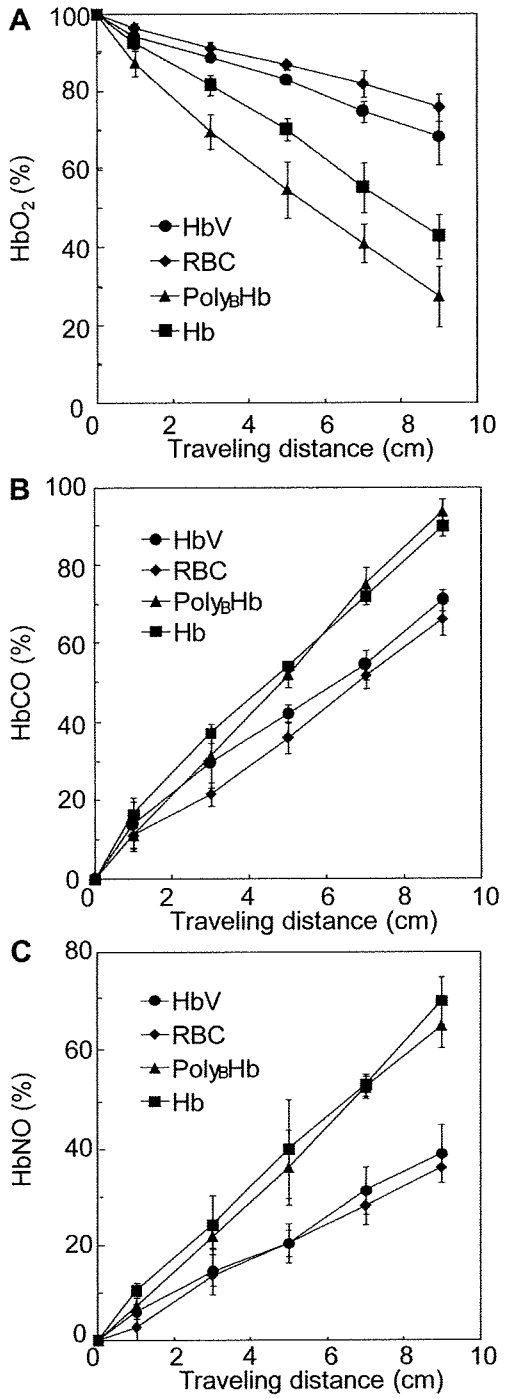

Fig. 4. The change of the level of ligand reactions of the Hb-containing fluids, HbV (●), $Poly_BHb$ solution (▲), Hb solution (■), and RBC (♦) with traveling distance. *A*: levels of O_2 release. *B*: levels of CO binding. *C*: levels of NO binding. The levels of the reactions are calculated from the data in Fig. 3.

cell-free Hb solution distributes homogeneously in the plasma phase and facilitates gas reactions (18, 27). The HbV is larger than these cell-free Hbs, but it is much smaller than RBCs and distributes homogeneously in the tube. However, the HbV suspension showed no such facilitated reactions. In our laboratory's previous paper, we included speculation that the discrepancies were attributable to marked differences in the Hb and HbV sizes, which might influence the diffusion of particles and the stirring effect when the suspension flows in the tubes (39). In the present study, we clarified from the computer simulation a marked difference between Hb and HbV in the diffusion profile. The simulation showed that small Hb diffuse laterally and mix efficiently during the perfusion that facilitated the gas reactions.

Even though the apparent NO-binding rate constant [$k_{on}^{\prime(NO)}$], the apparent CO-binding rate constant [$k_{on}^{\prime(CO)}$], and the apparent O_2-releasing rate constant [$k_{off}^{\prime(O_2)}$] differ significantly between HbV and RBC, as shown in Table 1, the HbV fluid and RBC fluid perfused through the narrow tube showed almost identical reaction profiles. The reaction rate constants were obtained by stopped flow-rapid scan spectrophotometry, where the test solution was extremely diluted, to observe the change of absorption spectroscopy ([Hb] = 0.0024–0.032 g/dl) (34, 42). In the present experiment, the [Hb] values of the test fluids are 10 g/dl, a more practical concentration. The results imply that the size and the diffusion of particles at a high concentration would be more important than the reaction rates of the individual particles. For particles of HbV size and/or larger, including RBCs, the lateral diffusion is considerably small. For flowing RBCs, the peripheral cell-free layer can be a diffusion barrier. The slightly higher viscosity of HbV than RBC would lower the lateral diffusion of HbV. Consequently, no remarkable difference was observed between HbV and RBC in this study. It is particularly interesting that the $k_{on}^{\prime(CO)}$ values of Hb and HbV measured using a stopped-flow spectrophotometer are almost identical. However, in this study, the CO-binding profile differs among the concentrated solutions. This also implies that the lateral diffusiveness of particles is more important in the tube than the individual binding rate constants of the particles in a cuvette. The NO and CO molecules, which diffuse through the tube wall and enter the lumen, would immediately react with Hb-containing solutions at the interface. Therefore, the fast mixing would be effective to create more binding site of these gas molecules. In the case of O_2 release, the O_2 can be removed more easily at the tube wall, where the O_2 concentration gradient is the greatest. The fast mixing would create a higher concentration gradient and fast O_2 transfer.

$Poly_BHb$ is larger than Hb and is expected to show a lower rate of O_2 release. However, the result was that $Poly_BHb$ showed a faster O_2 release rate than Hb. $Poly_BHb$ shows similar viscosity with Hb (1.48 cP for $Poly_BHb$; 1.35 cP for Hb) and contains many nonpolymerized tetrameric $_BHb$ (37.2%). The possible reason is that oxygen affinity of $Poly_BHb$ (54 Torr at 37°C) is much lower than that of human Hb with PLP (26 Torr), and the larger $k_{off}^{\prime(O_2)}$ value of $Poly_BHb$ than that of human Hb. It is expected that the gas reaction rates of cell-free Hb solutions, including $Poly_BHb$, can be reduced by increasing the solution viscosity or particle size and reducing D_i, according to the Stokes-Einstein equation (*Eq. 5*). In addition, an increased fluid viscosity would create a higher shear stress on the vascular wall and increase the production of vasorelaxation factor, such as NO. A combination with a high viscosity hydroxyetyl starch, or a conjugation with polyethylene glycol should be examples to retard the gas reactions and improve microcirculation (7, 54).

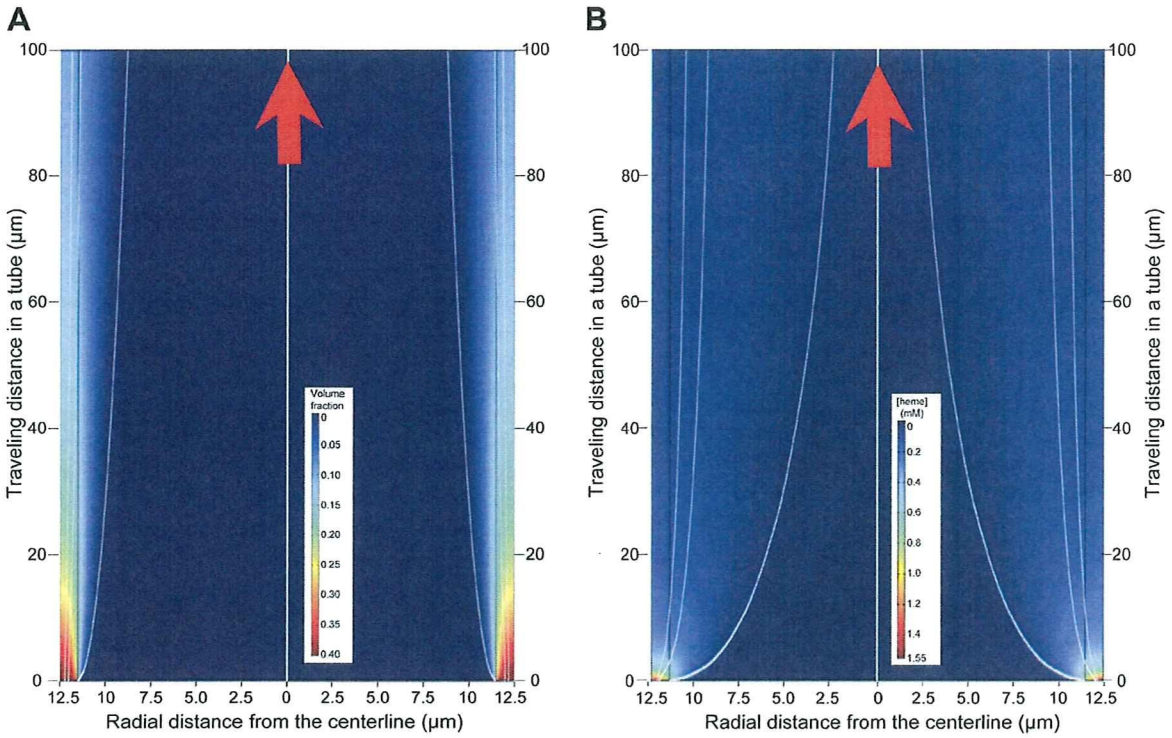

Fig. 5. Schematic representation of the simulated density distribution and track of Hb molecule in a narrow tube (<100 μm traveling distance). We assumed that two different solutions with the same physicochemical properties enter and flow through the same tube. The radius of the tube was 12.5 μm: *component 1* (blue color) enters the core of the tube (radial distance from the centerline, 0–11 μm), and *component 2* (red color) enters near the wall (radial distance from the centerline, 11–12.5 μm). Finally, both components are mixed completely, but the rate is dependent on the physicochemical properties. *A*: diffusivity of HbV particles as *component 2*. The concentration of the particle (HbV) is expressed as volume fraction (volume fraction is 0.4 at Hb concentration, [Hb], = 10 g/dl). *B*: diffusivity of Hb molecules as *component 2*. The color gradation reflects the change of the *component 2* concentration; the white lines represent tracking data of representative particles. The [Hb] is expressed as heme concentration = 1.55 mM at [Hb] = 10 g/dl.

The limitations of our study are that *1*) the gas inlet and outlet of the tube are dependent on the gas permeability of the plastic tube wall and the applied gas concentration in the outer medium; *2*) the inner tube diameter was limited to 25 μm, and the tube length was as long as 12 cm; and *3*) the concentrations of NO and CO are markedly higher than the physiological condition. However, these conditions are necessary to determine the difference using absorption spectrophotometry under a steady flow. A considerable amount of oxygen is known to be released from RBCs at a microvessel level. In this sense, the tube diameter, 25 μm, is appropriate because it is close to the diameter of arterioles that regulate peripheral blood flow in response to the oxygen transfer and other stimuli, such as shear stress and NO concentration. Page et al. (27) measured the O_2-releasing rate using gas-permeable silicone tube (25 μm) in only 4-mm traveling distance and in <1 s. Even though they observed Soret band in the absorption spectroscopy of HBOCs, no spectra was reported in the paper. McCarthy et al. (18) measured the O_2-releasing rate indirectly by observing the reduction of Po_2 from inlet to outlet, without observing the flow patterns in the microscope. Our group is the first to measure the NO- and CO-binding rates of HBOCs in the artificial narrow tube by directly observing the change of absorption spectra.

One question is whether the different reaction profiles between cellular HbV and cell-free Hbs in this study are sufficient to explain the absence or presence of vasoconstriction. We infer the presence of a threshold particle diameter, not only in terms of diffusiveness in the plasma phase, as discussed herein, but also in terms of penetration across the perforated endothelial cell layer to approach a space (such as the space of Disse near the sinusoidal endothelial layer in a hepatic microcirculation, or the space between the endothelium and the smooth muscle). At that space, CO or NO is produced as a vasorelaxation factor to bind to soluble guanylate cyclase, which catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (8, 17, 22, 32, 49). As summarized by Olson et al. (26), both the retardation of the NO reaction (reduced NO affinity) (6) and the larger particle diameter are inferred to be keys to suppression of vasoconstriction and hypertension induced by HBOCs.

Collectively, we clarified that one physicochemical property of the HBOCs, lateral diffusivity in the tube, can explain the differences in the rates of gas binding and releasing reactions that might directly be related to the mechanism of vasoconstriction. The difference would be magnified if we additionally consider the morphological structure of in vivo vascular walls and the effect of extravasation. Several biological mechanisms of vasoconstriction other than gas reactions are reported, such as induction of hypersensitivity of adrenergic receptors and plasma enthothelin-1 increase (9). Our present results cannot explain the physiological responses entirely. Nevertheless, they can at least support previous reports describing the presence and absence of HBOC-induced vasoconstriction related to

gaseous molecules after intravenous administration. In our study, the gas reactions of RBCs are always slowest. RBCs are evolutionally designed to maintain toxic Hb molecules in corpuscles and to retard all of the gas reactions. It is an important physicochemical requirement to design an HBOC.

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DISCLOSURES

H. Sakai, S. Takeoka, and E. Tsuchida are holders of patents on the liposomes (Hb-vesicles).

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Prognostic value of plasma high-sensitivity C-reactive protein levels in Japanese patients with stable coronary artery disease: The Japan NCVC-Collaborative Inflammation Cohort (JNIC) Study

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ABSTRACT

High-sensitivity C-reactive protein (hsCRP) levels can predict cardiovascular events among apparently healthy individuals and patients with coronary artery disease (CAD). However, hsCRP levels vary among ethnic populations. We previously reported hsCRP levels in Japanese to be much lower than in Western populations. We investigated the prognostic value of hsCRP levels in Japanese patients with stable CAD. The hsCRP levels were measured in 373 Japanese patients who underwent elective coronary angiography and thereafter decided to receive only medical treatment. Patients were followed up for 2.9 ± 1.5 years for major cardiovascular events (death, myocardial infarction, unstable angina, stroke, aortic disease, peripheral arterial disease, or heart failure). The median hsCRP level was 0.70 mg/l. During the follow-up, cardiovascular events occurred in 53 (14%) of the 373 patients. Compared with 320 patients without events, 53 with events had higher hsCRP levels (median 1.06 vs. 0.67 mg/l, $P < 0.05$). To clarify the association between hsCRP levels and cardiovascular events, the 373 study patients were divided into tertiles according to hsCRP levels: lower (<0.4 mg/l), middle (0.4-1.2 mg/l), and higher (>1.2 mg/l). The Kaplan–Meier analysis demonstrated a significant difference in the event-free survival rate between higher vs. middle or lower tertiles ($P < 0.05$). In multivariate Cox regression analysis, the hsCRP level of >1.0 mg/l was an independent predictor for cardiovascular events (hazard ratio, 2.0; 95%CI, 1.1–3.4; $P < 0.05$). Thus, in Japanese patients with stable CAD who received only medical treatment, higher hsCRP levels, even >1.0 mg/l, were found to be associated with a significantly increased risk for further cardiovascular events.

1. Introduction

Inflammation has been recognized to play an important role in the development of atherosclerotic disease, such as coronary artery disease (CAD) [1,2]. High-sensitivity C-reactive protein (hsCRP) levels, which are one of the biomarkers of systemic inflammation, have been reported to predict cardiovascular events among apparently

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healthy individuals [3–5] and patients with CAD [6–8]. However, hsCRP levels vary remarkably among different ethnic populations [9,10]. We [11] and others [12,13] reported serum hsCRP levels in Japanese populations to be much lower in comparison to those in Western populations. We also showed plasma hsCRP levels in Japanese patients with stable CAD to be very low [14]. However, the cut-off point of hsCRP levels for high-risk of cardiovascular events in Japanese patients with stable CAD has not been elucidated yet. Recently, the Hisayama Study [15] demonstrated that hsCRP levels were associated with future coronary events in a general population of Japanese, as reported in Western populations. They also showed the hsCRP cut-off point for high-risk of CAD in the Japanese population to be >1.0 mg/l, which is much lower than that for Western populations. The present study therefore investigated the prognostic value of plasma hsCRP levels and the hsCRP cut-off point for high-risk of cardiovascular events in Japanese patients with stable CAD.

2. Methods

2.1. Study patients

We investigated hsCRP levels in 373 consecutive Japanese patients (mean age, 64 ± 9 years; male, 79%) who underwent elective coronary angiography for suspected or known CAD and thereafter decided to receive only medical treatment at the National Defense Medical College Hospital, National Cardiovascular Center, and Kumamoto Medical Center in Japan. Any patients who decided to receive either percutaneous coronary intervention (PCI) or coronary artery bypass surgery (CABG) following angiography were excluded from our study. In addition, any patients with acute coronary syndrome within 3 months, those with a history of PCI within 6 months, those with a history of CABG, those with cardiomyopathies or valvular heart disease, or those with any inflammatory disease or malignancy were excluded. Our study was approved by the institutional ethics committees of the three hospitals. After written informed consent was obtained from all the study patients, blood samples were taken in a fasting state on the morning of the day when coronary angiography was performed. Serum lipid levels were measured by standard laboratory methods. Plasma hsCRP levels were measured using a BNII nephelometer (Dade Behring, Tokyo, Japan). The measurement of hsCRP in the three hospitals was standardized, as we previously reported [16]. The inter-assay and intra-assay coefficients of variation (CV) values were 1.3% and 1.4%, respectively [11]. Of the 373 patients, 211 (57%) had hypertension (blood pressures of ≥140/90 mmHg or on drugs), and 177 (47%) had hyperlipidemia (a total cholesterol level of >220 mg/dl or on drugs), of whom 162 (43%) were taking statins. Diabetes mellitus (a fasting plasma glucose level of ≥126 mg/dl or on insulin or hypoglycemic drugs) was present in 71 (19%) patients, and 139 (37%) were smokers.

2.2. Clinical follow-up for cardiovascular events

On coronary angiograms, CAD was defined as at least one coronary artery having >50% luminal diameter stenosis, and the severity of CAD was represented as the number of >50% stenotic vessels. Of the 373 patients, 269 (72%) were found to have CAD and selected medical treatment over either PCI or CABG after the discussions with the physicians in charge of them. All the study patients were followed up for 2.9 ± 1.5 years for major cardiovascular events (death from all causes, myocardial infarction, need for revascularization procedures for unstable angina, hospitalization for stroke, peripheral arterial disease (PAD) or aortic diseases, or hospitalization for heart failure). This clinical outcome was evaluated by reviewing their medical records and supplemented by a telephone interview with the patients and their family.

2.3. Statistical analysis

Any differences between the two groups were evaluated by the unpaired *t*-test for parametric variables, by the Mann–Whitney *U*-test for nonparametric variables, and by the chi-square test for categorical variables. The correlation between hsCRP levels and the number of stenotic coronary vessels was evaluated by Spearman's rank correlation test. The event-free survival rate in patients with lower, middle and higher tertiles of hsCRP levels was analyzed using the Kaplan–Meier method and was compared by the log-rank test. A multivariate Cox proportional hazards regression analysis was used to identify independent predictors for cardiovascular events. The receiver operating characteristics (ROC) curve analysis was performed to determine the optimal cut-off point of hsCRP levels for cardiovascular events. All the statistical analyses were performed with the SPSS for Windows 11.0.1 software package (SPSS Japan Inc., Tokyo, Japan). A two-sided P value of <0.05 was considered to be statistically significant. The results are presented as the mean value ±SD, except for hsCRP levels that are presented as the median value.

3. Results

Table 1 shows the baseline characteristics of the 373 study patients. Of the 373 patients, 269 (72%) were found to have CAD (>50% stenosis) on coronary angiograms, of whom 124 had 1-vessel disease, 95 had 2-vessel disease, and 59 had 3-vessel disease. The median hsCRP level in the 373 study patients was 0.70 mg/l, and the median hsCRP levels in 269 patients with CAD and 104 without CAD were 0.70 and 0.73 mg/l, respectively. The hsCRP levels did not significantly correlate with the number of >50% stenotic coronary vessels (P = NS). Moreover, 162 (43%) patients were taking statins, and 83 (22%) had a history of myocardial infarction.

During the follow-up of 2.9 ± 1.5 years, major cardiovascular events occurred in 53 (14%) of the 373 study patients (6 death, 3 myocardial infarction, 29 unstable angina, 11 stroke, 2 PAD/aortic disease, and 2 heart failure). Compared with 320 patients without events, 53 with cardiovascular events were older (68 ± 7 vs. 63 ± 9 years) and had higher rates of hypertension (72% vs. 54%) and CAD (92% vs. 69%) (P < 0.05) (Table 1). The number of >50% stenotic coronary vessels was greater in patients with events than in those without events (1.69 ± 0.89 vs. 1.17 ± 1.01, P < 0.001). Notably, patients with events had higher hsCRP levels than those without events (median 1.06 vs. 0.67 mg/l, P < 0.05).

To clarify the association between hsCRP levels and cardiovascular events, the 373 study patients were divided into tertiles according to hsCRP levels: lower (<0.4 mg/l), middle (0.4-1.2 mg/l), and higher (>1.2 mg/l) tertiles. The Kaplan–Meier analysis demonstrated a significant difference in the event-free survival rate for patients in the higher tertile compared with those in the middle or lower tertiles (P < 0.05), but no significant difference was observed in event-free survival rate between the middle and lower tertiles (P = NS) (Fig. 1). In a multivariate Cox proportional hazards analysis, compared with those in the lower tertile, patients in the middle tertile had an adjusted hazard ratio (HR) of 0.9 (95%CI, 0.4–2.1, P = NS), and patients in the higher tertile had an adjusted HR of 2.3 (95%CI, 1.3–4.0, P < 0.01). To determine the optimal cut-off point of hsCRP levels, the ROC curve analysis was performed. As shown in Fig. 2, the optimal cut-off point of hsCRP was found to be around 0.95 mg/l. Because the Centers for Disease Control and Prevention and the American Heart Association categorized patients by the hsCRP cut-off points of <1.0, 1.0–3.0, and >3.0 mg/l into low-, moderate-, and

Table 1
Clinical characteristics of patients with and without cardiovascular events.

	All (n = 373)	Events (+) (n = 53)	Events (−) vs. (+)	Events (−) (n = 320)
Age (years)	64 ± 9	68 ± 7	<0.001	63 ± 9
Gender (male)	294 (79%)	42 (79%)	NS	252 (79%)
Hypertension	211 (57%)	38(72%)	<0.05	173(54%)
Systolic blood pressure (mmHg)	132 ± 18	140 ± 18	<0.01	131 ± 18
Hyperlipidemia	177 (47%)	27(51%)	NS	150(47%)
Total cholesterol (mg/dl)	200 ± 33	203 ± 33	NS	199 ± 34
HDL-cholesterol (mg/dl)	51 ± 15	50 ± 14	NS	51 ± 15
Statin use	162(43%)	25(47%)	NS	137(43%)
Diabetes mellitus	71 (19%)	14(26%)	NS	57(26%)
HbA1c (%)	5.6 ± 1.0	6.0 ± 1.3	<0.01	5.6 ± 0.9
Obesity	139(37%)	23(43%)	NS	116(36%)
Smoking	251(67%)	38(72%)	NS	213(67%)
CAD	269(72%)	49(92%)	<0.001	220(69%)
1-vessel disease	124(33%)	17(32%)		107(33%)
2-vessel disease	95(25%)	22(42%)		73(23%)
3-vessel disease	50(13%)	10(19%)		40(13%)
The number of >50% stenotic coronary vessels	1.24 ± 1.01	1.69 ± 0.89	<0.001	1.17 ± 1.01
History of myocardial infarction	83(22%)	13(25%)	NS	70(22%)
HsCRP (mg/l)	0.70	1.06	<0.05	0.67

Data are presented as the mean value ±SD or the number (%) of patients, except for the hsCRP levels that are presented as the median value. Obesity was defined as a body mass index of >25.0.

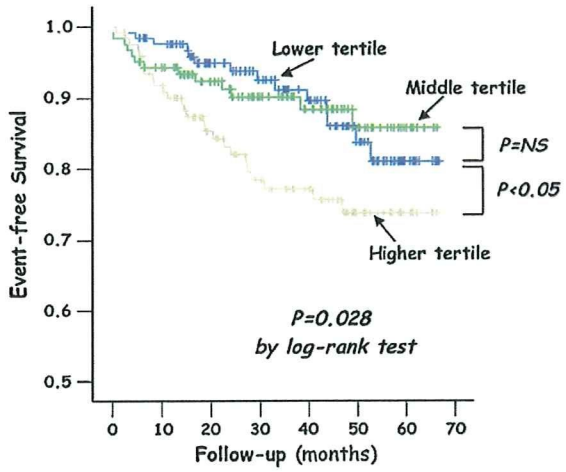

Fig. 1. Event-free survival from major cardiovascular events. The Kaplan–Meier analysis demonstrated a lower event-free survival rate in patients in the higher hsCRP tertile (>1.2 mg/l) than those in the middle (0.4–1.2 mg/l) and lower (<0.4 mg/l) tertiles (P < 0.05), but no significant difference was observed in event-free survival rate between the middle and lower tertiles (P = NS).

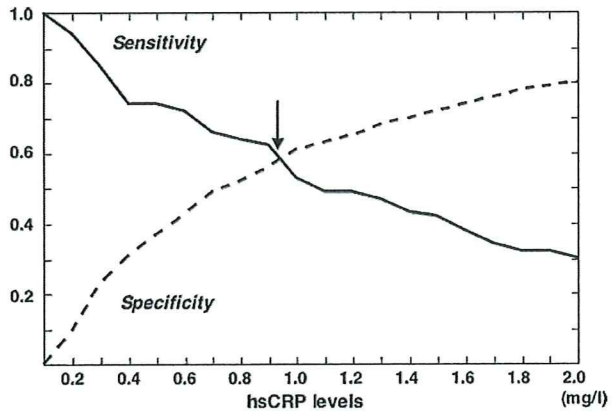

Fig. 2. Receiver operating characteristics (ROC) curves of cut-off points for hsCRP levels. The ROC curves indicated that the optimal cut-off point of hsCRP for major cardiovascular events was around 0.95 mg/l.

high-risk categories [32], we decided to use >1.0 mg/l as the hsCRP cut-off point. In a multivariate Cox proportional hazards analysis, an hsCRP level of >1.0 mg/l as well as age and the number of stenotic coronary vessels were found to be independent predictors for cardiovascular events (Table 2). The HR for cardiovascular events was 2.0 (95%CI, 1.1–3.4) for the hsCRP level of >1.0 mg/l (P < 0.05).

4. Discussion

The present study investigated the prognostic value of hsCRP levels in 373 Japanese patients who underwent elective coronary angiography. As a result, in Japanese patients with stable CAD who had only medical treatment, higher hsCRP levels, even >1.0 mg/l, were found to be associated with a significantly increased risk for further cardiovascular events.

Many studies have reported hsCRP levels to predict cardiovascular events among patients with CAD [6–8,17–28] as well as apparently healthy individuals [3–5]. However, as shown in Table 3, most of studies reporting the prognostic values of hsCRP in patients with CAD included a mixture of patients with unstable angina and those with stable angina [6–8,17–22]. Since patients with unstable angina are known to have higher hsCRP levels than those with sta-

Table 2
Factors associated with major cardiovascular events (Multivariate Cox proportional hazards analysis in the 373 study patients).

Variables	Hazard ratio (95%CI)	P value
hsCRP level >1.0 mg/l	2.0 (1.1–3.4)	<0.05
Number of stenotic coronary vessels	1.5 (1.1–2.0)	<0.01
Age (10-year increase)	1.9 (1.3–2.7)	<0.01

The dependent variable was major cardiovascular events. This analysis included age, gender, hypertension, hyperlipidemia, diabetes, smoking, body mass index, the number of >50% stenotic coronary vessels, statin, antiplatelet drugs, ARB/ACEI, and hsCRP >1.0 mg/l.

Table 3
Reported hsCRP levels for high-risk of cardiovascular events in patients with CAD.

Study	Reference numbers	Study patients	Treatment strategy	Endpoints	hsCRP
Unstable or stable angina					
Liuzzo et al.	[6]	31 Patients with UAP	Medically or PCI	Death or MI	>3.0 mg/l
Haverkate et al.	[7]	2121 Patients with UAP or SAP	Medically, PCI, or CABG	Death or MI	>3.6 mg/l
Zebrack et al.	[8]	2554 Patients with UAP or SAP	Medically, PCI, or CABG	Death or MI	>10.0 mg/l
Khor et al.	[17]	2254 Patients with UAP or SAP	Medically, PCI, or CABG	Death or MI	>12.0 mg/l
Horne et al.	[18]	985 Patients with UAP, AMI, or SAP	Medically, PCI, or CABG	Death	>12.0 mg/l
Buffon et al.	[19]	121 Patients with UAP or SAP	PCI (POBA)	Cardiovascular events*	>8.0 mg/l
de Winter et al.	[20]	1458 Patients with UAP or SAP	PCI (POBA or stenting)	Death or MI	>7.0 mg/l
Walter et al.	[21]	276 Patients with UAP, AMI, or SAP	PCI (stenting)	Cardiovascular events*	>5.0 mg/l
Zairis et al.	[22]	483 Patients with UAP, AMI, or SAP	PCI (stenting)	Death, MI, or UAP	>6.8 mg/l
Stable CAD					
Leu et al.	[23]	75 Non-DM patients with stable CAD	Medically	Cardiovascular events**	>1.0 mg/l
Sabatine et al.	[24]	3771 Patients with stable CAD	Medically	Death, MI, or stroke	>1.0 mg/l
Speidl et al.	[25]	124 Patients with stable CAD (<50 years)	Medically or PCI	Cardiovascular events***	>1.6 mg/l
Ikonomidis et al.	[26]	100 Patients with stable CAD	Medically, PCI, or CABG	Death, MI, or UAP	>2.5 mg/l
de Winter et al.	[27]	501 Patients with stable CAD	PCI (POBA or stenting)	Death, MI, or UAP	>3.0 mg/l
Dibra et al.	[28]	1152 Patients with stable CAD	PCI (stenting)	Death or MI	>5.0 mg/l

The hsCRP level indicates the cut-off point for high-risk of cardiovascular events. UAP, SAP, AMI, and POBA indicate unstable angina, stable angina, acute myocardial infarction, and balloon angioplasty.

* Cardiovascular events: death, MI, or coronary revascularization.

** Cardiovascular events: death, MI, coronary revascularization, UAP, stroke, or peripheral artery disease.

*** Cardiovascular events: death, MI, coronary revascularization, or UAP.

ble angina [6,29], the reported cut-off points of hsCRP for high-risk of cardiovascular events seem to be high in a mixture of patients with unstable and stable angina. In contrast, the cut-off point of hsCRP for high-risk was relatively low in patients with stable CAD, especially in those who selected only medical treatment over either PCI or CABG [23–28]. Treatment strategies, medical treatment or PCI/CABG, would also affect hsCRP cut-off points for risk stratification. Recently, Sabatine et al. [24] have reported an elevated hsCRP level, even >1.0 mg/l, to be a significant predictor of cardiovascular events in 3771 patients with stable CAD who had only medical treatment. Leu et al. [23] also showed an hsCRP level of >1.0 mg/l to be a predictor of cardiovascular events in 75 non-diabetic patients with stable CAD who had only medical treatment. In the present study, we investigated hsCRP levels in patients with stable CAD who selected only medical treatment over either PCI or CABG and demonstrated that an hsCRP level of >1.0 mg/l was associated with a significantly increased risk for further cardiovascular events in Japanese patients with stable CAD. Hence, the cut-off point of hsCRP for high-risk of cardiovascular events in patients with stable CAD who had only medical treatment would be >1.0 mg/l in Japanese as well as in other ethnic populations.

It is well known that hsCRP levels vary remarkably among different ethnic populations [9,10]. A twin study demonstrated hsCRP levels to have a moderate degree of heritability [30], and some CRP gene polymorphisms were reported to influence hsCRP levels [31]. These suggest that some genetic factors may contribute to ethnic differences in hsCRP levels. The hsCRP levels in Japanese populations have been reported to be much lower than those in Western populations [11–13]. In the present study, the median hsCRP level in Japanese patients with stable CAD was 0.70 mg/l, which was much lower than those in other ethnic patients with stable CAD in the studies of Sabatine et al. [24] and Leu et al. [23] (1.71 and 1.02 mg/l). The hsCRP levels in Japanese patients with CAD seem to be lower than those in other ethnic patients with CAD. Therefore, the cut-off point of hsCRP for high risk in patients with stable CAD should be much lower in Japanese than in other ethnic patients. However, in patients with stable CAD who selected only medical treatment over either PCI or CABG, the hsCRP level of >1.0 mg/l was found to be a significant predictor of further cardiovascular events in the present study as well as in both studies of Sabatine et al. [24] and Leu et al. [23]. The Centers for Disease Control and Prevention and the American Heart Association categorized patients by the hsCRP cut-off points of <1.0, 1.0–3.0, and >3.0 mg/l into low-, moderate-, and high-risk categories, respectively [32]. Therefore, in patients with stable CAD who received only medical treatment, the hsCRP level of >1.0 mg/l, which corresponds to the moderate-risk category, was found to be associated with a significantly increased risk for cardiovascular events in Japanese as well as in other ethnic populations. The hsCRP cut-off points should be determined in each patient group depending on unstable or stable CAD, treatment strategies (medical treatment or PCI/CABG), and ethnics.

Our study was not without limitations. First, our study was not a randomized trial, and, in our study patients, their medical treatment was selected over either PCI or CABG after the discussions with the physician in charge. These may have caused some selection bias and have confounded our results. Second, the number of our study patients was relatively small (373 patients), and only 53 patients had cardiovascular events. However, the sample size of our study (1082 person-years of follow-up) was found to be enough to detect a 2-fold higher risk of cardiovascular events in patients with high hsCRP level (>1.0 mg/l) with a statistical power of 80% and a alpha value of 0.05, because 935 person-years were estimated as the adequate size with the event rate of 0.049 per person-year in the present study. Finally, our study had only patients with stable CAD who received only medical treatment. Our study excluded any patient who received PCI or CABG following angiography. Therefore, our results cannot be applicable to patients who received PCI or CABG. To determine the prognostic value of pre-procedural hsCRP levels in Japanese patients with stable CAD undergoing PCI, another study is needed.

In conclusion, the present study showed that hsCRP levels in Japanese patients with stable CAD were low in comparison to those in Western populations. However, in patients with stable CAD who received only medical treatment, higher hsCRP levels, even >1.0 mg/l, were found to be associated with a significantly increased risk for further cardiovascular events in Japanese patients, as reported in other ethnic patients.

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Soluble Elastin Decreases in the Progress of Atheroma Formation in Human Aorta

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Background: The serum levels of soluble elastin increase in patients with aortic dissection, but its distribution and characteristics are unclear.

Methods and Results: The 173 aortic specimens were categorized into 4 groups under microscopy (non-atherosclerotic aorta, n=13; fiber-rich plaque, n=77; lipid-rich plaque, n=66; ruptured plaque, n=17). Soluble elastin was abundant within the intima of both the non-atherosclerotic aorta and fiber-rich plaque, rather than in the media, and was decreased within the intima of lipid-rich and ruptured plaques. Soluble elastin levels decreased with progress of atherosclerosis (6.0±0.3μg/mg protein in non-atherosclerotic aorta; 5.8±0.2μg/mg protein in fiber-rich plaque; 4.9±0.2μg/mg protein in lipid-rich plaque; 2.8±0.4μg/mg protein in ruptured plaque, P<0.05). As well, both matrix metalloprotease-9 activity and elastin mRNA expression showed inverse distribution against soluble elastin (r=−0.437, P<0.0001; r=−0.186, P<0.05, respectively). Multivariable analysis revealed a decrease in the level of soluble elastin in ruptured plaque (2.8±0.4μg/mg protein in ruptured plaque, n=18; 5.5±0.2μg/mg protein in non-ruptured plaque, n=155, P<0.01). Furthermore, western blot showed soluble elastin consists of heterogeneous molecular pattern proteins.

Conclusions: Both the synthesis and degradation of elastin may be enhanced in active atherosclerotic lesions. (*Circ J* 2009; **73:** 2154–2162)

Key Words: Atherosclerosis; Elastin; Pathology; Plaque

Atherosclerosis is a progressive disease characterized by the accumulation of lipids, foamy macrophages, vascular smooth muscle cells, and extracellular matrix, leading to the formation of plaques.[1] The pathobiological process of plaque rupture is highly complex, multifactorial, and not yet fully understood. Nevertheless, ruptured plaques have several histologically distinct features: a thin fibrous cap, a prominent collection of lipid, and abundant macrophages.[2–6]

Elastin is a major structural extracellular matrix constituent of the arterial wall. Mature elastin is composed of elastin subunits that are intermolecularly cross-linked into a fibrous network that creates a highly polymerized insoluble protein with elasticity, tensile strength, and stability.[7] Accelerated elastin degeneration and synthesis is apparently observed in atherosclerosis.[8–10] With regard to elastolysis, several matrix metalloproteases (MMPs), particularly MMP-9, are already known to be activated in the progress of atherosclerosis.[11,12] Lutton et al reported the potential role of MMP-9 in elastin formation in atherosclerosis using knockout mice.[13] Thus, the degradation of mature elastin may occur in the advanced stages of atherosclerosis, presumably via the action of specific proteolytic enzymes, including MMP-9.[12,13] Indeed, alteration and/or degradation of mature elastin may lead to arterial remodeling and modification of its mechanical properties, and may play a critical role in the progress of atherosclerosis.[12,14,15] However, it has been difficult to detect fragmentation of elastins in arterial tissues by immunohistochemistry or western blot analysis because antibody specifically corresponding to soluble elastin antigens has not been available.

Recently, we established mouse monoclonal IgG antibodies (HASG monoclonal antibodies (mAbs)) that can efficiently react with soluble elastin fragments in human biological fluids.[16] Using a sandwich enzyme-linked immunosorbent assay (ELISA) with 2 different HASG mAbs (clone nos. HASG-61-1 and HASG-30), we have demonstrated that the serum concentration of elastin-associated proteins that react with HASG mAbs gradually increases with aging in normal human subjects, and that there is a marked increase in patients with open-type acute aortic dissection.[17] Those results indicated the potential role of the elastin-associated proteins that react with HASG mAbs (namely, 'soluble elastin') as a new marker of elastin degradation and synthesis in human aortic specimens; however, as yet, pathologic investigation of soluble elastin has not been performed.

Therefore, the aim of the present study was to investigate the biochemical and topographical characteristics of soluble elastin in specimens of human aorta at different stages of

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atherosclerosis. To this end, we examined (1) the morphological characteristics of soluble elastin, (2) the relationship of the distribution patterns of soluble elastin and elastin mRNA expression, (3) the relationship between the level of soluble elastin and the histological features of atherosclerotic plaque, and (4) the relationship between the soluble elastin level and MMP-9 activity in atherosclerotic plaque.

Methods

Tissue Specimens

We obtained 173 fresh specimens of human aortic tissue from 9 autopsies (8 men, 1 woman; 64.0±3.4 years, range 53–84) within 12 h post mortem. The subjects consisted of 4 cancer cases and 5 non-cancer cases. The aorta was opened longitudinally, and each specimen was cut into 3 sections: the central section was reserved for histology, and the 2 adjacent sections were frozen in liquid nitrogen and stored at –80°C until use in protein assay and reverse transcriptase polymerase chain reaction (RT-PCR) study. The institutional review board approved the research protocol (approval no. 263).

Classification of Plaques

The specimens were diagnosed into 8 groups (types I, II, III, IV, Va, Vb, Vc, and VI) according to the classification issued by the Committee on Vascular Lesions set up by the Council on Atherosclerosis of the American Heart Association (AHA classification).[18] Briefly, type I has an adaptive intimal thickening without accumulated foamy macrophages; type II has accumulated foamy macrophages within the intima without extracellular lipid deposition; type III has accumulated foamy macrophages with small extracellular lipid depositions and necrotic tissue within the intima (lipid constituents); type IV has large amounts of lipid constituents within the intima, and has a superficial, thin fibrous cap; type Va has large amounts of lipid constituents within the intima, but has a superficial, thick fibrous cap; type Vb has large amounts of lipid constituents, but also has mineral deposition within the intima; type Vc has large amounts of vascular smooth muscle cells, collagen, and elastin fibers (fibrocellular constituents) instead of lipid constituents in the intima; type VI exhibits intraplaque hemorrhage, fibrin deposition, fissure of the fibrous cap or thrombus formation, in addition to the accumulation of lipid constituents in the intima.

After diagnosis based on the AHA classification, we further categorized each specimen into 4 groups:[19,20] 'non-atherosclerotic aorta' (type I), 'fiber-rich plaque' (types II, III, and Vc), 'lipid-rich plaque' (types IV, Va, and Vb), and 'ruptured plaque' (type VI). The intima of the fiber-rich plaque is basically composed of fibrocellular constituents, whereas that of the lipid-rich plaque is composed of lipid constituents.

In addition, each specimen was evaluated for the presence of 7 histological features, previously shown to be associated with plaque instability: plaque necrosis, macrophage infiltration into the fibrous cap, thin fibrous cap <65μm in thickness, intraplaque fibrin, intraplaque hemorrhage, plaque rupture, and mineral deposition.[1,3,4,20,21] Fibrin deposition or hemorrhage was defined as a red-colored non-structural substance or aggregations of red blood cells outside the intimal microvessels using the Masson trichrome procedure. All evaluations were performed more than 3 times independently by 2 observers who were blinded to the results of the biochemical analyses.

mAbs Against Solubilized Elastin

The 2 mouse mAbs (clone nos. HASG-61-1 and HASG-30) established according to standard hybridoma technology against highly purified human aortic soluble elastin (Elastin Products Co, Owensville, MO, USA) were used in the present study. To verify the specificity of each mAb against human soluble elastin antigen in absorption experiments, the tissue sections to be used for immunohistochemistry were treated with primary antibodies in the presence of antigen peptides (data not shown). These 2 mAbs display little cross-linkage with other fibers of associated proteins such as desmosin, collagens, fibrillin or microfibrils.[16]

Tissue Extraction for Protein Analysis

Specimens were weighed, crushed using scissors, and homogenized using ice-cold glass homogenizers into 10 volumes of extraction buffer[11] containing 10 mmol/L sodium phosphate (pH 7.2), 500 mmol/L sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.2% sodium azide, 2 mmol/L EDTA, and a designed protease inhibitor (1 tab/10 ml of extraction buffer; Complete Mini, Roche Diagnostics GmbH, Roche Applied Science, Penzberg, Germany). The protein level of each sample was determined using a BC protein assay system (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA for Soluble Elastin

The levels of soluble elastin in the protein extracts were measured by sandwich ELISA utilizing the 2 mAbs (HASG-61-1 and HASG-30) as previously described.[17] The standard curve was obtained using 8 points for concentrations ranging from 0.69 to 500 ng/ml of soluble human elastin fragments (Elastin Products Co). The minimum detection limit for soluble elastin concentration was 0.5 ng/ml.

MMP-9 Activity

The level of MMP-9 activity in the protein extracts was measured using an MMP-9 activity assay system kit (RPN2634; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, 100μl of protein extract was embedded in an immobilized 96-well microplate and incubated overnight at 4°C. After washing, 50μl of either p-aminophenylmercuric acetate solution or assay buffer was added to wells containing the standard or the protein extract, respectively. After incubation for 1.5 h at 37°C, 50μl of the detection reagent was embedded in all wells and the reaction was measured at 405 nm after 1 h of incubation.

Histological Staining

After fixation in 10% phosphate-buffered formalin (pH 7.3) for 24 h, histological specimens were paraffin-embedded, sectioned, and stained using hematoxylin–eosin, elastica van Gieson (EVG) or Masson trichrome.

Immunohistochemistry

Deparaffinized formalin-fixed sections were stained using the polymer–peroxidase method (EnVision+/HRP; DAKO Cytomation, Glostrup, Denmark) with mouse mAbs (CD68 for macrophages [Dako Cytomation; diluted 1:200], α-smooth muscle actin for vascular smooth muscle cells [Dako Cytomation; diluted 1:200], HASG-61-1 or HASG-30 for soluble elastin, each at a final concentration of 0.4μg/ml). In the absorption experiment, tissue sections were treated with each primary antibody in the presence of antigen

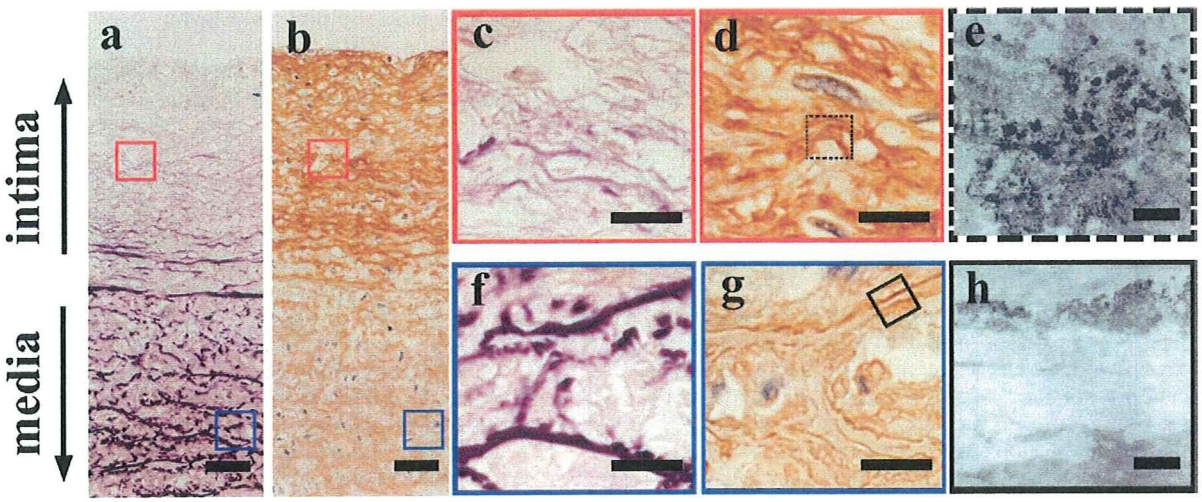

Figure 1. Pathologic characteristic of soluble elastin in non-atherosclerotic aorta. Elastica van Gieson stain (**a**, **c**, **f**), immunohistochemistry for soluble elastin with HASG-61-1 (**b**, **d**, **g**), and immunoelectron microscopy for soluble elastin with HASG-61-1 (**e**, **h**) in whole arterial wall (**a**, **b**), intima (**c–e**), and media (**f–h**) of non-atherosclerotic aorta. Scale bars: **a**, **b** = 50 μm; **c–f** = 10 μm; **g**, **h** = 500 nm. HASG, mouse monoclonal IgG antibody.

peptide (200 μg/ml). For the negative control, the incubation step with the primary antibody was omitted.

Immunoelectron Microscopy

Immunohistochemical electromicroscopic analysis was performed as previously described.[22] The sections were incubated with HASG-61-1 and with HRP-Linked F(ab')2 antibody (1:30; Amersham Bioscience) for 48 h at 4°C.

Total RNA Extraction and Semi-Quantitative RT-PCR for Elastin mRNA

RT-PCR was performed as previously described, using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied Biosystems, Alameda, CA, USA) with human elastin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers.[23] For elastin, the primers and TaqMan probe used were: 5'-GGC AAT TCC TGG AAT TGG A-3' (sense), 5'-TGC TTC TGG TGA CAC AAC CC-3' (antisense), and 5'-CAT CGC AGG CGT TGG GAC TCC-3' (TaqMan probe). The cDNA amplification product was predicted to be a 256-bp fragment from position 861–1116 in the cDNA of human elastin (GenBank M36860). The reaction master mix contained 900 nmol/L elastin primers and 200 nmol/L TaqMan probe.

In Situ Hybridization (ISH) for Elastin mRNA

ISH for elastin mRNA using deparaffinized formalin-fixed sections was performed as previously described.[23] The human elastin cDNA probe used was a 430-bp fragment (obtained from positions 298–727 in the cDNA of human elastin) subcloned into the EcoR I site of a pGEM-T Easy Vector (Promega, Madison, WI, USA). Antisense probes and corresponding sense probes were labeled with biotin using SP6 and T7 polymerases, respectively (RNA labeling kit; Boehringer Mannheim, Mannheim, Germany). Hybridization was performed overnight at 42°C in 50% (vol/vol) deionized formamide, 5×Denhardt's solution, 5% (W/vol) dextran sulfate, 2×SSC, 0.3 mg/ml human placenta DNA (D3287; Sigma-Aldrich), 20 mmol/L EDTA, and 2 μg/ml biotin-labeled probes.

Western Blot Analysis

Equal amounts (50 μg) of protein extracts were separated by 7.5% SDS-PAGE, and transferred to polyvinylidene difluroride membranes (Hybond-N+; Amersham Biosciences, Piscataway, NJ, USA) as described previously.[23] Each mAb (clone no. HASG-61-1 or HASG-30) was used at a final concentration of 0.4 μg/ml. In the absorption experiment, the transferred membrane was treated with each primary antibody in the presence of antigen peptide (200 μg/ml).

Statistical Analysis

Data are presented as mean±SEM. Comparisons between 2 groups were performed using the Mann-Whitney U-test. Comparisons among 4 groups were performed using ANOVA with a post-hoc Games-Howell test. Multivariable analyses were performed using multiple regression analysis. Differences were considered significant at $P<0.05$.

Results

Characteristics of Soluble Elastin in the Non-Atherosclerotic Aorta

The EVG staining and immunohistochemistry for soluble elastin using serial cross-sections and immunoelectron-microscopic analyses of non-atherosclerotic aortic specimens are shown in **Figures 1a–h**. Soluble elastin was present both within the intima (**Figures 1b, d**) and around mature elastin within the media (**Figures 1b, g**), showing that soluble elastin is distributed both within the extracellular matrix of the intima (**Figures 1a–e**) and around mature elastin within the media (**Figures 1a, b, f–h**). Soluble elastin appeared to be more abundant in the intima than in the media, and also appeared to be different from that of mature elastin itself (**Figures 1b, d, e, g, h**).

Distribution Patterns of Soluble Elastin in Plaques

Each specimen was diagnosed as 1 of 8 groups: type I, n=13; type II, n=34; type III, n=29; type IV, n=21; type Va, n=23; type Vb, n=22; type Vc, n=14; type VI, n=17. The specimens were then categorized as non-atherosclerotic

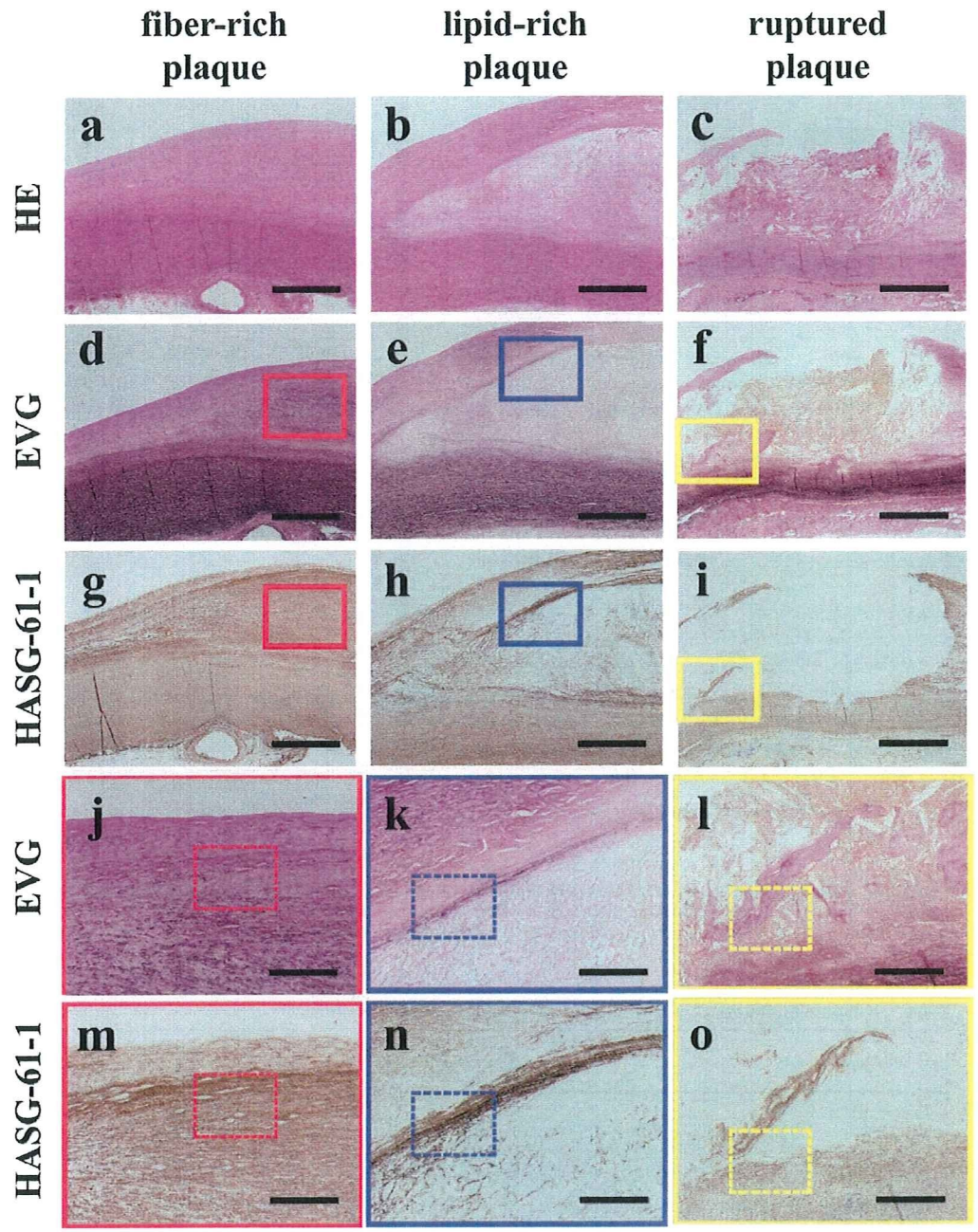

Figure 2. Pathological characteristics of soluble elastin in advanced atherosclerotic plaques. Hematoxylin–eosin stain (HE; **a–c**), elastica van Gieson stain (EVG; **d–f, j–l**), immunohistochemistry with HASG-61-1 (**g–i, m–o**). (**j–o**) High-power view of insets shown in **d–i**. Scale bars: **a–i**=1 mm; **j–o**=250 μm. HASG, mouse monoclonal IgG antibody.

aorta (n=13), fiber-rich plaque (n=77), lipid-rich plaque (n=66) or ruptured plaque (n=17).

Figure 2 is representative images of advanced atherosclerotic plaques (fiber-rich plaque in **a, d, g, j, m**; lipid-rich plaque in **b, e, h, k, n**; ruptured plaque in **c, f, i, l, o**). In the advanced atherosclerotic aorta, soluble elastin was also dense in the intima rather than in the media, as in non-atherosclerotic aorta, and was noted to be dense within the extracellular matrix of the intima in the fiber-rich plaque (**Figure 2g**) and in the fibrous cap of lipid-rich plaques and ruptured plaques (**Figures 2h, i**). As well, the immunoreactivity for soluble elastin within the intima was weaker in the core of both lipid-rich plaques (**Figure 2h**) and ruptured plaques (**Figure 2i**) compared with the fiber-rich plaque (**Figure 2g**). A focal area of strong staining was noted in the intima of the fiber-rich plaque (**Figure 2g**, inset high-power view: **Figure 2m**), and in the rim of the intima adjacent to the atheroma formation in lipid-rich plaque (**Figure 2h**, inset; high-power view: **Figure 2n**). The areas densely positive for soluble elastin mostly coincided with those stained with resorcin fuchsin with EVG (**Figures 2d, e, j, k**). In addition, there was less stained with resorcin fuchsin and with HASG-61-1 in the ulceration in ruptured plaque (**Figures 2f, i, l, o**).

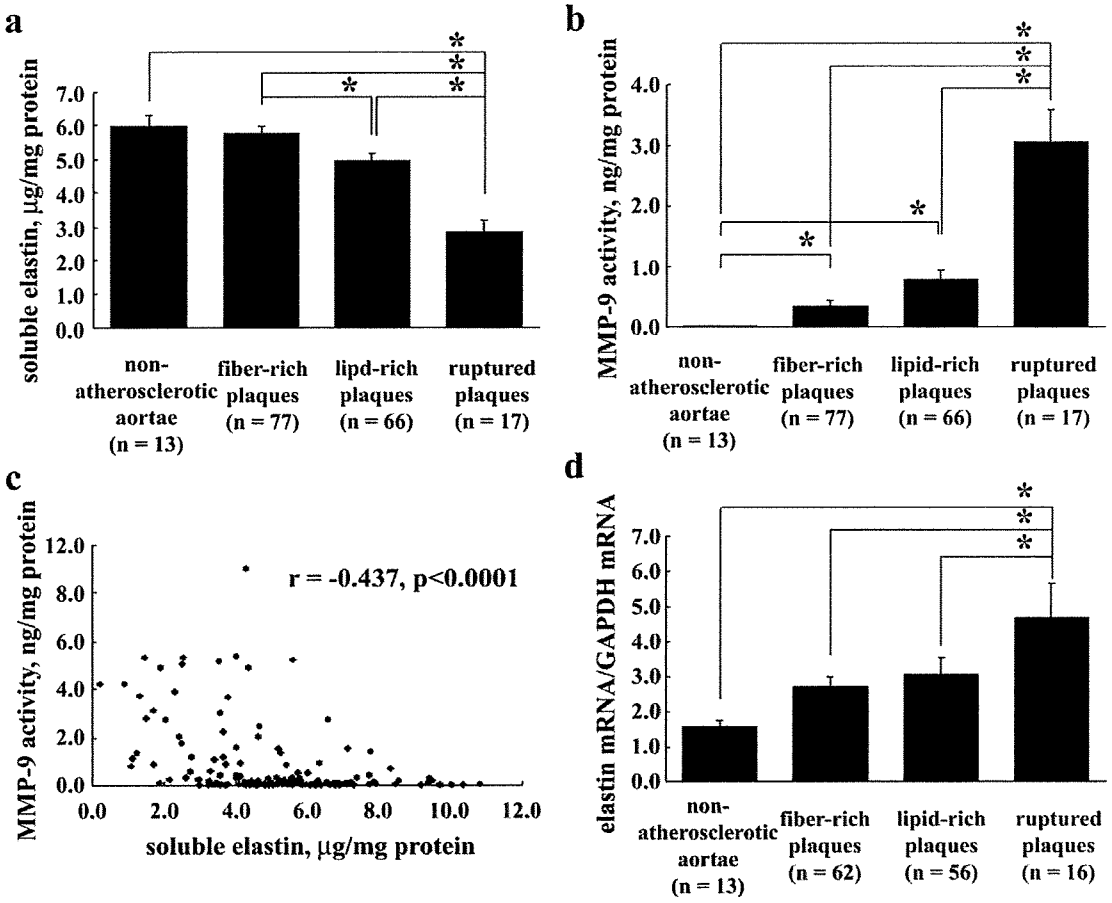

Figure 3. Ruptured plaque has lowest level of soluble elastin (**a**), but highest MMP-9 activity (**b**); that is, soluble elastin and MMP-9 activity shows an inverse distribution pattern in human aortic tissue (**c**). As with MMP-9 activity, elastin mRNA expression is highest in ruptured plaque (**d**). *P<0.05; data are mean±SEM. MMP, matrix metalloprotease.

Table. Relationship Between Level of Soluble Elastin and Histological Features

Histological feature	Soluble elastin level, μg/mg protein*				Univariate† P value	Multivariable†† P value
	Present	n	Absent	n		
Plaque necrosis	5.0±0.2	113	5.7±0.3	60	0.09	0.84
Macrophage infiltration into fibrous cap	4.8±0.2	95	5.8±0.2	78	0.01	0.17
Thin fibrous cap‡	4.8±0.2	76	5.6±0.2	97	0.03	0.24
Intra-plaque fibrin‡‡	4.2±0.3	53	5.6±0.2	120	<0.01	0.09
Intra-plaque hemorrhage‡‡	2.9±0.6	16	5.5±0.2	157	<0.01	0.45
Plaque rupture	2.8±0.4	18	5.5±0.2	155	<0.01	0.01
Mineral deposition	4.8±0.4	44	5.4±0.2	129	0.13	0.48

*Data are mean±SEM.
†Univariate, Mann-Whitney U-test; ††multivariable, multivariable regression analysis; ‡fibrous cap was considered to be thin when thickness <65 μm; ‡‡fibrin deposition or hemorrhages were recognized as red-colored non-structural substances or aggregations of red blood cells outside the intimal microvessels (Masson trichrome procedure).

Level of Soluble Elastin in Protein Extracts

In the ELISA examination of 173 specimens, soluble elastin levels showed a stepwise decrease during the progress of atherosclerosis. The soluble elastin levels in ruptured plaques (2.8±0.4 μg/mg protein, n=17) were significantly lower than those in the non-atherosclerotic aorta (6.0±0.3 μg/mg protein, n=13), fiber-rich plaque (5.8±0.2 μg/mg protein, n=77) or lipid-rich plaque (4.9±0.2 μg/mg protein, n=66; P<0.05 in each case; **Figure 3a**).

Level of MMP-9 Activity in Protein Extracts

The level of MMP-9 activity was significantly higher in ruptured plaque (3.0±0.6 ng/mg protein, n=17) than in the non-atherosclerotic aorta (0.0±0.0 ng/mg protein, n=13), fiber-rich plaque (0.3±0.1 ng/mg protein, n=76) or lipid-rich plaque (0.8±0.2 ng/mg protein, n=67; P<0.05 in each case; **Figure 3b**). Thus, a stepwise increase in the MMP-9 activity was seen during the progress of atherosclerosis. Indeed, the plot of MMP-9 activity against soluble elastin level showed an inverse distribution (**Figure 3c**).

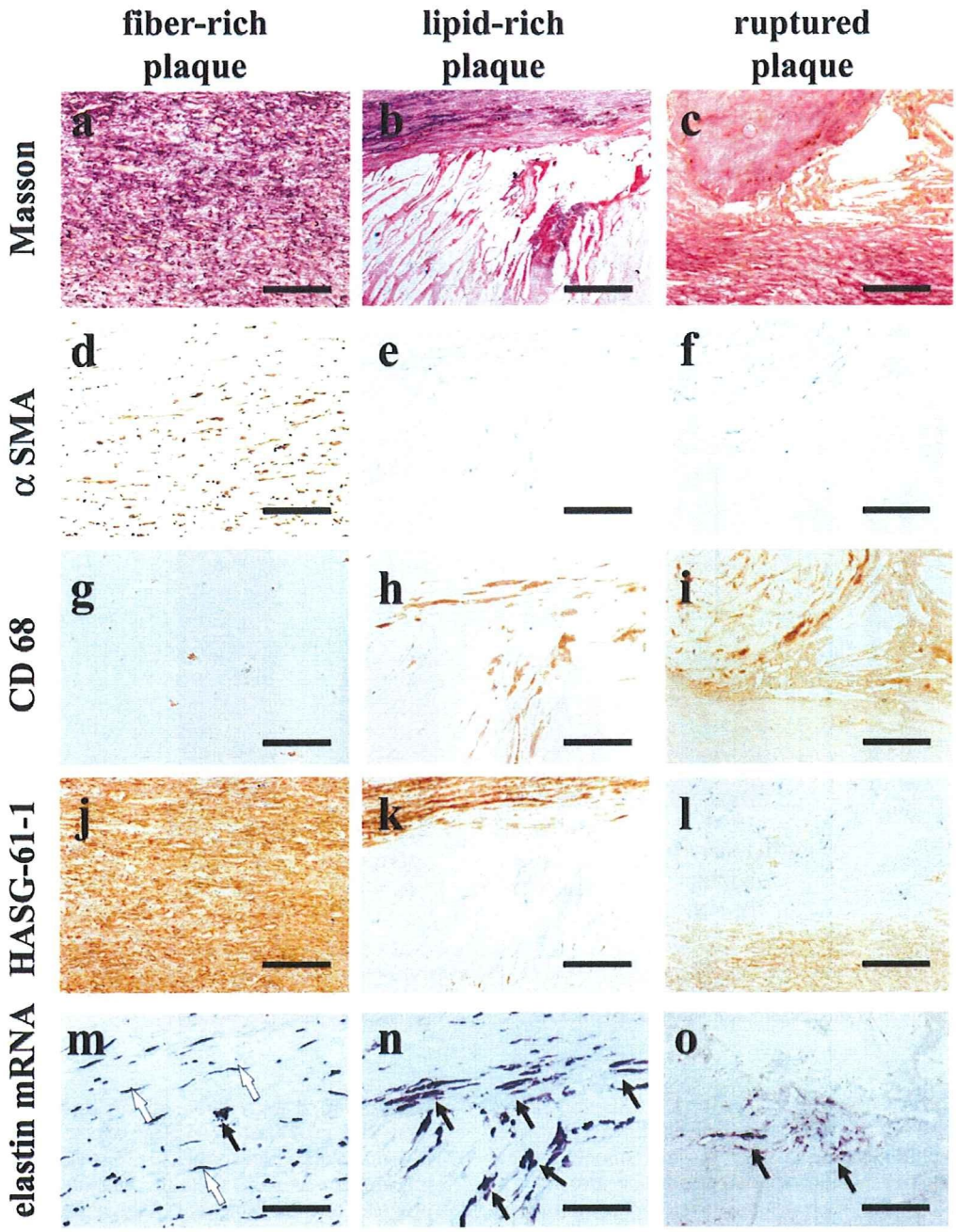

Figure 4. High-power view of insets shown in **Figure 2j–o**. Elastica van Gieson stain (**a–c**), immunohistochemistry for vascular smooth muscle cells with α-smooth muscle actin (**d–f**), immunohistochemistry for macrophages with CD68 (**g–i**), immunohistochemistry for soluble elastin with HASG-61-1 (**j–l**) and in situ hybridization for elastin mRNA (**m–o**). Scale bars=100 μm. HASG, mouse monoclonal IgG antibody.

Relationship Between Level of Soluble Elastin and Individual Histological Features of Plaque

Univariate analysis showed that soluble elastin levels were significantly lower in plaque with histological features associated with instability (viz. macrophage infiltration into the fibrous cap, thin fibrous cap, intraplaque fibrin, intraplaque hemorrhage, and plaque rupture) than in plaques without these features (**Table**). There was no significant difference in soluble elastin levels between plaques with and without plaque necrosis or between those with and without mineral deposition. Multivariable analysis between soluble elastin level and histological features showed that the soluble elastin level was significantly lower in plaques showing evidence of rupture than in those without this feature (**Table**, P=0.01).

ISH and Semi-Quantitative RT-PCR for Elastin mRNA

Representative high-power images of serial sections of fiber-rich plaque, lipid-rich plaque and ruptured plaque are shown in **Figure 4** (Masson trichrome procedure, **a–c**; immunohistochemistry for vascular smooth muscle cells, **d–f**; immunohistochemistry for macrophages, **g–i**; immu-

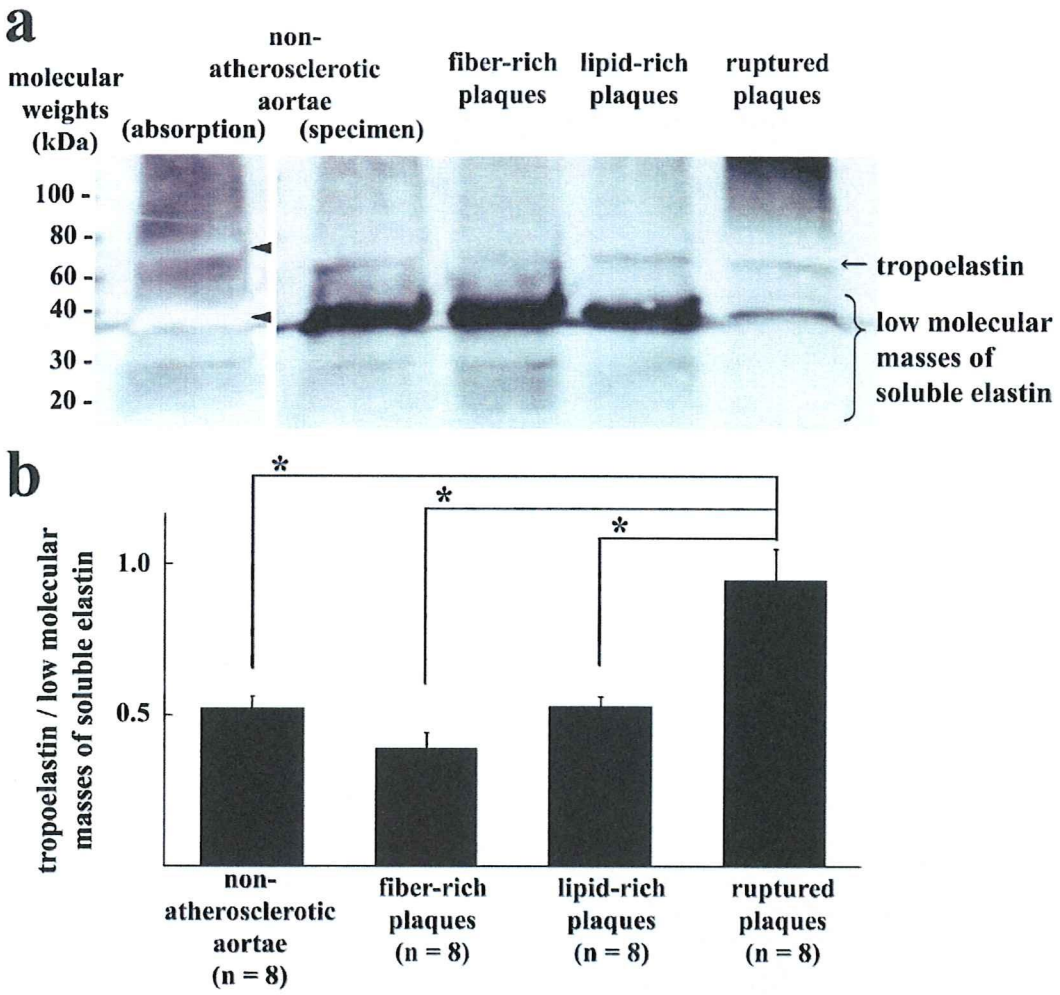

Figure 5. Western blot analysis shows soluble elastin has at least 5 bands of proteins with heterogeneous molecular weights (**a**), including tropoelastin (65 kDa), and (**b**) low-molecular-mass soluble elastins ranging from 35 to 45 kDa. Data are mean±SEM. *P<0.05. Band at extreme left in (**a**) shows that detection of soluble elastin were almost negligible in the absorption experiment with non-atherosclerotic aorta on the same membrane (**arrowheads**).

nohistochemistry for soluble elastin, **j–l**; ISH for elastin mRNA, **m–o**). In the ISH, grains of elastin mRNA were observed within the cytoplasm of vascular smooth muscle cells in fiber-rich plaque (**Figure 4m**, white arrows) and macrophages in fiber-rich plaques (**Figure 4m**), in the rim of the intima adjacent to the atheroma formation in lipid-rich and ruptured plaque (**Figures 4n, o**).

Semi-quantitative RT-PCR for elastin mRNA was performed on 147 of the 173 samples. PCR products were detected at 256 bp (data not shown). Elastin mRNA expression showed evidence of a stepwise increase (as with MMP-9 activity) during the progress of atherosclerosis. The ratio of elastin mRNA over GAPDH mRNA was significantly higher for ruptured plaque (4.7±1.0, n=16) than for the non-atherosclerotic aorta (1.6±0.2, n=13), fiber-rich plaque (2.7±0.3, n=62), or lipid-rich plaque (3.1±0.5, n=56; P<0.05, in each case, **Figure 3d**).

Western Blot Analysis

Figure 5 shows the results of western blot analysis for soluble elastin. At least 5 bands ranging from 20 to 65 kDa, including tropoelastin, were noted in the 4 types of plaque (**Figure 5a**). Thus, soluble elastin consists of tropoelastin, a pre-cursor of elastin (65 kDa), and several heterogeneous low-molecular-mass proteins (35–45 kDa). It seemed that low-molecular-mass soluble elastins decreased in the progress of atherosclerosis. **Figure 5b** shows the ratio of low-molecular-mass soluble elastin over tropoelastin in the 4 types of plaque. The ratio of tropoelastin/low-molecular-mass soluble elastin was significantly higher in ruptured plaque (1.0±0.1) than in the non-atherosclerotic aorta (0.5±0.0), fiber-rich plaque (0.4±0.1) and lipid-rich plaque (0.5±0.0) (**Figure 5b**, P<0.05, all).

Discussion

Although elastin degeneration and synthesis are involved in atherosclerosis, there have been few reports investigating the relationship between elastin and atherosclerosis.[7–9] Recently, Krettek et al reported that macrophages, as well as vascular smooth muscle cells, express elastin mRNA.[10] Concerning elastolysis, MMP-9 is reportedly involved in elastin degeneration,[11–13] and Goncalves et al reported that intermediate fractions of elastin increase as atherosclerosis

progresses.[14] Those reports indicated that elastin degradation and/or degradation products increase during the progression of atherosclerosis, according to the presence of various kinds of proteases including MMP-9. However, the soluble elastin that we investigated in the present study of aortic specimens showed a decreasing pattern, which is different from the other elastin-associated proteins previously described.[14,24–28]

In the present study, we found that in the non-atherosclerotic aorta soluble elastin was (1) distributed both within the intima and around the mature elastin within the media, (2) different from mature elastic fiber (**Figure 1**), (3) had the lowest mRNA expression among the 4 histological groups (**Figure 3d**), (4) present in a low ratio to tropoelastin, which is conjectured to be a marker of elastin synthesis, compared with ruptured plaque (**Figure 5**), and (5) associated with the activity of MMP-9 among the 4 groups (**Figure 3b**). These results indicate that in the non-atherosclerotic aorta from subjects approximately 53–84 years of age, soluble elastin is densely distributed and neither endogenous elastogenesis or elastolysis is activated excessively, so soluble elastin may be deposited gradually and preserved within the intima and around the mature elastic fibers within the media over a number of years because the intima is not diseased. However, it needs to be clarified whether soluble elastin can be also detected in the non-atherosclerotic aorta of normal subjects younger than those entered in this study.

In advanced atherosclerotic lesions, soluble elastin was found to be present within the extracellular matrix of the intima in fiber-rich plaque (**Figures 2g,4j**) and the fibrous caps of lipid-rich plaque (**Figures 2h,4k**). In these plaques, moreover, focal areas of resorcin fuchsin staining with EVG (indicating the presence of mature elastin fibrils) mostly coincided with the areas that were strongly positive for soluble elastin (**Figures 2d,g,j,m**: fiber-rich plaque; **Figures 2e,h,k,n**: lipid-rich plaque). In those areas, elastin mRNA are also expressed in both vascular smooth muscle cells and macrophages in the fiber-rich plaque (**Figures 4d, g,m**), lipid-rich plaque (**Figures 4e,h,n**), and ruptured plaque (**Figures 4f,l,o**). In accordance with these histological observations, elastin mRNA expression showed a stepwise increase during the progress of atherosclerosis (**Figure 3d**). ELISA appeared to show less soluble elastin in fiber-rich and lipid-rich plaques than in the non-atherosclerotic aorta, although admittedly statistical significance was not reached (**Figure 3a**). MMP-9 activity, which indicates elastolytic activity in the arterial wall, displayed a pattern of change similar to that shown by elastin mRNA expression (**Figures 3b,d**) and an inverse relationship with soluble elastin (**Figure 3c**). Interestingly, western blot analysis showed the ratio of tropoelastin/low-molecular-mass soluble elastin was lowest in fibrous plaques and relatively higher in lipid-rich plaques, regardless there being no statistical significance (**Figure 5**). These results indicate that in both fiber-rich and lipid-rich plaques (1) the level of soluble elastin tended to decrease in response to increased activity of MMP-9, and (2) both elastogenesis and elastolysis may have been induced in activated vascular smooth muscle cells and macrophages.

In contrast, in the cores of both lipid-rich and ruptured plaques, soluble elastin (**Figures 2h,i,4k,l**) and mature elastin fibrils (**Figures 2e,f**) were both scarcely detected. ISH showed that elastin mRNA was abundantly expressed within vascular smooth muscle cells and macrophages in both lipid-rich and ruptured plaques (**Figures 4n,o**), whereas MMP-9 activity and elastin mRNA expression were highest in ruptured plaques among the 4 tissue groups (**Figures 3b, d**). The ratio of tropoelastin/low-molecular-mass soluble elastin was highest in ruptured plaque. In addition, our ELISA data demonstrated that the level of soluble elastin was lowest in ruptured plaque among the 4 groups (**Figure 3a**). Furthermore, multivariable analysis showed that soluble elastin was significantly reduced in plaque that showed histological evidence of rupture (**Table**). These results indicate that (1) soluble elastin is significantly decreased in the core of both lipid-rich plaque and ruptured plaque, (2) elastogenesis and elastolysis may be excessively upregulated in the shoulder of both lipid-rich plaques and ruptured plaques, (3) soluble elastin may be transferred rapidly from the arterial wall to the systemic blood circulation by plaque rupture, and (4) based of these phenomena, the turnover of elastin may be enhanced in active atherosclerotic lesions (supporting the basic view of atherogenesis as a chronic inflammatory–repair process). We used postmortem aortic specimens, which is the limitation of the present study because we could not completely exclude the influence of postmortem elastolysis; however, used tissue samples obtained within 12h of postmortem. Further study needs to be undertaken to investigate the possible biological functions of soluble elastin.

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Disclosure/Conflict of Interest

None.

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Cosmic Effect of Rosuvastatin in COSMOS

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Coronary atherosclerosis starts in the mid-20s in men and gradually progresses. When the coronary arteries are occluded more than 75%, effort angina can occur, so slowing the progression of coronary atherosclerosis is important for the secondary prevention of ischemic heart disease. If we can achieve sufficient regression of coronary atherosclerosis with medical therapy, we may avoid excessive intervention and be able to provide more conventional therapies for patients with chronic ischemic diseases.

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In this issue of the Journal, Takayama et al publish the results of the COSMOS study, the Multicenter Coronary Atherosclerosis Study Measuring Effects of Rosuvastatin using Intravascular Ultrasound in Japanese Subjects.[1] This study showed −5.1%±14.1% reduction in plaque volume and 7.3%±15.6% increase in lumen volume with rosuvastatin treatment (average dose, 16.9±5.3 mg/day for 76 weeks) in Japanese patients with ischemic heart diseases. That result is comparable with the ASTEROID trial in the USA.[2] In the ASTEROID study, patients were treated with very high-dose rosuvastatin (40 mg/day) and −6.7%±11.1% reduction in plaque volume was obtained. Notably, a similar reduction in plaque volume was obtained with approximately half the dose of rosuvastatin, indicating the required high-dose statin therapy aiming the coronary regression in Japanese patients.

Previous reports showed decreases in coronary plaque volume with statins. The ESTABLISH study demonstrated 13% reduction in plaque volume with atorvastatin in patients with acute coronary syndrome (ACS),[3] but COSMOS enrolled only 7.9% patients with unstable angina and no patients with acute myocardial infarction. The atherosclerotic region contains lipid-rich, yellow plaque in ACS and in such cases, lipid-lowering therapy with statins can reduce plaque volume much more effectively than in the white atherosclerotic plaques in patients with stable angina. Considering secondary prevention and treatment of chronic ischemic heart diseases, the reduction in plaque in stable regions might be a more important long-term effect of statin treatment. Further clinical studies without ACS cases are awaited to establish the effects of statins on the regression of stable coronary atheroma.

The COSMOS study also included patients receiving prior lipid-lowering therapy if the plasma low-density lipoprotein (LDL) levels were ≥100 mg/dl or the total cholesterol levels were ≥180 mg/dl. Importantly, rosuvastatin can decrease plasma LDL levels to −33.5%±16.1% in patients receiving prior lipid-lowering therapy and to −52.5%±9.6% in those who did not. According to "the lower is better" theory, the effect of rosuvastatin on coronary plaques may be because of its stronger lipid-lowering effects. For that reason, strong statins, such as rosuvastatin and atorvastatin, have been in used in various trials for coronary regression. However, the regression of atherosclerosis with rosuvastatin cannot be simply explained by its effects in lowering plasma LDL levels in the COSMOS trial. Although rosuvastatin decreased plasma LDL levels more in the patients who did not receive prior conventional lipid-lowering therapy, the regression of plaque did not differ between those who did or did not receive prior lipid-lowering therapy. Moreover, the reduction in plaque volume and the reduction in the plasma LDL level were not significantly correlated. These results indicate that effects beyond lowering plasma LDL levels affected the regression of atherosclerosis in the COSMOS trial. Interestingly, rosuvastatin increased plasma high-density lipoprotein (HDL) levels to a similar degree in both groups (+20.3%±23.9% in patients receiving prior lipid-lowering therapy, +18.3%±20.3% in patient not receiving prior lipid-lowering therapy). The regression in total atheroma volume was weakly but significantly correlated with the plasma HDL level and LDL/HDL ratio. Thus, an increase in the plasma HDL level may be an important factor in the decrease in plaque volume by rosuvastatin. High-dose statins are required for regression of coronary atherosclerosis and we raise the question that pleiotropic effects of statins, especially those not related to lipid metabolism, might have affected the results of the COSMOS trial.

Metabolic disorders, inflammation, and oxidative stress are the most important factors promoting vascular diseases. In the Jupiter trial, rosuvastatin decreased cardiovascular events in elder people with high plasma levels of high-sensitivity C-reactive protein (hs-CRP) and the beneficial effect of rosuvastatin was associated with anti-inflammatory effects, as indicated by decreasing plasma hs-CRP levels.[4] We previously reported that after exclusion of patients with statin treatment, plasma hs-CRP levels are associated with the extent of coronary stenosis in patients undergoing coronary angiography.[5] These findings suggest that lowering plasma hs-CRP levels with statins significantly affects coronary atherosclerosis. In the COSMOS study, rosuvastatin did not significantly decrease the plasma hs-CRP level (3,326±7,823 at baseline vs 933±1,549 ng/ml, P=0.49),

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which may be related to the large variation in the data. Other inflammatory factors may be also involved in coronary atherosclerosis, such as interleukin (IL)-1, IL-6, IL-18, TNF-α, MMP or osteopontin.[6] Further analysis may be required to clarify the anti-inflammatory effects of rosuvastatin in patients with stable coronary atherosclerosis.

Although no direct evidence has been found, antioxidants effects of rosuvastatin might affect the regression of atherosclerosis. Statins are known to inhibit small G proteins, such as Ras, Rho, and Rac, by preventing isoprenoid modifications. A recent report by Rashid et al showed that the clinical relevant dose of statins could not inhibit Ras and Rho, but did effectively inhibit Rac1.[7] Rac1 is assembled with NADPH oxidases, the major vascular oxidases, and the inhibition of Rac1 is involved in various cardioprotective effects associated with the reduction of reactive oxygen species (ROS). The modulation of coronary ROS levels by rosuvastatin may not only cause plaque regression, but also enlarge the lumen or preserve endothelial function. We previously reported that atorvastatin induced regression of atherosclerosis in the thoracic aorta (−14%), but not in the abdominal aorta (+2%), as assessed by magnetic resonance imaging during a 2-year follow-up.[8] The different effects of statins on regression of atherosclerosis may be related to variable levels of ROS production in the vessel walls. ROS production per surface area is greater in the carotid and abdominal aorta than in the thoracic aorta in rabbits,[9] where peroxynitrite may regulate vascular tonus.[10] In the coronary arteries, endothelium-derived hydrogen peroxide is much more important for regulating vascular tonus than in the thoracic aorta.[11] Thus, the involvement of ROS in vascular tonus may be more prominent in the coronary arteries than in the thoracic aorta, which may affect the degree of regression with statin therapy. High-dose rosuvastatin decreased only 5% of the plaque volume, and 34.7% of patients were dropped from the trial, mostly because of laboratory data abnormalities. Additional therapies to modulate plasma HDL-levels, inflammation, and small GTPases, as well as excessive oxidative stress, may help to achieve the ideal regression of coronary atherosclerosis in the future.

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