[7,15,16] The expression of ICAM-1 on endothelial cells is increased by inflammatory and immune cytokines[17] and is upregulated in regions overlying atheromatous lesions.[18] The expression of ICAM-1 on endothelial cells is considered important for the atherosclerotic processes, and a recent study revealed that the administration of an anti-ICAM-1 monoclonal antibody significantly attenuated neointimal formation in rats after balloon injury.[19]

The ICAM-1 molecule is expressed not only on the surface of endothelial cells but also on the surface of neutrophils, and its expression is augmented by stimulation with endotoxin (lipopolysaccharide) and tumor necrosis factor-α.[20] However, both the relationship between the severity of coronary atherosclerosis and the behavior of ICAM-1 expressed on neutrophils during their passage through the coronary circulation remain unknown. To clarify the role of neutrophil ICAM-1 in human coronary artery disease, we evaluated changes in the expression of ICAM-1 on neutrophils in the coronary circulation by using flow cytofluorometry. We also examined alterations in the levels of the circulating form of ICAM-1 (cICAM-1) and other adhesion molecules in the coronary circulation.

METHODS

Study patients

Eighteen patients (14 men, 4 women; mean age 65 years, range 41 to 74) who underwent coronary angiography for the diagnosis of chest pain or the further investigation of an ischemic cardiac event were enrolled in the study. Informed consent was obtained from each patient. Patients with unstable angina, recent myocardial infarction, valvular heart disease, or clinical evidence of heart failure were excluded. Eight patients had angiographically normal coronary arteries (4 patients) or only mild stenosis in the left coronary arteries (4 patients) (group A), and the other 10 patients had severe stenosis in the left coronary arteries (group B).

The clinical characteristics of the patients, including risk factors for coronary artery disease, and angiographic findings are listed in Table 1. There were no significant differences in gender or in coronary risk factors between groups A and B; however, the mean age was higher in group B than in group A (70 ± 1 vs 59 ± 3 years, respectively; $p<0.01$). In group A, 4 patients had previously undergone angioplasty of the right coronary artery at the time of the prior myocardial infarction.

Angiographic assessment

Cardiac catheterization and quantitative coronary angiography were performed using a femoral approach and a digital angiographic system (Philips Integris H3000, Philips Medical Systems Corp.) as described previously.[21] Standardized angiographic projections that showed the coronary stenosis at its greatest severity were obtained in each patient. The percentage of coronary narrowing was calculated by comparison of the minimum diameter of the involved segment to the diameter of an adjacent angiographically normal coronary segment, Normal coronary arteries were defined as the absence of coronary narrowings at both right and left coronary angiography. Mild coronary stenosis was defined as the presence of stenosis producing a $< 30\%$ reduction in the diameters of the left coronary arteries. Severe stenosis was defined as a $\geqq 70\%$ narrowing.

Flow cytofluorometric analysis

FITC-conjugated anti-LFA1β (CD18), Leu8 (CD62L, L-selectin), control IgG1, and PE-conjugated anti-Leu54 (CD54, ICAM-1), and control IgG1 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). Blood samples were obtained from the ostium of the left coronary artery (LCA) and the coronary sinus (CS) after an intravenous bolus of 5,000 IU of heparin was administered. Blood was collected in a tube containing acid citrate dextrose (ACD). Whole blood cells were directly stained using monoclonal antibodies, and analyzed after treatment using lysing solution (Becton Dickinson) to examine the expression of CD18, L-selectin, and ICAM-1 on neutrophils. This procedure does not utilize any steps of centrifugation before antigen labeling using a monoclonal antibody and, therefore, minimizes the activation of the cells in the samples.[8,22,23] Cells were analyzed with a FACScan™ Flow Cytometer and Lysis II™ software (Becton-Dickinson). At least 10,000 neutrophils were acquired using a gate set on light scatter dot plotgrams as described previously.[23] The relative mean fluorescence intensity (MFI) was obtained from the histograms as a parameter of the level of antigen expression. To standardize patient-to-patient variation, the percentage of change in the venous (from the CS) minus arterial (from the ostium of the LCA) difference of the expression of neutrophil adhesion molecules in the coronary circulation was calculated as: (venous MFI-arterial MFI) × 100/arterial MFI.

Table 1. Clinical profiles of patients

Patient	Age (years) and sex	Risk factors				Clinical diagnosis	Angiographic findings Coronary segment* (% DS)
		HT	HC	DM	SM		
Group A							
1	58M	No	Yes	No	Yes	OMI	#2(43%)
2	64M	Yes	Yes	No	No	Atypical chest pain	Normal coronary artery
3	41F	Yes	Yes	No	No	OMI	#1(40%), #6(24%)
4	72M	No	No	No	Yes	Atypical chest pain	Normal coronary artery
5	57M	Yes	No	No	Yes	Atypical chest pain	Normal coronary artery
6	65M	No	No	No	Yes	Atypical chest pain	Normal coronary artery
7	56M	No	Yes	No	Yes	OMI	#3(40%), #7(23%)
8	60F	Yes	Yes	Yes	No	OMI and Angina pectoris	#1(73%), #13(27%)
Group B							
1	71M	Yes	Yes	No	Yes	Angina pectoris	#7(47%), #13(99%)
2	74M	Yes	Yes	No	Yes	Angina pectoris	#6(72%), #9(55%)
3	67M	No	No	No	Yes	Angina pectoris	#7(65%), #9(70%)
4	72F	No	No	No	No	OMI and Angina pectoris	#1(70%), #7(100%), #9(72%), #11(65%), #13(99%)
5	71F	Yes	No	No	No	Angina pectoris	#1(73%), #6(72%), 14 (61%)
6	72M	Yes	No	No	Yes	Angina pectoris	#7(56%), #13(74%)
7	65M	Yes	Yes	Yes	Yes	Angina pectoris	#4(50%), #7(80%), #9(75%), #10(59%), #12(69%)
8	74M	No	Yes	No	Yes	OMI and Angina pectoris	#3(100%), #7(70%), #11(55%), #12(99%)
9	67M	No	No	No	Yes	OMI and Angina pectoris	#2(100%), #13(71%)
10	62M	Yes	Yes	Yes	No	OMI and Angina pectoris	#8(62%), #13(99%)

M, male; F, female; HT, hypertension; HC, hypercholesterolemia (Total cholesterol>220 mg/dl); DM, diabetes mellitus, SM: smoking habit, *, location of stenosis in coronary arteries according to the American Heart Association Classification, % DS, % diameter Stenosis.

In vitro stimulation of neutrophil activation

To simulate the alteration of antigen expression on activated neutrophils, white blood cells obtained from three healthy male volunteers (aged 30 to 33 years) were stimulated *in vitro*. Normal white blood cells were obtained from 5 ml of heparinized peripheral blood by density centrifugation using Histopaque-1119 (Sigma). The cells were washed once with Ca, Mg-free PBS, resuspended in 15 ml of PBS, divided into three aliquots in separate tubes, and incubated in a water bath for 15 min at 37℃ under three different conditions. One tube was incubated without any activator as a control, the second had formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Chemical Co., St. Louis, MO) added to a final concentration of 1 μmol/L for 5 min, and the third had cytochalasin B (Sigma) added to a final concentration of 5 μg/mL for 10 min, followed by fMLP 5 min before the end of the incubation.[22,24] The incubation was stopped by the addition of an excess of ice-cold PBS, after which the samples were kept in an ice bath for at least 10 min and then analyzed to evaluate the expression of antigens on neutrophils as described above.

ELISA assay of cICAM-1

Blood samples for the measurement of cICAM-1 were collected in plastic tubes. Serum was separated within an hour and stored at −20℃ until the assay. Serum levels of cICAM-1 were measured by an enzyme-linked immunosorbent assay system (sICAM-1 ELISA kit; Immunoteck, Marseilles, France) according to the supplier's instructions. The assay was performed in triplicate, which showed less than 10% variability.

Statistics

Unless specified, values are expressed as means ± SEM. Statistical comparisons between the two groups of patients were conducted using an unpaired *t*-test. Categorical variables were compared using chi-square analysis. Relationships between levels of cICAM-1 and MFI of cell surface ICAM-1 were examined by linear regression analysis. A p value<0.05 was considered statistically significant.

RESULTS

In vitro activation of neutrophils from normal volunteers

The effects of *in vitro* stimulation by fMLP alone or cytochalasin B followed by fMLP on adhesion molecules expressed on neutrophils collected from healthy subjects are shown in Fig. 1. The MFIs of CD18, L-selectin, and ICAM-1 are shown in Table 2. Stimulation with fMLP alone, as well as with cytochalasin B followed by fMLP, increased the expression of CD18 and decreased that of L-selectin. The expression of ICAM-1 was not affected by the addition of fMLP alone, but decreased after stimulation with cytochalasin B followed by fMLP.

Expression of neutrophil adhesion molecules from patients

Fig. 2 shows typical histograms of CD18, L-selectin, and ICAM-1 observed in the samples collected from the ostium of the LCA and the CS in one patient (No. 10) of group B. The expressed ICAM-1 decreased in the CS, which may indicate the release of ICAM-1 from neutrophils during coronary circulation in this individual with severe stenosis in the LCA. However, the fluorescence intensity of CD18 and L-selectin did not change during passage through the coronary circulation.

The MFIs of CD18, L-selectin, and ICAM-1 on neutrophils collected from the ostium of the LCA and the CS in the two groups are shown in Table 3. There were no differences between the two groups in the MFIs of the adhesion molecules at the two collection sites. As shown in Fig. 3, the percentages of change in CD18 and L-selectin levels across the coronary circulation did not significantly differ between groups A and B (CD18, −3.8±4.3% vs −3.1±2.0%; L-selectin, −1.5±4.5% vs −6.0±3.6%, respectively). On the other hand, the expression of ICAM-1 on neutrophils passing through the coronary circulation was significantly decreased in group B compared with group A (−9.7±2.9% vs 1.0±4.0%, respectively;

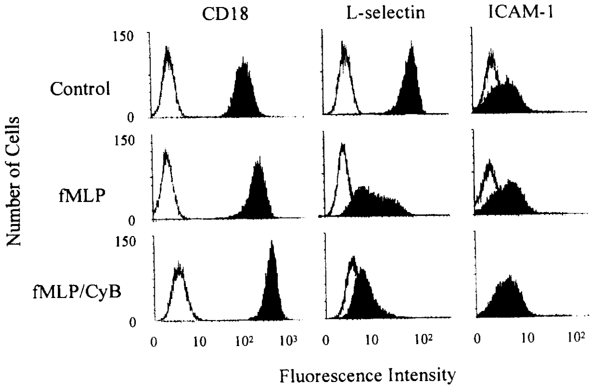

Fig. 1 Alterations in the expression of intercellular adhesion molecule-1 (ICAM-1) on neutrophils and in the serum level of the circulating form of ICAM-1 in the coronary circulation of patients with coronary artery disease.

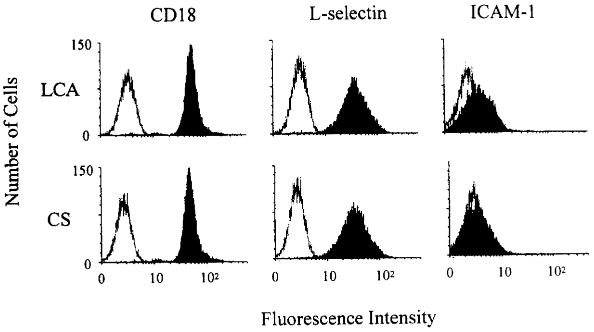

Fig. 2 Alterations in the expression of intercellular adhesion molecule-1 (ICAM-1) on neutrophils and in the serum Level of the Circulating Form of ICAM-1 in the coronary circulation of patients with coronary artery disease.

Table 2. Expression of CD18, L-selectin and ICAM-1 on neutrophils

	MFI of neutrophils at LCA			MFI of neutrophils at CS		
	CD18	L-selectin	ICAM-1	CD18	L-selectin	ICAM-1
Group A	79 ± 7	59 ± 10	17 ± 11	73 ± 6	58 ± 10	5 ± 1
Group B	73 ± 4	80 ± 9	7 ± 1	70 ± 4	76 ± 9	6 ± 1

MFI, mean fluorescence intensity; LCA, ostium of left coronary artery; CS, coronary sinus.

$p < 0.05$).

Serum levels of cICAM-1

There were no significant differences between groups A and B regarding the serum levels of cICAM-1 at the ostium of the LCA (520±31 μg/L vs 552±48 μg/L), in the CS (524±40 μg/L vs 589±55 μg/L), or in the peripheral vein (523±33 μg/L vs 574±55 μg/L). The percentage of change in the venous-arterial difference of cICAM-1 level was significantly higher in group B compared with group A (6.3±1.9% vs 0.5±1.7%, respectively; $p < 0.05$). Therefore, the expression of ICAM-1 on neutrophils decreased and the serum level of cICAM-1 increased in the coronary circulation in group B in comparison with group A. However, there was no correlation between the decrease in expression of neutrophil surface ICAM-1 and the increased level of cICAM-1 ($r = 0.08$, $p = 0.76$).

DISCUSSION

This study shows that ICAM-1 expressed on neutrophils is decreased during passage through the coronary circulation in patients with severe stenosis of the coronary arteries. ICAM-1 is expressed not only on the surface of endothelial cells, but also on circulating leukocytes to interact with β-integrins.[15,16] Although there are some reports of changes in the expression of adhesion molecules on neutrophils in the coronary circulation, they concern alterations in CD18, CD11b, and L-selectin after angioplasty.[25-27] To our knowledge, our study is the first to demonstrate changes in ICAM-1 expression on neutrophils passing through the coronary circulation.

This study also showed that the serum level of cICAM-1 is increased during passage through the coronary circulation in patients with severe stenosis of the coronary arteries. Miwa et al.,[28] studied the level of circulating ICAM-1 in patients with stable effort angina and a significant (>75% luminal narrowing) organic stenosis of either coronary artery. They found no significant changes in the level of cICAM-1 between the aortic root and coronary sinus in patients with stable coronary artery disease. They also found no significant difference in the levels of cICAM-1 in the coronary sinus and aortic root between patients with stable angina and control subjects. Our findings in this study are not comfirmed by their study. However, Haught et al.[29] demonstrated that the level of cICAM-1 was higher in the peripheral blood of patients with stable angina than in control subjects. Thus, the effects of coronary stenosis to the level of cICAM-1 remain controversial.

In patients with severe coronary stenosis, the level of cICAM-1 was increased and the expression of ICAM-1 on neutrophils was decreased during passage through the coronary circulation, even during the attack-free period. However, there was no correlation between the increase in cICAM-1 and the decrease in neutrophil ICAM-1. We presume that the change in cICAM-1 is not directly related to the decreased expression of ICAM-1 on neutrophils. Some studies have revealed elevated cICAM-1 levels

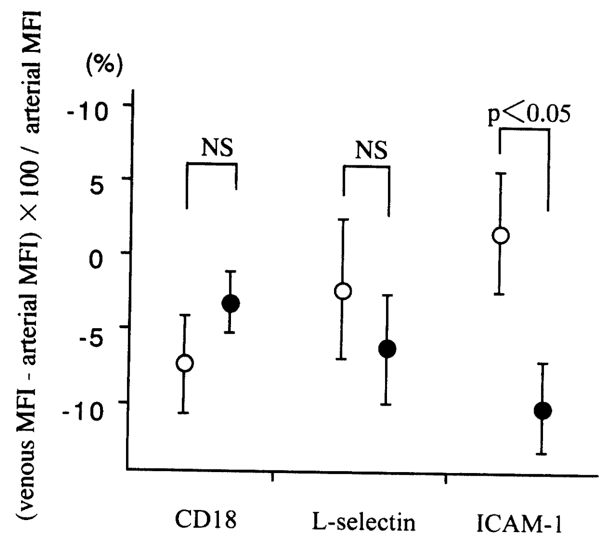

Fig. 3 Alterations in the expression of intercellular adhesion molecule-1 (ICAM-1) on neutrophils and in the serum level of the circulating form of ICAM-1 in the coronary circulation of patients with coronary artery disease.

Table 3. Expression of CD18, L-selectin and ICAM-1 on neutrophils from patients

	MFI of neutrophils at LCA			MFI of neutrophils at CS		
	CD18	L-selectin	ICAM-1	CD18	L-selectin	ICAM-1
Group A	73 ± 6	71 ± 7	5 ± 0	70 ± 5	69 ± 7	5 ± 0
Group B	73 ± 4	80 ± 9	7 ± 1	70 ± 4	76 ± 9	6 ± 1

Data are expressed as mean ± SEM.

MFI, mean fluorescence intensity; LCA, ostium of left coronary artery; CS, coronary sinus.

in inflammatory conditions in which the cxpression of adhesion molecules on endothelial cells is increased,[30,31] and, although the origins of cICAM-1 are still unclear, at least some may arise from shedding or proteolytic cleavage from endothelial cells.[32,33] Therefore, the increase in cICAM-1 through the coronary circulation in patients with coronary stenosis may be a result of an increased expression of ICAM-1 on endothelial cells overlying stenotic lesions. We suggest that the expression of ICAM-1 on neutrophils may change through contact with activated endothelial cells on stenotic lesions in the coronary arteries. ICAM-1 expressed on neutrophils may also interact with β-integrins expressed on other neutrophils, and may influence the aggregation of the cells and formation of coronary atherosclerosis.

Our study showed that the expression of ICAM-1 on neutrophils changed during passage through the coronary circulation in patients with severe coronary stenosis, whereas the changes in the expression of CD18 and L-selectin were not significant. Stimulation of neutrophils *in vitro* with fMLP resulted in an increased expression of CD18 and decreased expression of L-selectin. Stimulation by cytochalasin B plus fMLP, which is a stronger stimulation than fMLP alone,[22] resulted in a decreased expression of both ICAM-1 and L-selectin. The changes in adhesion molecules on neutrophils passing through coronary vessels were basically similar to those changes observed *in vitro* after stimulation with cytochalasin B plus fMLP. Various levels of *in vitro* stimulation of neutrophils caused differential changes in the expression of adhesion molecules, and such differential changes were also observed during the passage of neutrophils through the coronary circulation.

The reduction in ICAM-1 expression of neutrophils from patients with severe coronary stenosis indicates a persistent stimulation of leukocytes in such patients. From our results, we suggest that the ICAM-1 expressed on neutrophils was digested and released into the plasma during coronary circulation in the presence of severely stenotic coronary arteries. This finding provides further evidence that coronary artery disease involves a chronic inflammatory process. Among the adhesion molecules expressed on neutrophils, ICAM-1 seems to be the molecule most sensitive to the activity of the coronary atherosclerotic process. An analysis of ICAM-1 may be useful to predict the progression of the disease.

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