± 0.04 , $P = 0.0002$ (residual); triglyceride: TT 1.26 ± 0.03 mmol/l, TC 1.15 ± 0.04 , CC 0.95 ± 0.09 , $P < 0.0001$ (residual)]. *IMS-JST005603* corresponds to the *HaeIII* (C317T) polymorphism described in a previous paper (Groenendijk et al. 2001b).

The *ApoE* polymorphism was also strongly associated with the levels of total cholesterol and HDL-C [total cholesterol: E2 5.13 ± 0.06 mmol/l, E3 5.37 ± 0.02 , E4 5.41 ± 0.05 , $P = 0.0002$ (residual); HDL-C: E2 1.67 ± 0.03 mmol/l, E3 1.56 ± 0.01 , E4 1.52 ± 0.02 , $P < 0.0001$ (residual)].

Accordingly, we evaluated the effect of the *ABCA1* polymorphisms on the HDL-C level adjusted for the

Table 1 Linkage disequilibrium between SNPs in the 5'-flanking region and exon 1 of the *ABCA1* gene. I/D#1 GTTTTGT(TTTT(-752)

Genotype	G(-1498)C	T(-1423)C	T(-1387)C	AT(-1019)(-)	G(-926)T	G(-790)A	I/D#1	C(-559)T
G(-1498)C		0.01976	0.41818***	1***	0.41818***	0.00047	0.67347***	0.22034**
T(-1423)C			0.04726	0.01976	0.04726	0.01003	0	0.06087
T(-1387)C				0.41818***	1***	0.00111	0.67347***	0.65714***
AT(-1019)(-)					0.41818***	0.00047	0.67347***	0.22034**
G(-926)T						0.00111	0.67347***	0.65714***
G(-790)A							0.14667*	0.16483**
I/D#1								1***
C(-559)T								
G(-402)C								
C(-297)T								
G(-273)C								
I/D#2								
G(-99)C								
C(-14)T								
CS2A								
T313C								
G380T								

R^2 values are shown in the upper right, and **bolded values** indicate $r^2 > 0.5$. Absolute D' -values are shown in the lower left, and **bolded** significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

ApoA1 IMS-JST005603 and *ApoE* polymorphisms in addition to standard factors, including sex, age, BMI, smoking, and consumption of alcohol.

Association study of *ABCA1* (Suita population)

The association between the *G*(-273)C polymorphism and the lipid level in the Suita population is presented in Table 2. The genotype frequency of the *G*(-273)C polymorphism in the Suita population was not deviated from the Hardy-Weinberg equilibrium. The HDL-C level adjusted for age, sex, BMI, smoking, and consumption of alcohol was significantly associated with the *G*(-273)C polymorphism ($P = 0.0148$). The *G*(-273)C polymorphism was even more tightly associated with the HDL-C level when adjusted for the *ApoE* and *ApoA1*(IMS-JST005603) genotypes in addition to the standard factors ($P = 0.0074$). The *C*(-297)T polymorphism was also associated with the HDL-C level ($P = 0.0455$ adjusted for age, sex, BMI, smoking, and consumption of alcohol; $P = 0.0195$ when also adjusted for the *ApoE* and *ApoA1* genotypes). The effect of the *C*(-297)T polymorphism on the HDL-C level may be, at least in part, explained by its linkage with the *G*(-273)C polymorphism ($r^2 = 0.46667$, D' value = 1, $P < 0.0001$). *G*(-790)A was not associated with the lipid levels. Among the polymorphisms selected from JSNPs, including R219K and I823M, only the IMS-JST071749 polymorphism was associated with the HDL-C level ($P = 0.0060$ adjusted for age, sex, BMI, smoking, and consumption of alcohol; $P = 0.0093$ when also adjusted for the *ApoE* and *ApoA1* (IMS-JST005603) genotypes). The R219K and I823M polymorphisms were not associated with the HDL-C level [$P = 0.3877$ (R219K) and $P = 0.2286$ (I823M) adjusted for age, sex, BMI, smoking and consumption of alcohol; $P = 0.1926$ (R219K) and $P = 0.1209$ (I823M) when also adjusted for the *ApoE* and *ApoA1* genotypes].

Association study of *ABCA1* (HTN group)

To reconfirm the association between the *G*(-273)C, *C*(-297)T, and IMS-JST071749 polymorphisms and the HDL-C level, we determined the genotypes in the HTN group. As shown in Table 3, the *G*(-273)C polymorphism was associated with the residual HDL-C level ($P = 0.0310$). The genotype frequency of the *G*(-273)C polymorphism in the HTN group was in accordance with Hardy-Weinberg equilibrium and did not differ from that of the Suita population ($P = 0.2953$). The *C*(-297)T ($P = 0.1829$) and IMS-JST071749 ($P = 0.4130$) polymorphisms were not associated with the residual HDL-C level. Thus, a positive association was observed between *G*(-273)C and the HDL-C level in two groups: the Suita population and the HTN group.

Association between *ABCA1* *G*(-273)C and incidence of MI

We next evaluated whether the *ABCA1* *G*(-273)C polymorphism was associated with the incidence of MI. The HDL-C level in the male MI group (1.09 ± 0.01 , $P < 0.0001$) was significantly lower than that in the male Suita subjects (1.44 ± 0.02). The effects of this genotype on the HDL-C level were not observed in this group, probably because a substantial proportion of this group had dyslipidemia and had been treated with hypolipidemic drugs.

No significant association was observed between the *ABCA1* *G*(-273)C polymorphism and the incidence of MI [the MI group: GG $n = 212$ (38.6%), GC $n = 289$ (45.2%), CC $n = 130$ (16.2%); the Suita population: GG $n = 309$ (35.5%), GC $n = 362$ (48.3%), CC $n = 130$ (16.2%), $P = 0.4443$].

(-), I/D#2T GGGG(-226)(-)

G(-402)C	C(-297)T	G(-273)C	I/D#2	G(-99)C	C(-14)T	C52A	T313C	G380T
0.22034**	0.73333***	0.22034**	0.73333***	0.29781***	0.55012***	0.01524	0.55012***	0.52781***
0.06087	0.01449	0.06087	0.01449	0.06636	0.10559*	0.21726***	0.10559*	0.11538*
0.65714***	0.30667***	0.65714***	0.30667***	0.71214***	0.37882***	0.04726	0.37882***	0.36111***
0.22034**	0.73333***	0.22034**	0.73333***	0.29781***	0.55012***	0.01524	0.55012***	0.52781***
0.65714***	0.30667***	0.65714***	0.30667***	0.71214***	0.37882***	0.04726	0.37882***	0.36111***
0.16483**	0.07692	0.16483**	0.07692	0.15119**	0.09502*	0.01003	0.09502*	0.09582*
1***	1***	1***	1***	0.40741***	1***	0.06158	1***	1***
1***	0.46667***	1***	0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
	0.46667***	1***	0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
		0.46667***	1***	0.21839**	0.80952***	0	0.80952***	0.7978***
			0.46667***	0.46798***	0.57647***	0.06087	0.57647***	0.55981***
				0.21839**	0.80952***	0	0.80952***	0.7978***
					0.26978***	0.06636	0.26978***	0.25325***
						0.10559*	1***	1***
							0.10559*	0.11538*
								1***

values indicate $D' > 0.5$. All values refer to the variant allele indicated in the table

Table 2 Lipid levels in the *ABCA1* G(-273)C genotypes (Suita population). Subjects who were receiving anti-hyperlipidemic medication were excluded. Values are mean \pm SEM. *P*-values calculated by ANOVA

Factors	GG	GC	CC	<i>P</i> -value
<i>n</i> (male/female)	306/291	358/415	127/129	
Age (y)	64.1 \pm 0.5	63.7 \pm 0.4	63.9 \pm 0.7	0.7934
BMI (kg/m ²) ^a	22.7 \pm 0.1	22.4 \pm 0.1	22.9 \pm 0.2	0.0607
Smoking (cigarettes/day)	9.2 \pm 0.5	8.5 \pm 0.5	8.6 \pm 0.8	0.5806
Alcohol consumption (ml/week)	85.7 \pm 5.5	80.1 \pm 4.9	71.3 \pm 8.5	0.3597
Total cholesterol (mmol/l)	5.31 \pm 0.03	5.36 \pm 0.03	5.38 \pm 0.05	0.3559
HDL ^b cholesterol (mmol/l)	1.53 \pm 0.02	1.58 \pm 0.01	1.60 \pm 0.03	0.0258
Triglycerides (mmol/l) ^c	1.25 \pm 0.04	1.15 \pm 0.03	1.18 \pm 0.05	0.2583
Residual HDL cholesterol (mmol/l) ^d	-0.03 \pm 0.01	0.00 \pm 0.01	0.05 \pm 0.02	0.0148
Residual HDL cholesterol (mmol/l) ^e	-0.03 \pm 0.01	0.01 \pm 0.01	0.05 \pm 0.02	0.0074

^aBody-mass index

^bHigh-density lipoprotein

^cTest performed on log-transformed values

^dResidual HDL cholesterol was adjusted for sex, age, body-mass index, smoking, and alcohol consumption

^eResidual HDL cholesterol was adjusted for sex, age, BMI, smoking, alcohol consumption, *ApoE* genotype, and *ApoA1* genotype (*JST-IMS005603*)

Table 3 Lipid levels in the *ABCA1* G(-273)C genotypes (hypertension group). Values are mean \pm SEM. *P*-values calculated by ANOVA

Factors	GG	GC	CC	<i>P</i> -value
<i>n</i> (male/female)	165/128	196/141	58/47	
Age (y)	64.5 \pm 0.6	65.6 \pm 0.6	65.3 \pm 1.1	0.4561
BMI (kg/m ²)	24.1 \pm 0.3	23.8 \pm 0.3	23.3 \pm 0.4	0.2766
Smoking (cigarettes/day)	11.6 \pm 0.9	10.9 \pm 0.9	12.1 \pm 1.6	0.7828
Drinking habit (I/II) ^a	117/170	154/180	41/60	0.3460
Total cholesterol (mmol/l)	5.18 \pm 0.05	5.28 \pm 0.05	5.33 \pm 0.09	0.2316
HDL cholesterol (mmol/l)	1.31 \pm 0.02	1.36 \pm 0.02	1.44 \pm 0.04	0.0259
Triglycerides (mmol/l) ^b	1.54 \pm 0.07	1.52 \pm 0.07	1.64 \pm 0.12	0.9429
Residual HDL cholesterol (mmol/l) ^c	-0.04 \pm 0.02	0.02 \pm 0.02	0.07 \pm 0.04	0.0310

^aDrinking habit: I subjects with drinking habit, II subjects without drinking habit

^bTest performed on log-transformed values

^cResidual HDL cholesterol was adjusted for sex, age, BMI, smoking, and drinking habit

Discussion

In the present study, we evaluated the effects of polymorphisms in *ABCA1* on the HDL-C level using a

large cohort representing the general population in Japan (the Suita Study). To evaluate the genetic influence of *ABCA1* polymorphisms on HDL-C level, the HDL-C level was adjusted not only for standard

factors but also for other important genetic factors including the *ApoA1* and *ApoE* polymorphisms. Moreover, we reconfirmed the effects of *ABCA1* G(-273)C polymorphism on HDL-C in the HTN group. We next investigated the association between the *ABCA1* G(-273)C and the incidence of MI, but did not observe any association.

The present study is distinguished by three main features: (1) an association study using a large cohort study (the Suita population), (2) taking into account of the influence of the *ApoA1* and *ApoE* polymorphisms, and (3) a confirmation of the association using another set of subjects (the HTN group).

We found that three SNPs were associated with the HDL-C level in 14 SNPs of the *ABCA1* gene in the Suita population. However, if we applied Bonferroni's correction for multiple tests, three SNPs might not be considered significantly associated with the HDL-C level [G(-273)C, $P=0.1036$; C(-297)T, $P=0.273$; *IMS-JST071749*, $P=0.1302$, P values are corrected by multiplying with 14 (14 SNPs)]. Thus, we verified this positive association in another set of subjects (the HTN group). This association study revealed that G(-273)C, but not C(-297)T or *IMS-JST071749*, was associated with the HDL-C level. Thus, it is highly likely that *ABCA1* G(-273)C was truly associated with the HDL-C level.

Since the *ABCA1* G(-273)C polymorphism is in the promoter region, it is likely that this polymorphism may alter the expression level of *ABCA1*. However, this polymorphic site had no consensus sequence for transcriptional factors. The TGGGG(-226)(-) insertion-deletion polymorphism, which is one of the polymorphisms in LD with the G(-273)C polymorphism ($r^2=0.46667$), was in the middle of the consensus sequence of the ZNF202 binding site (GnT repeat)(Porsch-Ozcuremez et al. 2001). The insertion allele, which mainly corresponds to the (-273)C allele, should disrupt this binding site and may be associated with higher transcriptional activity of the *ABCA1* gene, which may lead to higher HDL cholesterol levels. However, the C(-297)T polymorphism, which was in more tight LD with the TGGGG(-226)(-) insertion-deletion polymorphism, appeared to have less effect on the HDL cholesterol level than the G(-273)C polymorphism. It remains to be determined whether this discrepancy merely reflects a statistical error or if the G(-273)C polymorphism might have additional functional significance. A more detailed promoter analysis will be needed to determine which polymorphisms are functionally important.

The present study revealed that the *ABCA1* I823M polymorphism was not associated with the HDL-C level, inconsistent with a previous report (Harada et al. 2003). This discrepancy may be due to the study design, since a small-scale association study has relatively weak statistical power. In the present study, the sample power was 0.77 for the distribution, sample size, frequencies of the alleles, and α value (0.05, two-tailed).

The sample size in the previous study ($n=410$) does not seem to be sufficient to give adequate statistical power. Moreover, the frequency of the I823 allele in the previous study (allele frequency 0.492) was different from that in the Suita population (0.36) and JSNP information (0.38). Thus, the subjects in the previous study did not seem to be representative of the general Japanese population, as noted by Harada et al. (2003).

Recently, the polymorphisms in the promoter region of *ABCA1*, which corresponds to C(-559)T in the present study and seems to be in tight linkage with G(-273)C ($r^2=1$, D' -value=1), was found to be modestly, but not significantly ($P=0.09$), associated with the HDL-C level using LCAS subjects (Lutucuta et al. 2001). The effect of the *ABCA1* G(-273)C polymorphism on the HDL-C level was significant, but still relatively weak ($r^2=0.0050$). Accordingly, the sample size ($n=372$) in the previous study (Lutucuta et al. 2001) seems to have been too small to detect the effect of polymorphisms on the HDL-C level clearly.

While the *ABCA1* G(-273)C polymorphism was associated with HDL-C level, it was not found to be associated with the incidence of MI. The *ApoE* polymorphism (E2, E3, and E4) had the greatest influence on the HDL-C level among the three polymorphisms, *ABCA1* G(-273)C ($r^2=0.0050$), *ApoA1* JST-IMS005603 (0.0100), and *ApoE* (0.0118). However, the *ApoE* polymorphism was only weakly associated with the incidence of MI ($P=0.0840$). Thus, *ABCA1* G(-273)C may have too weak an influence on the HDL-C level to alter the incidence of MI through a reduction of the HDL-C level. More large numbers of MI subjects might be necessary to detect the influence of the *ABCA1* G(-273)C polymorphism on MI incidence.

In summary, the present study provides the first evidence that the common *ABCA1* G(-273)C polymorphism in the promoter region is significantly associated with the level of HDL cholesterol in the Japanese.

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Lesion Severity and Hypercholesterolemia Determine Long-Term Prognosis of Vasospastic Angina Treated With Calcium Channel Antagonists

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Although patients with medically treated vasospastic angina have a good outcome, few data exist regarding the role of underlying lesion severity associated with or without hyperlipidemia in the prognosis. Therefore, the aim of the present study was to assess the relationship between the long-term outcome of vasospastic angina and the factors influencing its prognosis. A total of 256 patients (219 men, 37 women; mean age, 54.1±9.2) who had coronary spasm with or without underlying lesions and were being treated with calcium channel antagonists were enrolled and followed for 13.6±3.7 years. Cardiac events consisted of cardiac death and ischemic events, which included acute myocardial infarction and unstable angina. Cox analysis selected coronary artery stenosis (CAS, ≥50%) and risk factors such as age, hypertension, diabetes mellitus, low-density lipoprotein-cholesterol (LDL-C), sex and smoking. There were 19 cases of cardiac death (7.4%) and 58 of ischemic events (22.7%) during the follow-up period. The presence of significant CAS was an independent predictor of event-free survival (hazard ratio (HR)=2.84, 95% confidence interval (CI)=1.79–4.52, $p<0.0001$). In 193 patients without significant CAS, there were 10 cases of cardiac death (5.2%, $p<0.05$) and 34 of ischemic events (17.6%, $p<0.01$). In that group, high LDL-C was the independent predictor of event-free survival (HR=3.89, 95% CI=1.20–12.6, $p=0.02$). Kaplan-Meier survival analysis revealed significantly lower event-free survival in patients with than in those without lesions ($p<0.0001$ by log-rank test). These results demonstrate that the most important factor for long-term prognosis of vasospastic angina treated with calcium channel antagonists is significant CAS. High LDL-C, which might alter the underlying coronary endothelial function and/or accelerate atherosclerotic lesions, could also contribute to the occurrence of cardiac events, particularly in patients without significant CAS. (Circ J 2003; 67: 1029–1035)

Key Words: Calcium channel antagonist; Coronary artery disease; Long-term prognosis; Vasospasm

Coronary spasm provokes the myocardial ischemia associated with angina pectoris, acute myocardial infarction and sudden death.^{1,2} Calcium channel antagonists, nitrates or a combination of both drugs have been used effectively to prevent coronary spasm.^{3–6} Indeed, the overall cardiac mortality is relatively low and the prognosis seems to be good for medically treated patients with coronary spasm.^{7–9} Coronary spasm frequently occurs in minimally narrowed coronary segments,^{10,11} suggesting a pathophysiologic correlation between coronary spasm and atherosclerosis, which has been demonstrated in an experimental animal model.¹² Several studies have examined the relationship between the clinical characteristics and prognosis of this type of angina, and demonstrated that preexist-

ing atherosclerosis could be an important risk factor for cardiac death and myocardial infarction during the relatively early phase.^{5–9} However, there is little information regarding the long-term prognosis of medically treated coronary spasm with or without atherosclerotic risks, such as underlying coronary artery stenosis (CAS) and intrinsic hyperlipidemia. Such data should be important for not only preventing vasospasm, but also preventing the development of atherosclerosis. The purpose of this study was, first, to determine the event-free survival rate in patients with angiographically documented coronary spasm, and second, to identify the clinical predictors of cardiac events, particularly in patients with and without significant CAS.

Methods

Patient Population

Of 2,740 patients who underwent diagnostic coronary angiography for suspected ischemic heart disease between 1977 and 1987, 284 consecutive patients with coronary spasm were enrolled and followed. During the follow-up, 21 patients went missing. Patient enrollment was completed in 1987 when 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, which might have altered the long-term prognosis,¹³ was not generally available. Therefore, no patient had been given HMG-CoA reductase

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Table 1 Baseline Clinical and Angiographic Characteristics

	Total (n=256)	Without CAS (n=193)	With CAS (n=63)	p value
Age (years)	54.1±9.2	54.4±8.0	55.0±10.3	NS
Male sex (%)	219 (85.5)	163 (84.5)	56 (88.9)	NS
Coronary risk factors				
Hypertension (%)	115 (44.9)	91 (47.2)	24 (38.0)	NS
High LDL-C (%)	61 (23.8)	39 (20.2)	22 (34.9)	NS
Diabetes mellitus (%)	7 (2.7)	3 (1.6)	4 (6.3)	NS
Smoking (%)	174 (66.8)	129 (66.8)	45 (71.4)	NS
Arrhythmia during angina (%)	10 (3.9)	10 (5.2)	0 (0)	NS
Multivessel spasm (%)	18 (7.0)	17 (8.8)	1 (1.6)	NS

CAS, coronary artery stenosis; LDL-C, low-density lipoprotein-cholesterol.

Table 2 Incidence of Cardiac Events During Follow-up Period

Cardiac event	Total (n=256)	Without CAS (n=193)	With CAS (n=63)	p value
Cardiac death (%)	19 (7.4)	10 (5.2)	9 (14.3)	0.017
Sudden death (%)	8 (3.1)	3 (1.6)	5 (7.9)	0.024
Myocardial infarction (%)	3 (1.1)	2 (1.0)	1 (1.6)	NS
Heart failure (%)	5 (2.0)	4 (2.1)	1 (1.6)	NS
Others (%)	3 (1.2)	1 (0.5)	2 (3.2)	NS
Ischemic event (%)	58 (22.7)	34 (17.6)	24 (38.1)	<0.01
Nonfatal myocardial infarction (%)	15 (5.9)	8 (4.1)	7 (11.1)	0.041
Unstable angina (%)	29 (11.3)	21 (10.9)	8 (12.7)	NS
PTCA (%)	12 (4.7)	5 (2.6)	7 (11.1)	0.011
CABG (%)	2 (0.8)	0 (0)	2 (3.2)	NS
Time of event from registration (years)	4.9±4.7	4.6±4.3	5.3±5.3	NS

CABG, coronary artery bypass grafting; CAS, coronary artery stenosis; PTCA, percutaneous transluminal coronary angioplasty.

inhibitor at the time follow-up began, but 7 patients were unexpectedly given this drug during the follow-up period, particularly after 1990. Therefore, these 28 patients were excluded, and a total of 256 patients were analyzed in this study. There were 219 men and 37 women aged 54.1±9.2 years. All the patients had chest pain at rest and/or on exertion. ECG changes such as ST elevation or depression during chest pain attacks were demonstrated in 154 patients.

Cardiac Catheterization Procedures

Written informed consent was obtained from all patients for cardiac catheterization and the provocation of coronary spasm. All patients were fasted and received 3,000–5,000 U heparin intravenously before the procedure. Calcium channel antagonists, nitrates, β -blockers and other anti-anginal drugs were discontinued at least 9 h before procedures, because these drugs could alter the basal coronary tone associated with the occurrence of spasm.

After control coronary angiography, the provocative tests were performed. We initially used intravenous ergonovine to induce spasm,¹⁴ but since 1986, the intracoronary ergonovine test has been used for safety reasons.¹⁵ When coronary spasm was not provoked, ergonovine maleate was administered until the total dose reached 0.4 mg in the intravenous test, or 0.04 mg in the intracoronary test. Under these conditions, the standard 12-lead ECG was continuously monitored to record ST change (≥ 0.1 mV) and associated arrhythmias. When chest pain or significant ST-segment changes were observed, selective coronary angiography was immediately performed. Coronary spasm was considered positive when there was luminal narrowing $\geq 75\%$; however, diffuse spasm $\geq 75\%$ without signs of myocardial ischemia was not considered positive, because

it is difficult to differentiate real spasm from a pharmacological reaction.¹⁶ Multi-vessel spasm was defined as vasospasm in more than one major coronary artery.

After provocation, 0.25–0.5 mg (0.5 mg/ml) nitroglycerin was given to obtain maximal coronary dilation, and coronary angiography was again performed in multiple projections to evaluate the extent and severity of CAS. Left ventriculography was performed to calculate the left ventricular ejection fraction by the area–length method. Coronary angiography and left ventriculography were evaluated by at least 2 angiographers who were independent of each other. Significant CAS was defined as lumen stenosis $\geq 50\%$. In the case of repeated angiography, progression of lesion severity was defined as an increase of $\geq 20\%$ in a preexisting stenosis of $\geq 50\%$, and an increase of $\geq 30\%$ in a stenosis $< 50\%$, or any increase in lesion severity that resulted in total coronary occlusion. New stenoses were defined as stenoses $\geq 20\%$ that developed at a site that was previously angiographically normal.¹⁷

Assessment of Coronary Risk Factors

The coronary risk factors at baseline were hypertension, diabetes mellitus, high low-density lipoprotein-cholesterol (LDL-C) and history of smoking. Blood sugar, total cholesterol, LDL-C, high-density lipoprotein-cholesterol, and triglycerides were measured on the morning of angiography while the patient was still in a fasting state. In patients who could be followed up for more than 6 months, these variables were re-examined as frequently as possible during the follow-up period. In patients in whom LDL-C had not been measured and the triglyceride concentration was < 300 mg/dl, the LDL-C concentration was calculated using the following equation: LDL-C=(total cholesterol–

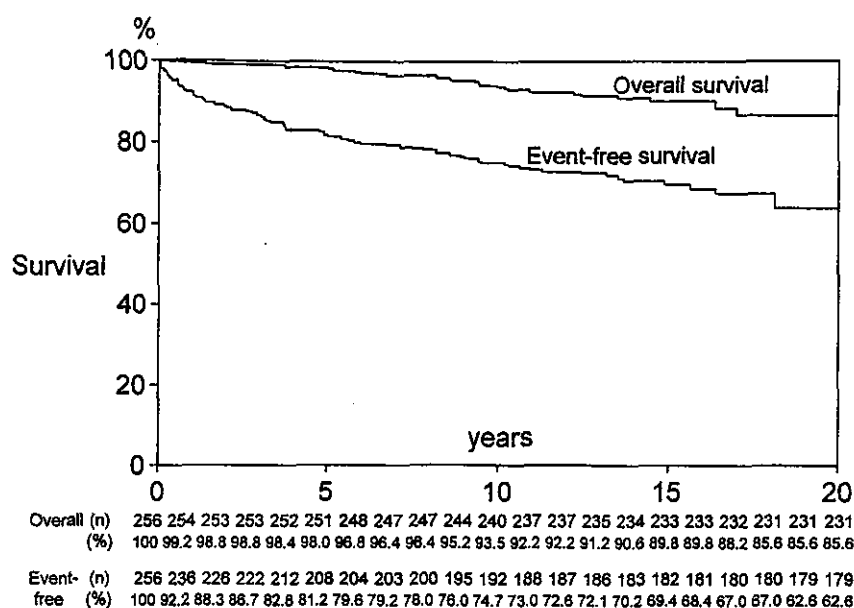


Fig 1. Kaplan-Meier analysis of overall mortality and event-free survival rate for entire patient group. Vertical and horizontal axes represent % survival rate and time from registration, respectively.

HDL-C-1/5 triglycerides). Hypertension was defined as systolic blood pressure >140 mmHg, diabetes mellitus was defined as fasting glucose >140 mg/dl, and high LDL-C was defined as serum LDL-C concentration >140 mg/dl. A smoking history was obtained from all patients: smokers were defined as patients who had smoked more than 20 cigarettes per day for >20 years. We also evaluated the contribution of the patients' sex and age to the prognosis.

Long-Term Follow-up

The cardiac events included cardiac death and ischemic events. Cardiac death consisted of sudden death, which was defined as death within 1 h after collapse, and death associated with acute myocardial infarction, heart failure, or other cardiovascular disease. Ischemic events consisted mainly of non-fatal myocardial infarction, unstable angina and the need for percutaneous transluminal angioplasty (PTCA) or coronary artery bypass surgery (CABG). Unstable angina was defined as worsening of chest symptoms,¹⁸ and except for emergency procedures the indication for PTCA and CABG was considered by at least 2 cardiologists independent of this study.

Myocardial infarction was diagnosed on the basis of the development of a new Q wave in the ECG and elevation of serum concentrations of cardiac enzymes. Unstable angina was defined as repeated anginal attacks at rest. Non-fatal myocardial infarction was defined as survival of the patient who could then be discharged from hospital. In this study, if a patient died of cardiac death and had suffered an ischemic event before death, the cardiac event was not defined as cardiac death, but as an ischemic event. Accordingly, a cardiac event in this study meant the first cardiac event that occurred during follow-up.

All patients were given at least either dihydropyridine or diltiazem hydrochloride. Drug therapy was started immediately after angiographic diagnosis and continued by the individual physicians until the end-point. The end-points of follow-up were the first cardiac event and non-cardiac death without a cardiac event. The follow-up period of each patient was calculated from the date of the initial diagnostic angiography. Those who did not visit for follow-up examination and checking the status of medical compliance were

Table 3 Predictors of Cardiac Events in All Patients During Follow-up Period

	Hazard ratio	95% CI	p value
CAS	2.84	1.79-4.52	<0.0001
High LDL-C	2.21	0.79-6.21	0.079
Diabetes mellitus	1.72	0.94-3.16	0.16
Hypertension	1.21	0.71-2.00	0.45
Sex	1.18	0.59-2.37	0.65
Age	0.99	0.97-1.03	0.89
Smoking	0.99	0.58-1.68	0.96

CAS, coronary artery stenosis; CI, confidence interval; LDL-C, low-density lipoprotein-cholesterol.

followed by telephone interviews with the patient or the family, from whom the status of smoking was carefully elicited. When anginal attacks became frequent or were not relieved by medical treatment, the patient was admitted and coronary angiography was performed again to determine alternative methods of management.

Statistical Analysis

All values are expressed as mean \pm SD. Continuous variables were compared by unpaired Students' t-test. Categorical variables were compared by chi-square test (with Fisher's exact test, as appropriate for smaller sample size). Multivariate analysis with Cox proportional-hazards regression analysis was used to assess the independent significance of prognostic factors for cardiac events. Event-free survival rate was calculated using the Kaplan-Meier method, and differences between survival curves were assessed by the log-rank test.¹⁹ Values of $p < 0.05$ were considered significant. Analyses were performed using StatView 4.5 statistical software (Abacus Concepts, Berkeley, CA, USA).

Results

Baseline Data Collection (Table 1)

Coronary spasm was angiographically demonstrated during spontaneous angina in 40 patients and in 216 patients by provocation tests. Spasm in more than one

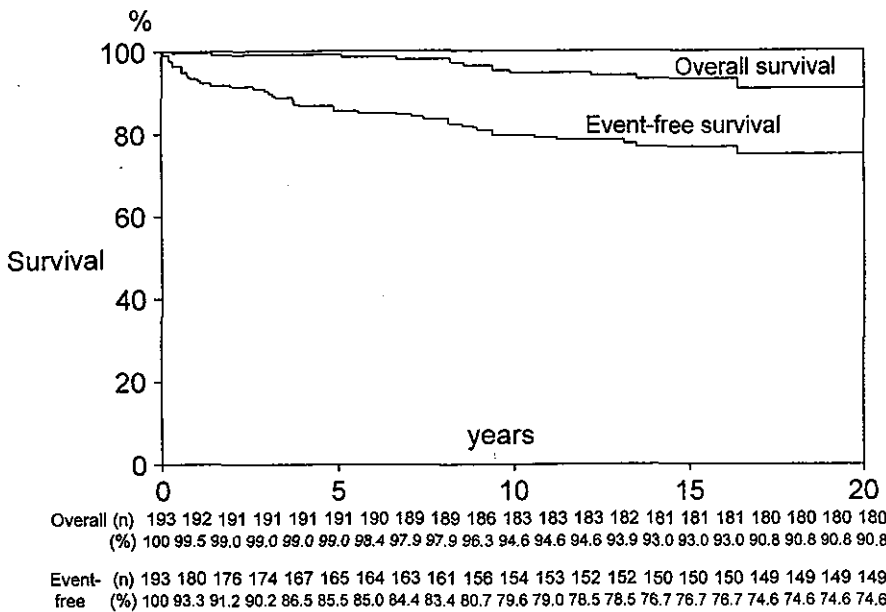


Fig2. Kaplan-Meier analysis of overall mortality and event-free survival in patients without significant coronary artery stenosis. Vertical and horizontal axes represent % survival rate and time from registration, respectively.

Table 4 Predictors of Cardiac Events in Patients Without Significant CAS During Follow-up Period

	Hazard ratio	95% CI	p value
High LDL-C	3.89	1.20-12.6	0.02
Sex	1.51	0.64-3.59	0.35
Smoking	1.14	0.57-2.28	0.71
Age	0.99	0.95-1.03	0.51
Hypertension	0.95	0.50-1.82	0.87
Diabetes mellitus	0.97	0.13-7.41	0.98

CAS, coronary artery stenosis; CI, confidence interval; LDL-C, low-density lipoprotein-cholesterol.

major coronary artery was demonstrated in 18 patients. Of the 256 patients, 63 had significant CAS; 53 with single-vessel disease, 8 with double-vessel disease, and 2 with triple-vessel disease. Among the remaining 193 patients who did not have significant CAS, 99 exhibited normal coronary angiography after nitroglycerin administration.

ECG during anginal attacks showed ST elevation in 123 patients, depression in 51 patients and no significant ST change in 82 patients. Ventricular tachycardia was recorded in 6 patients, and second- or third-degree atrioventricular block in 4 patients during provoked coronary spasm. Left ventriculography was normal in 200 patients; segmental wall motion abnormalities were observed in 25 patients. Left ventriculography was not performed in 31 patients because previous echocardiography had shown normal wall motion and cardiac systolic function. Ninety-nine patients were treated with a calcium channel antagonist such as dihydropyridine (30-60 mg/day) or diltiazem hydrochloride (90-120 mg/day), and 157 patients with a combination of calcium channel antagonist and nitrate (40-80 mg/day). In addition to these medications, β -blockers were used in 9 patients. The dose of each drug was calculated to prevent the occurrence of angina during daily activities.

Incidence of Cardiac Events During Follow-up (Table 2)

The mean duration of follow-up was 13.6 ± 3.7 years (range 0.3-20 years). The first cardiac event occurred at 4.9 ± 4.7 years from registration. Of the 46 patients who

died during follow-up, 19 died of cardiac death (8 sudden death, 3 myocardial infarction, 5 unexpected heart failure, 3 unknown cause). The other 27 patients died from non-cardiac causes. Ischemic events occurred in 58 patients (non-fatal acute myocardial infarction in 15, unstable angina in 29, PTCA in 12, CABG in 2). The coronary lesions causing the ischemic events were confirmed to coincide with the previous sites of vasospasm in 30 patients and could not be confirmed in the remaining 28 patients.

The overall survival rate was 99.2% at 1 year, 98.0% at 5 years, 93.5% at 10 years and 85.6% at 20 years. Under these conditions, the event-free survival rate was 92.2%, 81.2%, 74.7%, and 62.6%, respectively (Fig 1). When all of the 7 variables were analyzed, the presence of CAS was shown to be an independent predictor of cardiac events (hazard ratio (HR)=2.84, 95% confidence interval (CI)=1.79-4.52, $p < 0.0001$). There was no difference in survival rate between the patients with CAS in the left coronary and right coronary arteries. High LDL-C and diabetes mellitus had relatively high hazard ratios (2.21 and 1.72, respectively) without statistical significance. Other factors were not significant predictors of cardiac events (Table 3).

In patients without CAS, Kaplan-Meier survival analysis revealed an event-free survival rate of 85.5% at 5 years, 79.6% at 10 years, 76.7% at 15 years and 74.6% at 20 years (Fig 2). Under these conditions, high LDL-C was the only independent predictor of event-free survival (HR=3.89, 95% CI=1.20-12.6, $p=0.02$, Table 4). As for the relationship between ST deviation and occurrence of cardiac events, cardiac events occurred in 56 of 174 patients (32.2%) with ST deviation, elevation or depression, during vasospasm and in 21 of 82 patients (25.6%, NS) without ST deviation. In 99 patients with normal angiography after nitroglycerin, the incidence of cardiac events and the event-free survival rate were not different from those of the remaining 94 patients with minimal and not significant lesion ($p=0.074$).

Association Between CAS and Cardiac Events

Although there were no differences in the baseline clinical characteristics between patients with and without CAS

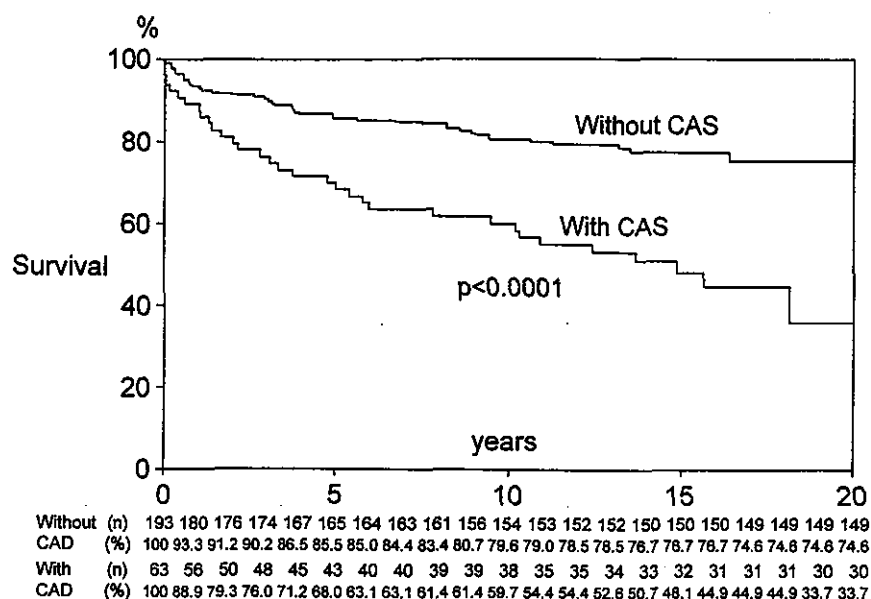


Fig 3. Comparison of event-free survival curves between patients with and without significant coronary artery stenosis (CAS). Vertical and horizontal axes represent % survival rate and time from registration, respectively.

(Table 1), the overall rate of cardiac events was significantly lower in patients without CAS (22.8%) than in those with CAS (52.4%, $p<0.01$). As for cardiac deaths, 9 of 63 patients with CAS died and only 10 of 193 patients without CAS, yielding a significant difference in cardiac mortality between patients with and without CAS ($p=0.017$). Also, there was a significant difference in the incidence of sudden death between these subgroups ($p=0.024$).

Eight of 15 patients with non-fatal myocardial infarction did not have CAS whereas the remaining 7 patients did. Thus, the incidence of non-fatal myocardial infarction in patients without CAS was significantly lower than that in patients with CAS ($p=0.041$). However, there was no significant difference in the time interval between registration and the first cardiac events in patients without (4.6 ± 4.3 years) and with (5.3 ± 5.3 years) CAS. There was a marked difference in the event-free survival rate between the subgroups with and without CAS ($p<0.0001$, Fig 3).

Coronary angiography was repeated in 45 of the 256 patients. Progression of coronary atherosclerosis was observed in 10 patients with CAS (45.5%) and in 13 patients without CAS (56.5%, NS). It was interesting that progression of coronary atherosclerosis was more frequently observed at previous sites of spasm (17 patients or 37.8%) than at non-spastic sites (7 patients or 15.5%, $p=0.016$).

Discussion

Prognostic Value of CAS and High LDL-C

One of the important findings of the present study is that the presence of significant CAS was an independent predictor of event-free survival, as well as the simple survival, in patients with vasospastic angina treated with calcium channel antagonists. The local effect of more complete occlusion during coronary spasm may predispose to malignant arrhythmia or severe myocardial ischemia, although there was no difference in the incidence of cardiac events, including sudden death, in patients with and without ST deviation that may represent the severity of transient myocardial ischemia. Treatment with calcium channel antagonists could reduce this transient ischemia in both patients with and without significant CAS.

Coronary spasm is a possible mechanism for the progression of atherosclerosis.^{17,20,21} Indeed, in the present study repeated coronary angiography revealed that approximately 40% of patients had progression of coronary atherosclerosis at the sites of previously demonstrated vasospasm. We speculate that coronary spasm accelerates the progression of atherosclerosis, resulting in sudden death and other cardiac events. Under these conditions, elevation of the LDL-C concentration might play an important role in aggravating atherosclerosis through the disintegration of endothelial function.²² However, high LDL-C was second to CAS in terms of hazard ratio in all the present patients. It is possible that a relatively close relation of high LDL-C to significant CAS might make it difficult to differentiate LDL-C as an independent factor in the presence of CAS. Actually, this was shown to be the most important independent factor for cardiac events in patients without CAS. Based on an animal model, an important early step in the development of atherosclerotic lesions is endothelial dysfunction associated with high cholesterol, leading to abnormal vessel tonus characterized by paradoxical constriction to physiologic and pathologic stimuli.²³ Vasospasm may be an extreme of this phenomenon and related to lesion development. From this point of view, it is interesting to speculate that high LDL-C may be related to impairment of coronary endothelial dysfunction,²⁴ although recent work does not support this hypothesis.²⁵

Previous studies suggest that the occurrence of coronary spasm is independent of plasma cholesterol concentrations; in the present study, two-thirds of the patients had normal cholesterol levels. However, under treatment with calcium channel antagonists, which effectively prevent spasm, conventional risk factors such as hypercholesterolemia might be important for the development of atherosclerotic lesions associated with cardiac events. Also it was unclear in the previous studies whether or not the LDL-C concentration was high, even though the total cholesterol concentration was within normal range.

The finding that the incidence of sudden death was lower than in previous studies may be explained by the small number of patients with life-threatening arrhythmias in the present patient group; Millar et al reported that nearly 80%

of patients who died from sudden death had a history of ventricular tachyarrhythmias²⁶ Even so, 8 patients died suddenly during the follow-up period. It is unclear whether the presence of CAS is a major determinant of sudden death, because sudden death also occurred frequently in patients without CAS.²⁷ Although a previous study suggested that spasm in multiple vessels could be an important determinant of prognosis,²⁸ the present study could not confirm this finding because of the small number of patients with multi-vessel spasm. One of the reasons why there were relatively few instances of multi-vessel spasm was the different way of provoking spasm. Selective intracoronary injection of ergonovine might not have induced multi-vessel spasm in some patients in the present study.

Prognostic Value of Other Risk Factors

Except for high LDL-C, there was no statistically significant difference between the other coronary risk factors and cardiac events, although smoking is considered to be one of the major risk factors for the occurrence of vasospastic angina.²⁹ The patients with a history of smoking were carefully questioned about whether they had discontinued smoking during the follow-up period, but it is difficult to confirm the actual status of smoking just by questionnaires at the out-patient clinic. Discontinuation or, at least, a reduction in smoking might result in statistical insignificance of the history of smoking as a risk factor for cardiac events and sudden death.

Coronary spasm without CAS has a fairly good long-term clinical outcome, but even so, high LDL-C was an independent prognostic factor in this subgroup. This suggests that for patients with coronary spasm, manipulation of the therapeutic regimen to prevent not only the occurrence of vasospasm, but also the progression of atherosclerosis should be considered. Calcium channel antagonists such as nifedipine or nicardipine do not reduce cardiac events^{30,31} and therefore, a combination with other agents, such as an HMG-CoA reductase inhibitor, that may stabilize the atherosclerotic plaque would further reduce cardiac events in vasospastic angina;³ although the present study excluded patients in whom HMG-CoA reductase inhibitors were given.

The finding that other traditional risk factors were not significant for long-term prognosis can be explained by the specific situation of vasospastic angina. Indeed, previous studies demonstrated that coronary spasm could occur in the absence of hypertension and diabetes mellitus.²⁹ Magnesium deficiency, which was not determined in the present study, might be another risk factor for prognosis of coronary spasm, because 50% of patients with recent myocardial infarction have been shown to have coronary spasm associated with it.³²

Study Limitations

We previously reported that even in the absence of angiographic coronary disease, atherosclerosis can be demonstrated by intravascular ultrasound at the sites of spasm.^{33,34} Therefore, we could not precisely evaluate the progression or regression of coronary disease in all patients. Prospective follow-up of patients using ultrasound may resolve this problem. Whether patients consistently complied with medication during the follow-up period is important in considering the long-term prognosis. However, the mid-term prognosis of the present study was similar to those of others,^{8,9} which suggests that there was accepta-

ble drug compliance during the follow-up period. The use of different kinds of calcium channel antagonists for treatment may have had a small effect on the long-term prognosis, although dihydropyridines and diltiazem have a similar effect on coronary spasm in the relatively acute phase.³⁻⁶

Although it is clinically interesting to consider the relationship between the frequency of anginal attacks and long-term prognosis, we could not determine the significance, because of difficulty in quantitatively estimating the frequency. For the same reason, it was also difficult to correlate the presence of CAS with anginal frequency.

To further understand the role of high LDL-C in the long-term prognosis of vasospastic angina, the medical regimen of HMG-CoA reductase inhibitors in addition to calcium channel antagonists should be challenged. It is also necessary to compare the long-term prognosis between CAS patients with and without spasm. However, practically, it is difficult to enroll stable patients without coronary spasm, because PTCA or CABG rather than medical treatment is now the primary treatment.³⁵

Finally, the present study dealt only with Japanese patients with coronary spasm. However, despite the racial difference in coronary vasomotor reactivity,³⁶ the clinical outcome of the present patients is similar to the results of studies in Europe and North America. Therefore, the present data provide important information on the long-term prognosis of medically treated coronary spasm patients with or without CAS.

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Reactive oxygen species regulate FLICE inhibitory protein (FLIP) and susceptibility to Fas-mediated apoptosis in cardiac myocytes

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Abstract

Objective: Fas ligand (FasL) is a key cytokine which initiates apoptosis when FasL binds to its receptor, Fas. Cardiac myocytes are generally resistant to Fas-induced apoptosis. However, sublethal dose of doxorubicin (Dox) can sensitize cardiac myocytes to Fas-induced apoptosis. We investigated the molecular mechanism by which Dox sensitizes cardiac myocytes to Fas-induced apoptosis. FLICE inhibitory protein (FLIP) is a key molecule for blocking Fas-induced apoptosis by functioning as a caspase-8 dominant negative. **Methods and results:** FLIP was constitutively expressed in cultured neonatal rat cardiac myocytes. FLIP protein levels were markedly down-regulated by Dox in a time-dependent and dose-dependent manner. Next, we examined the relation of reactive oxygen species (ROS) by Dox to the expression of FLIP. Both of *N*-acetylcysteine (NAC) and the combination of superoxide dismutase and catalase restored the decreased FLIP in Dox-treated cardiac myocytes to the basal level. NAC also restored the increased formation of thiobarbituric acid-reactive substance after Dox-treatment. Concurrently, the susceptibility to Fas-mediated apoptosis disappeared with the treatments of the antioxidant agents. Hydrogen peroxide down-regulated FLIP in a dose-dependent fashion and also sensitized cardiac myocytes to Fas-induced apoptosis. **Conclusions:** FLIP, an inhibitor of apoptosis induced by cytokines of TNF family, contributes at least partly to Dox-induced sensitization to Fas-mediated apoptosis in cardiac myocytes. The expression of FLIP in cardiac myocytes is regulated by ROS.

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Keywords: Apoptosis; Cardiomyopathy; Cytokines; Free radicals; Myocytes

1. Introduction

Doxorubicin, an anthracycline antibiotics, has been used as a powerful drug in the fight against neoplastic diseases. However, its practical use is sometimes limited by acute and chronic cardiotoxicities [1]. The cardiotoxicity is dose-dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy with fatal congestive heart failure [1]. The exact causal mechanism of doxorubicin-induced cardiomyopathy remains unclear, but most of the evidence indicates that free radicals are involved [2,3]. The chemical structure of doxorubicin is prone to the

generation of free radicals, and the oxidative stress correlates with cellular injury [4]. Increased oxidative stress may lead to a variety of subcellular changes in the myocardium.

Apoptosis plays a pivotal role in loss of cells not only during physiological phenomena such as normal turnover of tissues, but also in many pathophysiological phenomena. Evidence is accumulating that the apoptotic mechanism is involved in various heart disorders [5–8]. Fas/Fas ligand system is one of the key regulators of apoptosis [9]. Fas/CD95 is the cell-surface receptor expressed in many cell types including cardiac myocytes [10]. Fas/CD 95 ligand (FasL) is expressed on the membrane of cells, such as activated T cells [11], NK cells [12], endothelial cells

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[13] or immune privileged sites [14]. Both the membrane-bound FasL and its cleaved form, soluble FasL, can induce apoptosis in vitro and in vivo [15–17], while the lethal ability is more potent in the membrane-bound FasL [18]. The Fas/FasL system has been reported to be activated in human heart failure [19–21]. Doxorubicin-induced deterioration in left ventricular performance in rat was inhibited by neutralizing anti-FasL antibody [22], suggesting an important role of Fas/FasL system in doxorubicin cardiomyopathy. However, it is obscure how administration of doxorubicin renders the myocardium prone to Fas-mediated apoptosis. We previously observed that cardiac myocytes were generally resistant to Fas-mediated apoptosis in vitro. However, after treatment of sublethal dose of doxorubicin, cardiac myocyte apoptosis was dramatically facilitated by recombinant FasL [23]. This finding is intriguing, because the doxorubicin-induced sensitivity to Fas in cardiomyocytes can be induced by a molecule which may be a target for treating doxorubicin-associated cardiomyopathy.

Recently, FLICE-inhibitory protein (FLIP), a molecule with sequence homology to caspase-8 (FLICE), was identified as an anti-apoptotic protein [24]. FLIP inhibits Fas- and TNF-mediated apoptosis [24,25]. When FasL binds to Fas, Fas-associated death domain (FADD) is activated and then, caspase-8 is cleaved and activated, leading to subsequent activations of caspase cascades. FLIP is capable of binding FADD, thereby preventing cleavage and activation of caspase-8, thus shutting off the initiation of the death pathway [24–26]. Although high levels of transcripts of FLIP are expressed in the heart compared to other organs [26], little is known about the basic mechanisms controlling the expression of FLIP. In this study, we investigated whether FLIP levels are related to the doxorubicin-induced sensitivity to Fas in cultured neonatal rat cardiac myocytes. Moreover, we examined the hypothesis that the expression of FLIP was down-regulated by oxidative stress.

2. Methods

2.1. Materials

Human soluble recombinant FasL (rFasL) [27] and human Fas chimeric protein (Fas-Fc) [28] were provided by Bioscience Research Laboratory, Mochida Pharmaceutical Co. (Tokyo, Japan). A polyclonal anti-FLIP antibody was generated in a Japanese white rabbit according to the protocol previously reported [26,29,30]. Anti-Fas and -FADD antibodies were purchased from Transduction Laboratory (Lexington, KY, USA). Doxorubicin, propidium iodide (PI), trypsin EDTA, superoxide dismutase, catalase and *N*-acetylcysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caspase-8 inhibitor (Z-Ile-Glu-Thr-Asp-CH2F or Z-IETD-FMK) was

purchased from Medical and Biological Laboratories Co. Ltd. (Nagoya, Japan). F-12 powder and fetal bovine serum (FBS) were from Cosmo Bio Co. Ltd. (Tokyo, Japan). Dulbecco's phosphate-buffered saline, transferrin, and insulin-selenite were from Gibco BRL (Grand Island, NY, USA). Collagenase and hydrogen peroxide (H_2O_2) were from Wako Pure Chemical Industry (Osaka, Japan).

2.2. Cell culture

The animals were handled according to the animal welfare regulations of Yamagata University, and the study protocol was approved by the Animal Subjects Committee of Yamagata University. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Cardiac myocytes were isolated from 0- to 1-day-old Wistar–Imamichi rats (Imamichi Institute for Animal Reproduction, Japan) by the methods previously described [23]. In brief, the ventricles were minced and digested with collagenase. After washing with MSS buffer (30 mmol/l HEPES, 4.1 mmol/l dextrose, 2 mmol/l KCl, 120 mmol/l NaCl, and 1 mmol/l KH_2PO_4), the digested fractions were filtered through a mesh. The pooled cells were resuspended in F-12 medium (10.62 g/l F-12 mixture, 15 mmol/l HEPES, 22 mmol/l $NaHCO_3$, 5 μ g/ml transferrin) with supplements of 5 μ g/ml insulin-selenite, 50 U/ml penicillin, 50 μ g/ml streptomycin and 10% FBS. After preplating in another flask to minimize non-myocyte contamination, cardiac myocytes were seeded onto fibronectin-coated dishes (5×10^5 cells/ml). Four days after seeding, the culture medium was changed to serum free F-12 medium containing transferrin, insulin and selenite. Doxorubicin treatment was started immediately after medium change. After treatment with or without doxorubicin (0.5 μ mol/l) for 12 h, rFasL was added onto the cell culture, and again incubated for 12 h.

2.3. Detection of apoptosis

TUNEL staining was performed with a commercially available kit for detection of end-labeled DNA according to the manufactures instructions (Mebstain Apoptosis Kit Direct, MBL, Nagoya, Japan). The cardiac myocytes were fixed, washed, and incubated with distilled water, and then incubated with TdT and FITC-dUTP. Counter staining was performed with propidium iodide (PI) by diluting with PBS to obtain a concentration of 1 μ g/ml. Approximately 800–1000 nuclei from random fields were analyzed for each sample. The apoptotic index was calculated as apoptotic nuclei/total nuclei $\times 100$ (%). The presence of apoptotic profiles was also confirmed by staining with Hoechst dye #33342.

2.4. Cell viability assay

Cell viability was analyzed by MTT assay [31]. Equal numbers of cardiomyocytes were plated on 96-well plates. MTT (3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide; 0.625 mg/ml) was added to each well. The plates were then analyzed with a multiwell plate reader at 590 nm.

2.5. Western blot analysis

Cultured cardiac myocytes were lysed for 30 min on ice in a lysis buffer containing 2% NP-40, 0.5% sodium deoxycholate, 0.2% SDS, and protease inhibitor mixture, and then centrifuged at 12 000 rpm for 10 min at 4 °C. The protein concentration of each sample was measured using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL, USA). BSA was used as a protein assay standard. For SDS-PAGE, 80 µg of protein of each sample was added on 10% polyacrylamide gel and then electrophoretically transferred to PVDF membrane. After blocking with a buffer containing 5% non-fat milk and 0.2% Tween-20 for 1 h at room temperature, the membrane was incubated with either anti-FLIP, anti-Fas, or anti-FADD antibody for 3 h at room temperature. After washing three times with PBS containing 5% non-fat milk, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG for 1 h at room temperature. The membrane was washed again and the signals were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech., Japan).

2.6. Measurement of lipid peroxidation

The extent of lipid peroxidation was determined by using a thiobarbituric acid reactive substance (TBARS) assay in cultured rat neonatal cardiac myocytes. Malondialdehyde (MDA) was used as standard and the results were expressed as nanomol MDA per milligram protein [32].

2.7. Caspase-8 protease assay

Activation of caspase-8 was detected by the caspase-8 colorimetric protease assay kit (MBL, Nagoya, Japan). The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (pNa) after cleavage from the labeled substrate IETD-pNa. IETD-pNa is a specific substrate for activated caspase-8, which has Ile-Glu-Thr-Asp amino acid sequence labeled with *p*-nitroanilide. The pNa light emission was quantified using a microtiter plate at 405 nm.

2.8. Data and statistical analysis

Data were expressed as mean ± S.E. The values were

tested by one-way analysis of variance (ANOVA), and was followed by the Scheffe's *F*-test. Differences were considered statistically significant at *P* < 0.05.

3. Results

3.1. Doxorubicin-induced sensitization to Fas-mediated apoptosis in neonatal rat cardiac myocytes

The apoptotic index had no significant change from control to treatments of recombinant human FasL (FasL) in neonatal rat cardiac myocytes (Fig. 1A). However, 12 h after pretreatment of sublethal dose of doxorubicin (0.5 µmol/l), FasL markedly increased the apoptotic index in a dose-dependent manner. The FasL-induced apoptosis was completely blocked by anti-Fas antibody and caspase-8 inhibitor (Z-IETD-FMK), indicating that Fas-mediated signaling was activated by FasL after pretreatment of doxorubicin. Similarly, although FasL alone did not affect the viability of cardiac myocytes, FasL reduced the viability of cardiac myocytes in the presence of sublethal dose of doxorubicin (Fig. 1B). Cardiac myocytes were stained with Hoechst dye (Fig. 1C,D). With 1 µg/ml of FasL after treatment of doxorubicin, nuclei of cells showed typical morphology of apoptosis such as nuclear condensation and fragmentation (Fig. 1D).

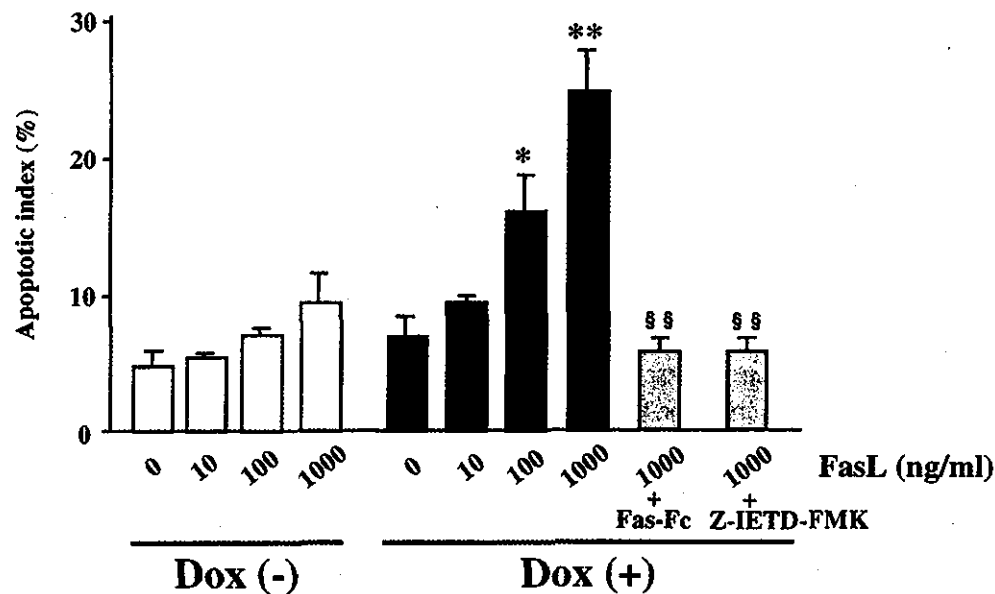
3.2. Down-regulation of FLIP protein levels by doxorubicin

FasL-induced activation of caspase-8 occurred only after treatment of sublethal dose of doxorubicin in cardiac myocytes [23] (also shown in Fig. 4). Fas protein was constitutively expressed at basal condition and slightly up-regulated by 24 h treatment of doxorubicin (data not shown). FADD protein levels were unchanged by the treatment (data not shown). We assessed expression levels of FLIP, a molecule with sequence homology to caspase-8 and capacity of preventing proteolytic activation of caspase-8. Immunoblotting revealed that untreated cardiac myocytes express a 55-kDa immunoreactive protein that has an identical mobility to the positive control, an extract from COS cells transfected with the FLIP_L expression plasmid [29]. Doxorubicin (0.5 µmol/l) strongly down-regulated the expression of the FLIP-immunoreactive protein in a time-dependent manner (Fig. 2A). When cardiac myocytes were treated with various concentrations of doxorubicin for 24 h, expression of FLIP protein was down-regulated in a dose-dependent manner (Fig. 2B).

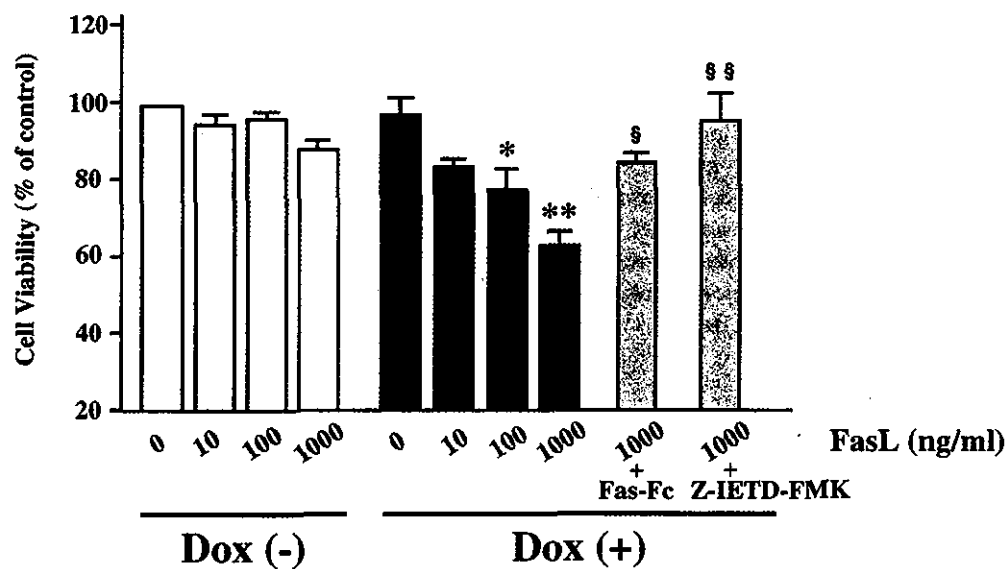
3.3. Antioxidants decrease doxorubicin-induced sensitivity to Fas and recover down-regulation of FLIP levels by doxorubicin

Antioxidants were used to determine whether reactive

A



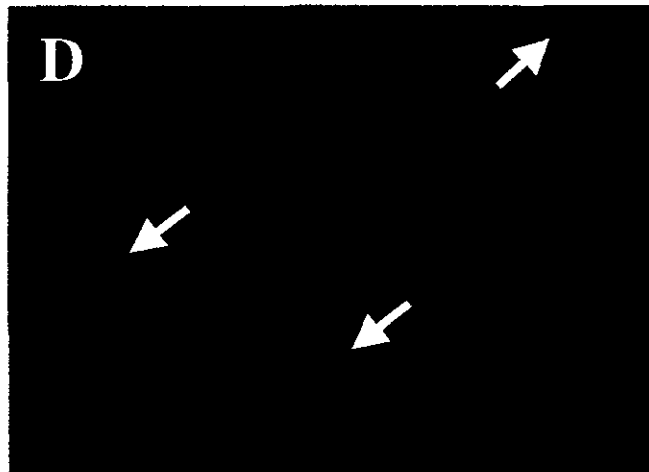
B



C



D



oxygen species can contribute to the sensitization to Fas after the treatment of doxorubicin in neonatal rat cardiac myocytes. In the presence of antioxidants, SOD and CAT, the apoptotic index was markedly reduced despite both treatments of doxorubicin and FasL (Fig. 3A). Similarly a scavenger of reactive oxygen species, *N*-acetylcysteine (NAC), decreased the apoptotic index induced by treatments of doxorubicin and FasL. The SOD and CAT recovered the decreased cell viability by doxorubicin and FasL, and NAC also restored the cell viability (Fig. 3B). The effects of these antioxidants indicate that reactive oxygen species are closely related to the doxorubicin-induced susceptibility to Fas in neonatal rat cardiac myocytes. Next, we examined whether the down-regulated FLIP levels by doxorubicin can be restored by the antioxidants (Fig. 3C). In the presence of NAC and the combination of SOD and CAT, the decreased levels of FLIP by doxorubicin were restored to the untreated levels. As expected with these findings, doxorubicin increased TBARS formation in the cultured cardiac myocytes, and NAC lowered the increased formation of TBARS with doxorubicin (Fig. 3D).

Activity of caspase-8 increased with the addition of FasL after pretreatments of doxorubicin. However, FasL or doxorubicin alone could not activate caspase-8 (Fig. 4). The SOD and CAT abolished the caspase-8 activation induced by FasL and doxorubicin. NAC also abolished the increased caspase-8 activity.

3.4. Hydrogen peroxide down-regulates FLIP levels and increases Fas-mediated apoptosis

To determine whether H_2O_2 inhibits the expression of FLIP, cardiac myocytes were incubated with exogenously administered H_2O_2 . As shown in Fig. 5, the levels of FLIP were decreased by incubation with H_2O_2 (50 $\mu\text{mol/l}$ for 12 h) in a dose-dependent manner. Moreover, FasL after pretreatment of H_2O_2 significantly decreased the cell viability of cardiac myocytes, whereas FasL or H_2O_2 alone had no significant change in the cell viability of cardiac myocytes (Fig. 5B).

4. Discussion

The mechanisms by which doxorubicin causes cardiotoxicity have been the subject of extensive investigation and debate for more than two decades [1,2]. It has been

suggested that apoptosis of cardiac myocytes is involved in the doxorubicin-induced loss of contractile function [22,33,34]. Recently, neutralizing anti-FasL antibody was reported to recover the doxorubicin-induced decrease of left ventricular performance in rat [22] suggesting that doxorubicin-induced apoptosis of cardiac myocytes may be executed through Fas-mediated pathway. Whereas cardiac myocytes were generally resistant to Fas-mediated apoptosis, cardiac myocytes became susceptible to Fas-mediated apoptosis after treatment of sublethal dose of doxorubicin [23]. Caspase-8 activity was markedly increased by FasL only after the pretreatment of doxorubicin indicating that doxorubicin modulates the signal from its receptor Fas to caspase-8. We focused on the behavior of FLIP, an anti-apoptotic molecule by inhibiting activation of caspase-8. Rat neonatal cardiac myocytes constitutively expressed FLIP protein, which is consistent with the resistance of cardiac myocytes to Fas-mediated apoptosis despite Fas expression in the untreated cardiac myocytes [10]. However, treatment of doxorubicin down-regulated the protein level of FLIP in neonatal rat cardiac myocytes in a time- and dose-dependent manner. Doxorubicin-induced downregulation of FLIP is one possible mechanism by which cardiomyocytes are prone to Fas-mediated apoptosis.

Doxorubicin has been reported to produce reactive oxygen intermediates including hydroxyl radicals and superoxide as well as hydrogen peroxide [4]. Doxorubicin also decreased protein levels of intracellular antioxidant enzymes, glutathione peroxidase (GSH) and manganese SOD [35]. The myocardial redox balance is markedly impaired by doxorubicin. Reactive oxygen species produced by doxorubicin play a role in cell death of cardiac myocytes [34]. Lower concentrations of doxorubicin produced apoptotic cell death of cultured cardiac myocytes [33,34,36] and there appears to be a switch from apoptosis to necrosis at doxorubicin concentrations of 2–10 $\mu\text{mol/l}$ [33,34]. In addition to doxorubicin concentration, cultured conditions, species and aging are important factors of cell survival of cardiac myocytes. In this study, 0.5 $\mu\text{mol/l}$ of doxorubicin did not induce an apparent decrease in cell viability of neonatal rat cardiac myocytes. Nevertheless, 0.5 $\mu\text{mol/l}$ doxorubicin down-regulated FLIP expression to a critical level at which cardiac myocytes became susceptible to FasL-induced apoptosis.

Although valuable information regarding signal transduction pathways leading to apoptosis has been obtained from the study using neonatal cardiac myocytes, there is

Fig. 1. Human recombinant Fas ligand (FasL) induced apoptosis in neonatal rat cardiac myocytes after treatment of doxorubicin. In the absence and presence of doxorubicin (Dox: 0.5 $\mu\text{mol/l}$) for 12 h, cardiac myocytes were incubated with various concentrations of FasL for additional 12 h. (A) Apoptotic index was calculated as a ratio of apoptotic cells to total cell number by in situ TUNEL staining. (B) Cell viability was determined by MTT assay. * $P < 0.01$, ** $P < 0.001$ vs. untreated cells; † $P < 0.05$, ‡ $P < 0.001$ vs. FasL 1 $\mu\text{g/ml}$ with doxorubicin. Results are expressed as mean \pm S.E.M. of five separate experiments. (C,D) Nuclear morphology of cardiac myocytes were stained with Hoechst 33248. Nuclei of untreated cardiac myocytes were shown (C). Cardiomyocytes were incubated with 1 $\mu\text{g/ml}$ of FasL after treatment of doxorubicin (D). Arrows indicate fragmented chromatin in nuclei of apoptotic cells.

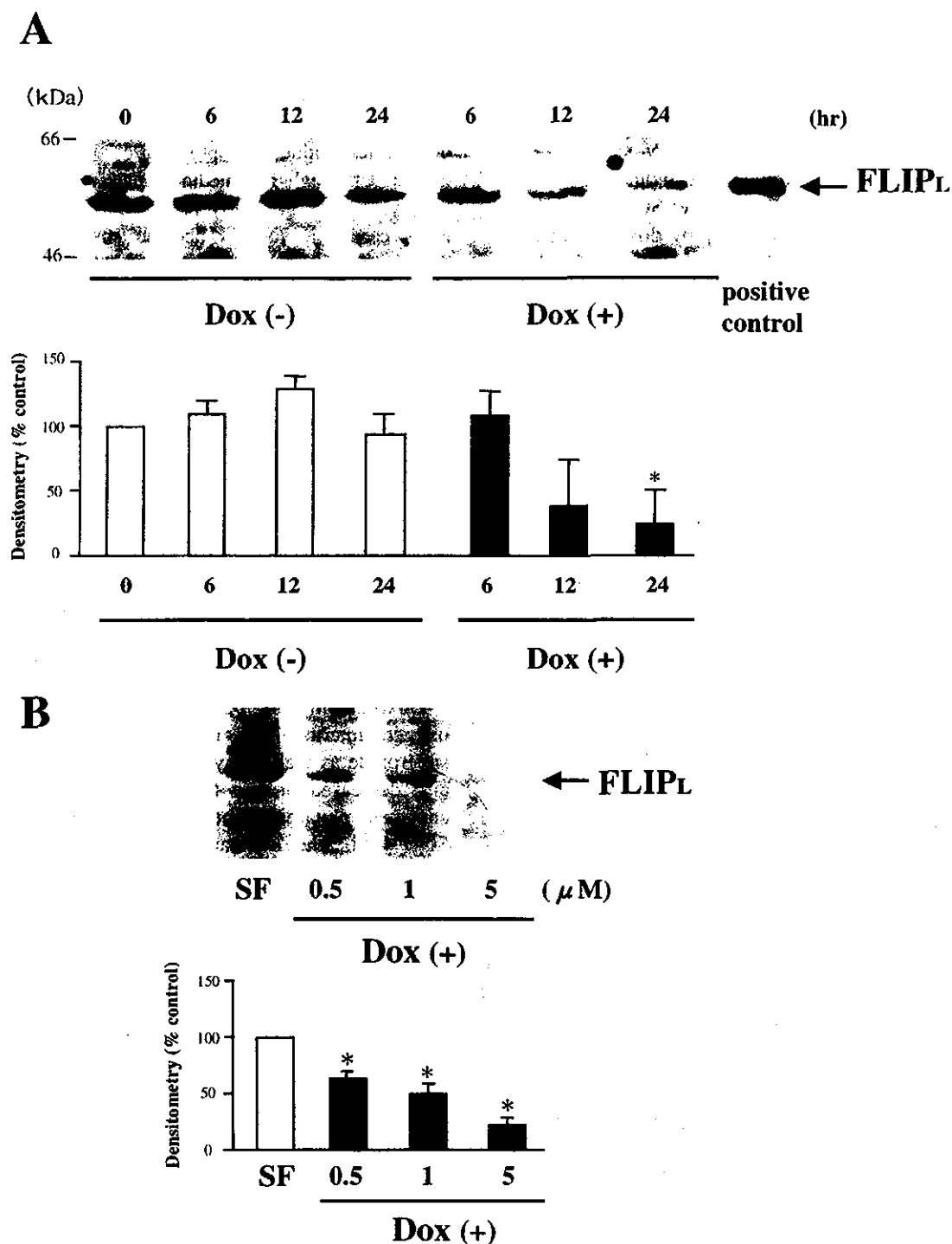


Fig. 2. Expression of FLIP on cardiac myocytes treated with doxorubicin. (A) The expression of FLIP_L (55 kDa) was determined by Western blot analysis in cardiac myocytes incubated with and without doxorubicin (Dox; 0.5 μmol/l) for the times indicated. FLIP_L expression was quantified by densitometry and presented as mean±S.E.M. from three independent experiments; * $P < 0.05$ vs. 0 h without Dox. (B) FLIP_L expressions of cardiac myocytes which were incubated for 24 h with various doses of doxorubicin. FLIP_L expression was quantified by densitometry and presented as mean±S.E.M. from three independent experiments; * $P < 0.01$ vs. serum free (SF).

little information regarding the mechanisms for apoptosis wherein this process occurs in the adult heart. Results from the in vitro study using neonatal cardiomyocytes can not always be applied to the adult intact heart.

One of the most important findings in the present study is the profound inhibition of FLIP expression by reactive oxygen species. Combination of superoxide dismutase (SOD) and catalase, which are antioxidant enzymes hydro-

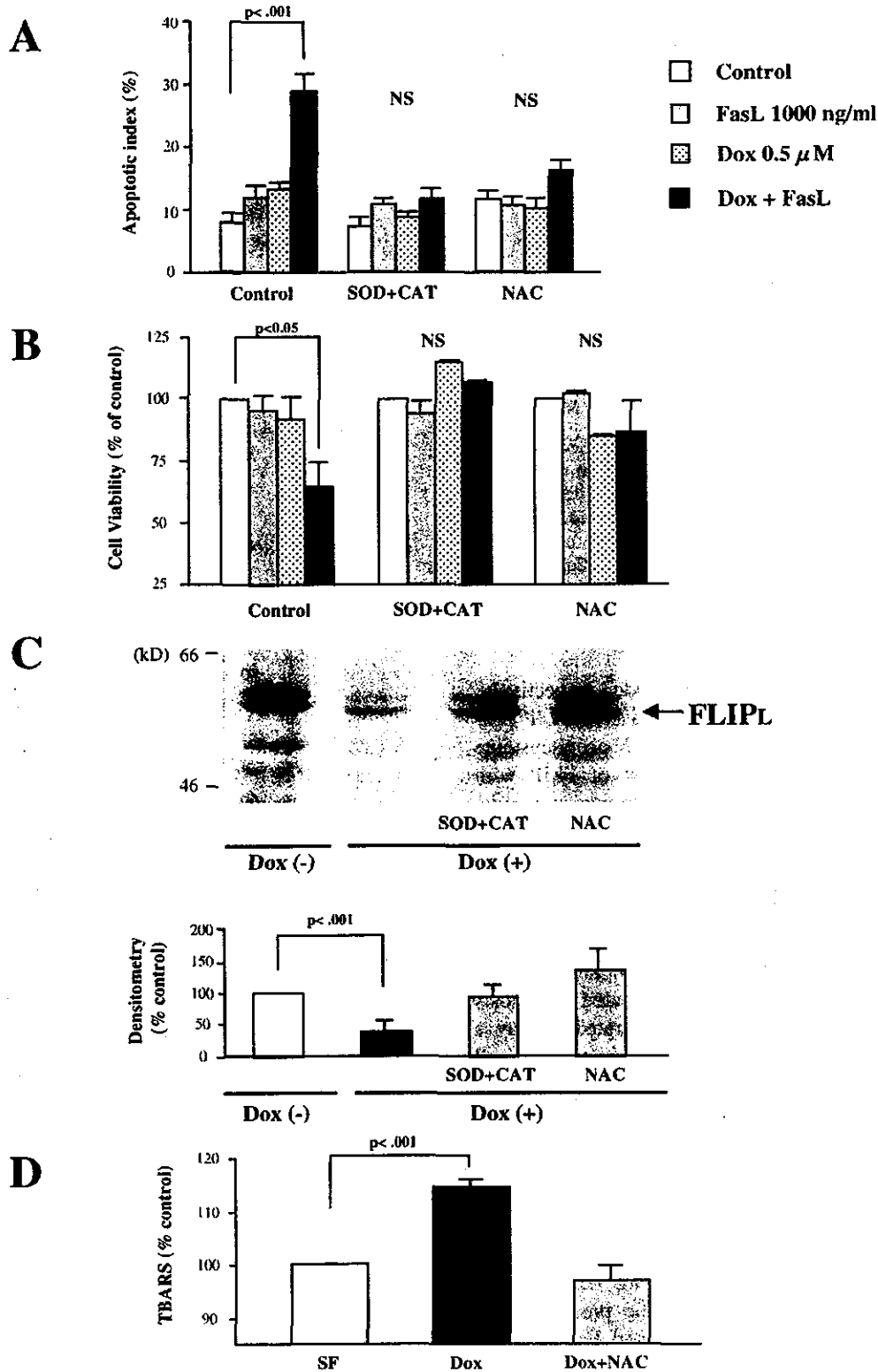


Fig. 3. Antioxidants abolished apoptosis of cardiac myocytes induced by recombinant Fas ligand (FasL) after treatment of doxorubicin (Dox; 0.5 μ mol/l). (A) Cardiomyocyte apoptosis was estimated by TUNEL staining in the presence of *N*-acetylcysteine (NAC) and the combination of superoxide dismutase (SOD) and catalase (CAT). Apoptotic index was calculated as a ratio of apoptotic cells to total cell number. (B) Cell viability was determined by MTT assay in the presence of NAC and the combination of SOD and CAT. Results are expressed as mean \pm S.E.M. of three separate experiments. (C) Antioxidants restored the expression of FLIP_L (55 kDa) in cardiac myocytes treated with Dox (0.5 μ mol/l). FLIP_L levels were determined by Western blot analysis in cardiomyocytes incubated with doxorubicin in the presence of NAC and the combination of SOD and CAT. FLIP_L expression was quantified by densitometry and presented as mean \pm S.E.M. from three independent experiments. (D) Oxidative stress by Dox was assessed by TBARS assay. NAC abolished the increase of MDA formation by Dox. Concentrations were as follows: SOD 500 U/ml, CAT 500 U/ml, and NAC 50 μ mol/l.