E-Selectin Polymorphism Associated With Myocardial Infarction Causes Enhanced Leukocyte-Endothelial Interactions Under Flow Conditions

Masayuki Yoshida, Yoshio Takano, Taishi Sasaoka, Toru Izumi, Akinori Kimura

Objective—Polymorphisms found in genes encoding adhesion molecules have been reported to be associated with atherosclerosis. We investigated the Ser128Arg polymorphism in the E-selectin gene in Japanese patients with myocardial infarction and its functional significance.

Methods and Results—Results from 135 patients with myocardial infarction and 327 control subjects revealed that the frequency of Arg128-positive was significantly higher in the patients than in controls (12.6% versus 6.7%; odds ratio, 2.0; 95% CI, 1.04 to 3.85), indicating that the Ser128Arg polymorphism was associated with myocardial infarction. We then generated a recombinant E-selectin adenovirus carrying a mutation (AdS128R-E) and compared it with its wild-type counterpart by evaluating the adhesion characteristics of transduced human umbilical vein endothelial cells under flow. AdS128R-E-transduced human umbilical vein endothelial cells supported significantly more rolling and adhesion of neutrophils and mononuclear cells compared with human umbilical vein endothelial cells transduced with AdWT-E (P < 0.001) and also exhibited significantly greater levels of phosphorylation of extracellular signal regulated kinase 1 and 2 and p38 mitogen-activated protein kinase, suggesting that an altered endothelial signaling pathway is associated with this polymorphism.

Conclusions—Our results suggest that the E-selectin Ser128Arg polymorphism can functionally alter leukocyte-endothelial interactions as well as biochemical and biological consequences, which may account for the pathogenesis of myocardial infarction. (Arterioscler Thromb Vasc Biol. 2003;23:783-788.)

Key Words: E-selectin polymorphism endothelium leukocyte adhesion mitogen-activated protein kinase

Leukocyte-endothelial interactions contribute to a variety of vascular disease processes, such as acute and chronic inflammation and atherosclerosis. Several soluble factors (eg, cytokines, chemokines, and growth factors), as well as cell surface adhesion molecules, which are expressed by both endothelial cells and leukocytes, interact in a complex fashion to efficiently mediate leukocyte recruitment. The selectin family of adhesion molecules shares a unique mosaic structure consisting of an amino-terminal lectin-like domain followed by an epidermal growth factor (EGF)-like domain, a variable number of complement regulatory repeats, a transmembrane domain, and a short cytoplasmic domain. E-selectin, one of the three members of this family, has been shown to support the rolling of leukocytes on activated endothelial cells and may also participate in the transition to stable adhesion that precedes transmigration.

Several recent findings regarding the genetic background of atherosclerosis have indicated that DNA polymorphisms in genes encoding adhesion molecules are associated with a higher risk of severe atherosclerosis. Moreover, it was recently shown in a German population that a serine to arginine exchange at the 128th amino acid in the EGF domain of the E-selectin gene has a possible association with severe atherosclerosis. Using a recombinant chimeric protein-based analysis, this mutation was reported to change the binding specificity of E-selectin; however, the physiological importance of this polymorphism in the pathogenesis of vascular diseases is not yet fully understood. Because E-selectin has a role in inflammation and atherosclerosis, we attempted to analyze the association of this mutation with myocardial infarction in Japanese patients as well as elucidate its functional consequences under more physiological conditions.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical veins and then cultured in 0.1% gelatin-coated tissue culture dishes in RPMI-1640 with 20% FCS (Life Technologies Oriental Inc), as described previously. HL60 cells

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Genetic Analysis
The study population was comprised of 135 unrelated Japanese individuals (103 male and 32 female) who had been admitted to Kitasato University Hospital with a diagnosis of myocardial infarction (MI) and given their informed consent to the study. The mean age of onset in all patients was 57.7±8.1 years. The diagnosis of MI was based on typical ECG changes as well as increased serum levels of creatinine kinase and lactate dehydrogenase and was confirmed by the presence of wall motion abnormalities or responsive stenosis in any of the coronary arteries, as documented by coronary angiography. Three hundred twenty-seven subjects (229 male and 98 female, mean age 47.6±9.0 years) with normal ECG results and no clinical signs of coronary artery disease were also used as control subjects. A blood sample was collected from all patients and control subjects, and genomic DNA was isolated with a DNA extraction kit (Qiagen, Hilden, Germany). After a 72-hour incubation at 37°C, HL60 cells, 2×10⁵/mL in RPMI-1640 containing 10% FBS and 20% FBS and twice with DPBS alone.

Western Blotting Analysis of MAPK
Endothelial monolayers in 6-well culture plates (Corning) were transduced with either AdE-sel or AdS128R-E at the indicated MOI. After a 72-hour incubation at 37°C, HUVECs (passages 2 and 3) were plated on 22-mm fibronectin-coated glass coverslips, as previously described. Anti-extracellular signal regulated kinase (ERK) 1/2, and anti-phospho-ERK1/2 (Thr202/Tyr204), and anti-p38 mitogen-activated protein kinase (MAPK) kinase were obtained from New England Biolabs (Beverly, Mass), whereas anti-phospho-p38 MAPK (Tyr180/182) antibodies were purchased from Biosource International (Camarillo, Calif).

Statistical Evaluation
Data are shown as mean±SD. The distribution of E-selectin allele frequencies was tested by a χ² analysis using a 2×2 table to find associations between MI and the variant allele. All statistical analyses considered P<0.05 to be statistically significant.

Results
First we investigated the possible association between an S128R mutation in the E-selectin gene and MI in a Japanese population to elucidate the contribution of this mutation toward the pathogenesis of severe coronary heart disease. One hundred thirty-five patients diagnosed with MI, based on criteria described in the Methods section, were used as subjects, after obtaining informed consent, and their results were compared with those from 325 controls. The S128R mutation of E-selectin was detected using a PCR–restriction fragment length polymorphism analysis of exon 3 of the

Adhesion Assay Under Laminar Flow
The parallel-plate flow chamber used in the present study has been described in detail previously. Briefly, endothelial monolayers on coverslips were transduced with AdE-sel and AdS128R-E at a multiplicity of infection (MOI) of 50, incubated for 72 hours at 37°C, and then positioned in a flow chamber mounted under an inverted microscope. In some experiments, HUVEC monolayers were incubated in the presence of RPMI-1640 with a saturating amount of the indicated mAb (10 µg/mL) for 20 minutes at 20°C just before the assay. The monolayers were perfused for 5 minutes with perfusion medium and then examined carefully to verify confluence. Indicated leukocytes were then diluted in the perfusion medium to 10⁶ cells/mL and drawn through the chamber at controlled flow rates to generate a calculated wall shear stress of 1.0 dyne/cm² for 10 minutes. The entire period of perfusion was recorded on videotape using a digital video recorder equipped with a time generator. Captured images were then transferred to a PC for image analysis to determine the number of rolling and adherent leukocytes in 5 to 10 randomly selected ×20 microscope fields during the final minute of each experiment.

Construction and Expression of Recombinant E-Selectin Adenovirus
The wild-type (WT) E-selectin adenovirus (AdWT-E) and a control adenovirus carrying a nuclear-targeted form of the wild-type (WT) E-selectin adenovirus (AdWT-E) and a control adenovirus carrying a nuclear-targeted form of E-selectin gene were amplified by polymerase chain reaction (PCR) using the following primer pair: F1, 5'-AGT AAT AGT CCT CCT CAT CAT G-3' and R1, 5'-ACC ATC TCA TCA GTT GAA GAA AGA G-3'. The resulting shuttle vector (pAdRSV4) plasmid vectors containing the entire E-selectin coding region (pAdRSV4) were used to generate a point mutation from A to C at the 561st nucleotide using a Quick Change Mutagenesis kit (Stratagene) with the following complement oligonucleotides: 5'-CAA TAC ATC TGG TGG CCA CGG T3' and 5'-ACC GTG GCC ACG GGA GTA TTT G3'. The resulting shuttle vector (pAdRSV128R-E) expressed an E-selectin protein that contained arginine, an amino acid, instead of serine at the 128th position. The recombinant adenovirus (AdS128R-E) was produced by transducing HL60 cells with an equal volume of 250 µL of ice-cold lysis buffer (200 µM HEPES [pH 7.4], 50 mM NaCl, 1% Triton X-100, 20 µg/mL leupeptin, 1 mM PMSF, 10 µg/mL aprotinin, and 1 mM sodium orthovanadate). After centrifugation at 13,000 g for 15 minutes, equal amounts (10 µg per lane) of the cell lysates were subjected to Western blotting analysis with anti-Erk1/2, anti-phospho-Erk1/2, anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies. Immune-reactive bands were visualized using horseradish peroxidase–conjugated secondary antibody with an enhanced chemiluminescent system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The activity of ERK1/2 kinase was measured using a p42/44 MAP Kinase Assay Kit (Cell Signaling Technology) according to the manufacturer’s protocol. Briefly, active MAPK was immunoprecipitated from HUVEC lysates transduced with AdWT-E or AdS128R-E. The resulting products were then incubated with an Elk-1 fusion protein in the presence of ATP, so that the active MAPK could phosphorylate Elk-1. The amount of phospho-Erk1-1 was then determined by Western blotting using a phospho-Elk1-1-specific antibody.

Concentration and Expression of Recombinant E-Selectin Adenovirus
AdWT-E and a control adenovirus carrying a nuclear-targeted form of β-galactosidase (AdRSV128R-E) have been previously described in detail. Anti-extracellular signal regulated kinase (ERK) 1/2, and anti-phospho-ERK1/2 (Thr202/Tyr204), and anti-p38 mitogen-activated protein kinase (MAPK) kinase were obtained from New England Biolabs (Beverly, Mass), whereas anti-phospho-p38 MAPK (Tyr180/182) antibodies were purchased from Biosource International (Camarillo, Calif).

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E-selectin gene. We found a significantly \((P<0.04)\) increased frequency of Ser128 to Arg mutations in the patient group (17 of 135, 12.6%) compared with the control group (22 of 327, 6.7%), as shown in Table 1. The frequency of E-sel 128Arg-positivity was 11.5% (age at onset equal or younger than 60 years, \(n = 73, 11\%\); older than 60 years, \(n = 31, 12.9\%\)) in male patients, whereas it was 15.6% (age at onset equal or younger than 60 years, \(n = 10, 20\%\); older than 60 years, \(n = 22, 13.6\%\)) in female patients. These findings suggested that the E-sel polymorphism might be a risk factor of MI in the Japanese population, especially in younger females. Furthermore, in accordance with previous reports of an association between this mutation and severe atherosclerosis in a white population, these also suggest a strong connection between the E-selectin polymorphism and severe vascular diseases, including MI, in various ethnic groups.

Next, we generated a recombinant adenovirus vector of human E-selectin carrying this mutation and examined its adhesion characteristics in comparison with its WT counterpart using an in vitro flow chamber system. In a previous study by another group,\(^{16}\) the binding strength of E-selectin to HL60 was reported to be significantly reduced for the mutant (S128R) compared with the WT (Ad-WT-E) and mutant (Ad-S128R-E) E-selectin in the immunoreactivity to these antibodies. Moreover, a fluorescent immunohistochemical analysis of the WT and mutant E-selectin failed to detect any significant difference in their distribution on endothelial surfaces (data not shown).

We then conducted a simulated in vitro flow assay using Ad-WT-E and Ad-S128R-E-transduced HUVEC monolayers. PMNs and PBMCs from healthy volunteers were isolated for use in flow assays. When PMNs and PBMCs were perfused at a shear stress of 1 dyne/cm\(^2\), Ad-S128R-E–transduced HUVECs supported significantly greater levels of rolling and adhesion of PMN than Ad-WT-E–transduced HUVECs (Figures 2A and 2B). Similar levels of enhanced rolling and adhesion were also seen when PBMCs were used in the assay (Figures 2A and 2B). In contrast, HUVECs transduced with AdRSVLacZ did not exhibit rolling or adhesion of PMNs and PBMCs (data not shown), which has also been previously described.\(^{9,11}\) Adhesion characterization of the WT and mutant E-selectin were additionally examined at shear stresses of 0.5, 1.0, and 2.0 dyne/cm\(^2\) using HL-60 cells. As shown in Figure 2C, Ad-S128R-E–transduced HUVECs supported significantly greater levels of adhesion under shear stresses of 0.5 and 1.0 dyne/cm\(^2\). Although not statistically significant, Ad-S128R-E–transduced HUVECs supported greater Hl60 cell adhesion compared with those transduced with Ad-WT-E at 2.0 dyne/cm\(^2\).

To examine the binding characteristics of WT-E- and S128R-E selecin, we used the E-selectin adhesion blocking mAb (7A9) on PMN adhesion to WT-E and S128R-E–selectin–transduced HUVEC. As shown in Figure 3, pretreatment of Ad-WT-E–transduced HUVECs with 7A9 partially blocked S128R-E selectin, we used the E-selectin adhesion blocking mAb (7A9) on PMN adhesion to WT-E and S128R-E–selectin–transduced HUVEC. As shown in Figure 3, pretreatment of Ad-WT-E–transduced HUVECs with 7A9 significantly blocked PMN rolling (77.5±42% inhibition versus control IgG, \(n = 8, P<0.003\)) as well as adhesion (87.0±7.7% inhibition, \(n = 8, P<0.0001\)), whereas pretreatment of Ad-S128R-E–transduced HUVECs with 7A9 partially blocked PMN rolling (46.7±16.7% inhibition, \(n = 7, P<0.003\)) and adhesion (51.0±9.6% inhibition, \(n = 7, P<0.0009\)). These results suggest that the S128R substitution in E-selectin dramatically conferred a ligand-binding affinity to E-selectin.

In addition to mediating leukocyte adhesion to vascular endothelium, recent studies have indicated that E-selectin may also transmit outside-in signals in vascular endothelium during leukocyte-endothelial interactions.\(^{9,17}\) Hu et al\(^{18}\) recently reported that E-selectin–dependent leukocyte adhesion induced activation of the MAPK pathway. To explore the functional significance of the S128R polymorphism in E-selectin–dependent signaling, Western blotting analysis was carried out using lysates prepared from HUVECs transduced with Ad-WT-E or Ad-S128R-E–selectin, and the phosphorylation of ERK and p38 MAPK was examined. As shown in Figures 4A and 4B, Ad-S128R-E–transduced HUVECs exhibited a constitutive phosphorylation of ERK and p38 MAPK kinase that was not observed in Ad-WT-E–transduced HUVECs. Adhesion of HL-60, a leukocyte cell
line possessing the E-selectin ligand, to HUVECs significantly induced the phosphorylation of ERK and p38 MAPK in Ad-WT-E-transduced HUVECs but not in Ad-S128R-E-transduced HUVECs (Figures 4A and 4B). This enhanced phosphorylation was observed in proportion to the virus titer and E-selectin expression levels (data not shown), suggesting that the constitutive activation of MAPK was an S128R polymorphism-dependent phenomenon. Moreover, direct measurement of ERK kinase activity, as shown in Figure 5, revealed a significantly greater quantity of phosphorylated-Erk-1 protein detected from Ad-S128R-E–transduced HUVECs compared with those transduced with Ad-WT-E in the absence of HL60. In addition, HL60-induced enhancement of ERK kinase activity, as observed in Ad-WT-E–transduced HUVECs, was not observed in Ad-S128R-E–transduced HUVECs (Figure 5).

### Discussion

Genetic mutations and polymorphisms are known to be risk factors for atherosclerosis and have been extensively studied for their potential association with atherogenic vascular diseases. Similarly, mutations in adhesion molecules, including P-selectin and E-selectin, and their association with cardiovascular diseases have recently been investigated. In the present study, we demonstrated that a Serine128Arg mutation in the EGF domain of E-selectin is a potential risk factor for genetic susceptibility to MI in the Japanese population. Considering the possibility of selection bias, which may have an influence on the results of association studies, we carefully examined the genetic and ethnic homogeneity of our study population along with the competency of the control group. Our entire study population resided in Tokyo and adjacent areas within Japan, where individuals are considered to have a homogeneous genetic background. Furthermore, the distribution of the E-selectin genotype in our control group showed Hardy-Weinberg equilibrium, which verified that the control group was statistically appropriate.

In a study of the association between the E-selectin gene and atherosclerotic vascular diseases, Ye et al. reported that the Ser128Arg mutation was associated with coronary artery disease in their patients. Wenzel et al. also found that both the Ser128Arg and Leu554Phe mutations in the transmembrane domain were related to early severe atherosclerosis in a German population. Results of these studies, conducted using subjects with different ethnic and genetic backgrounds, suggest the possibility that mutations found in the E-selectin gene (Ser128Arg and Leu554Phe) may play a functional role...
in the development of atherosclerosis. Notably, recent findings have also demonstrated that the association between the E-selectin S128R polymorphism and coronary artery calcification was prominent among younger women. We also previously demonstrated that the relationship between the E-selectin S128R polymorphism and MI was much stronger in women younger than 60 years of age. Together, these findings may indicate that an altered expression of E-selectin counteracts the inhibitory effects of estrogens to reduce the expression of adhesion molecules. Thus, as a coronary risk factor, the E-selectin S128R polymorphism may be of particular importance for younger women.

To functionally examine this issue, we created an experimental model that used vascular endothelium that overexpressed mutant E-selectin via a recombinant adenovirus. We found that the Ser128Arg mutation of E-selectin significantly enhanced its adhesion to leukocytes under physiological flow conditions, in contrast to Wentzel et al, who demonstrated that the Ser128Arg polymorphism reduces E-selectin binding to HL60 cells using COS-7 transfectants at 4°C. Our results make an interesting comparison to theirs, and we cannot exclude the differences seen in the expression profiles of the adhesion molecules and intracellular cytoskeleton between COS-7 cells and HUVECs, although the differences in assay conditions (static adhesion assay at 4°C versus physiological flow conditions at 37°C) may have had an influence.

It has also been reported that the Ser128Arg mutation totally confers carbohydrate specificity to E-selectin, and we also demonstrated such an effect by an anti-E-selectin mAb in Ser128Arg mutant E-selectin–transduced HUVECs in the present study. The inhibitory effect of the anti-E-selectin mAb on S128R-E-selectin–dependent PMN adhesion to HUVECs was not as dramatic as that on HUVEC transduced with its WT counterpart, although it was significant. Moreover, although E-selectin participates primarily in PMN adhesion rather than PBMNC adhesion to vascular endothelium, the S128R-E-selectin–transduced HUVEC exhibited a significantly greater level of PBMNC rolling and adhesion under flow conditions (Figure 2B). These data also strongly indicate an altered ligand specificity of S128R-E-selectin.

The mechanism by which this mutation causes enhanced binding activity under flow requires additional study. However, as revealed by a three-dimensional crystal-structural analysis of E-selectin, we now know that the position of the 128th amino acid in the EGF domain of E-selectin does not allow it to directly participate in the ligand-binding pocket. Recent observations suggest that the polymorphisms found in cell-surface receptors lead to a constitutive activation of the receptors in the absence of their ligands, possibly...
through constitutive oligomerization, as has been suggested for the EGF receptor, or via induction of a conformational change, as reported with CD16. Notably, polymorphic S128R-E-selectin exhibited constitutive phosphorylation of ERK1/2 and p38 MAPK without leukocyte adhesion, which indicates that a potential conformational change of E-selectin attributable to the S128R polymorphism may influence the intracellular signaling pathway of E-selectin. The presence of soluble ligands in the assay medium can be neglected, because an enhanced phosphorylation of ERK kinase was observed in the absence of serum in the media (data not shown). A chemical cross-linking approach for potential oligomerization of E-selectin failed to show the difference between WT- and S128R-E-selectin–transduced HUVECs (data not shown); therefore, the responsible mechanisms underlying the altered MAPK signaling pathway observed with S128R mutant E-selectin seem to involve complex processes, which we hope to focus on in a future project.

The participation of the ERK1/2 signaling pathway in E-selectin–dependent PMN adhesion to vascular endothelium was recently identified. Thus, the constitutive activation of ERK1/2 and p38 MAPK observed with S128R-E-transduced HUVECs may be a result of dysregulation of the E-selectin–dependent signaling in endothelial cells, which may play a role in the pathogenesis of MI. We have begun a series of experiments to elucidate the detailed molecular mechanisms involved in the constitutive phosphorylation of MAPKs.

Additional study of the pathological consequences of these disease-associated polymorphisms may provide a significant contribution to understanding the mechanisms of atherosclerosis as well as provide future diagnostic approaches to cardiovascular diseases.

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