P-Selectin Glycoprotein Ligand-1 (PSGL-1) Expressing CD4 T Cells Contribute Plaque Instability in Acute Coronary Syndrome

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Background: Adhesion molecules have essential roles in the development of atherosclerosis. We investigated whether P-selectin glycoprotein ligand-1 (PSGL-1)-expressing CD4 T cells contribute to plaque instability in acute coronary syndrome (ACS).

Methods and Results: We studied the adhesion molecules on CD4 T cells from consecutive patients with ACS treated with thrombus-aspirating device and compared them with healthy controls (n=48 each). Blood, thrombi, and plaque samples from the culprit coronary arteries were collected by thrombus aspiration performed during emergency coronary artery angiography. According to flow cytometry results, peripheral CD4 T cells from ACS patients strongly expressed PSGL-1 and integrin β2 (P<0.05 for both) more than those from controls; culprit coronary arteries contained an abundance of PSGL-1+ (P<0.001) but not integrin β2+CD4 T cells. In addition, immunohistochemical analysis of the thrombus-aspirating device samples revealed numerous PSGL-1+CD4 T cells in plaques from the culprit lesions. Results from the selectin-binding assay demonstrated that activated PSGL-1+CD4 T cells from ACS patients bound to P- or E-selectin after triggering the T-cell receptor, and adhered to endothelial cells under laminar flow conditions (P<0.05 and P<0.05, respectively), inducing their apoptosis (P<0.01) via activated caspase-3, which correlated with PSGL-1 expression (R=0.788, P=0.021) and was suppressed by application of a PSGL-1-specific antibody (P<0.05).

Conclusions: PSGL-1 contributed to cytotoxic CD4 T cell homing to the culprit coronary artery and promoted plaque instability in ACS.

Key Words: Acute coronary syndrome; Atherosclerosis; Cell adhesion molecules; Lymphocytes; Plaque vulnerability
in ACS, and analyzed thrombi and atherosclerotic plaques in samples from culprit coronary arteries obtained from thrombus-aspiration therapy.

**Methods**

**Study Population**

The study population consisted of 48 consecutive patients who underwent thrombus-aspiration therapy for ACS (73% male, 68±11 years old), including 27 cases of acute myocardial infarction and 21 cases of unstable angina pectoris. Blood was drawn from the peripheral vein at the time of admission to the Coronary Care Unit of Tokyo Women's Medical University, and blood, thrombus, and plaque samples from the culprit coronary arteries were collected at the time of thrombus-aspiration therapy during emergency coronary artery angiography (Table). ACS was defined according to criteria established by the American Heart Association. The control group consisted of 48 healthy patients (NC; 75% male, 51±9.0 years old) without cardiovascular disease, diabetes mellitus, hypertension, or hypercholesterolemia, and who were not smokers at the time of enrollment. Individuals with infectious, autoimmune, or neoplastic diseases were excluded from the study. The study was conducted in accordance with the Declaration of Helsinki. The Tokyo Women's Medical University Institutional Review Board approved all protocols, and informed consent was given by all subjects.

**Cell Culture**

Fresh peripheral blood mononuclear cells (PBMCs) were isolated from peripheral or culprit coronary arterial blood using Ficoll-Hypaque (Amersham Biosciences, Brown Deer, WI, USA). CD4 T cells were isolated (purity ≥95%) by negative selection using the Rosette Sep CD4 T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in the same medium as the primary CD4 cells. Human umbilical vein (HUVEC) ECs were purchased from Lonza (Walkersville, MD, USA) and propagated on collagen-coated tissue culture plates in EmGM-2 EC medium (Cambrex, Walkersville, MD, USA); cells from passages 4–6 were used.

**Flow Cytometry**

Cytometric analysis was performed by labeling PBMCs with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Cy5-conjugated monoclonal antibodies against the following human proteins: CD4 (FITC and PE-Cy5), CD69 (FITC), and PSGL-1/CD162 (PE) (all from BD Pharmingen, San Jose, CA, USA); L-selectin/CD62L (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and integrin β2/CD18 (FITC) and αM/CD11b (PE-Cy5) (both from BioLegend, San Diego, CA, USA). The expression levels of each molecule on the T cells were analyzed by flow cytometry (BD FACSCalibur, Franklin Lakes, NJ, USA), and data were analyzed using Win MDI software (Scripps Research Institute, La Jolla, CA, USA).

To investigate caspase-3-dependent apoptosis, freshly isolated CD4 T cells were cocultured on a HUVEC monolayer at an effector:target ratio of 5:1 with 50 ng/mL tumor necrosis factor (TNF)-α used as positive control for 3 h in RPMI medium with 2% FBS. CD4 T cells were then removed with phosphate-buffered saline (PBS) containing 10% FBS. HUVECs were collected by trypsinization and washed with cold PBS, then incubated in BD Cytofix/Cytoperm solution (BD Pharmingen) for 20 min on ice. HUVECs were pelleted by centrifugation, washed twice with BD Perm/Wash buffer (BD Pharmingen), stained with PE-conjugated rabbit anti-activated caspase-3 antibody (BD Pharmingen) in BD Perm/Wash buffer for 30 min at room temperature, and analyzed by flow cytometry.

**Selectin-Binding Assay**

To examine selectin binding, PBMCs were stimulated with and without the anti-human CD3 monoclonal antibody Orthoclone OKT3 (Janssen Pharmaceutical, Tokyo, Japan) for 48 h, then incubated with 50 ng/μL recombinant human E- or P-selectin/Fc chimera (R&D Systems, Minneapolis, MN, USA). Cells were then labeled with IgG F1-FITC (Sigma-Aldrich, St. Louis, MO, USA), CD4-PE-Cy5, CD69-FITC, or CD162-PE monoclonal antibodies followed by flow cytometric analysis.

**Adhesion Assay Under Laminar Flow**

The adhesion assay under laminar flow conditions was performed as previously described. Briefly, HUVECs were stimulated with TNFα (10 μg/mL) for 4 h on coverslips and then positioned in a flow chamber mounted on an IX80 inverted microscope (Olympus, Shinjuku, Tokyo). The stimulated HUVEC monolayer was immersed in a perfusion medium (0.2% FBS in PBS) for 3 min, after which CD4 T cells (1×10^6/mL) were drawn through the chamber using a syringe pump for 10 min at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². THP-1 cells (1×10^6/mL) were drawn through the chamber as a

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ACEI, angiotensin-converting enzyme; AMI, acute myocardial infarction; ARB, angiotensin II receptor blocker; BMI, body mass index; CAD, coronary artery disease.
by analyzing video images on a computer.

**Immunohistochemistry**

Samples obtained from the thrombus-aspirating device were fixed in a 4% paraformaldehyde neutral buffer solu-
PSGL-1+ CD4 T Cells Destabilize Plaques in ACS

To detect poly(ADP-ribose) polymerase (PARP) cleavage, fresh CD4 T cells were cocultured on a HUVEC monolayer at an effector:target ratio of 5:1 with 50 μg/mL TNF-α used as a positive control for 3 h and then washed with PBS, fixed in 3.7% formaldehyde, and treated with 0.1% Triton X-100 in 3% bovine serum albumin solution. HUVECs were then labeled with purified mouse anti-human PARP antibody (BD Pharmingen), PE-conjugated goat anti-mouse IgG, and DAPI for 1 h at room temperature, washed with PBS, and analyzed by fluorescence microscopy.

Statistical Analysis
Data were analyzed using Student’s t-test for independent or paired samples. In experiments with skewed distributions, data were analyzed with the Mann-Whitney U test. P-values <0.05 were considered statistically significant. Results are shown as the mean ± SD and as box plots with medians and 5th percentile ranges.

Results
PSGL-1-Expressing CD4 T Cells in Blood, Thrombi, and Plaques From the Culprit Coronary Arteries of ACS Patients
To investigate whether adhesion molecules on CD4 T cells differed between ACS patients and NC, the L-selectin, integrin β2, PSGL-1, and integrin αM expression on peripheral blood CD4 T cells was examined by flow cytometry. The ratio of integrin β2 and PSGL-1 expression on CD4 T cells was higher in ACS patients than in NC (P<0.05; Figure 1A). To determine whether adhesion mol-
Cells on CD4 T cells in ACS culprit coronary arteries have characteristic features. PBMCs were isolated from the culprit arteries using a thrombus-aspirating device and CD4 T-cell adhesion molecule expression was compared with that of peripheral blood CD4 T cells from the same patient. PSGL-1 was more strongly expressed on CD4 T cells in culprit coronary arterial blood than on those in the peripheral blood (P<0.001). In contrast, integrin β2 expression on CD4 T cells was comparable in both coronary and peripheral blood (Figure 1B).

To assess the clinical relevance of PSGL-1-expressing CD4 T cells in the culprit coronary artery, samples obtained from the thrombus-aspirating device were analyzed by immunohistochemistry. Cell morphology was evaluated by hematoxylin and eosin staining and by labeling with an antibody against the platelet marker CD41. Thrombi were covered with CD41-positive platelets, and necrotic material mixed with cholesterol crystals and variable amounts of inflammatory infiltrate were observed in the aspirates, which were histologically defined as atherosomatous plaque by a pathologist (Figure 2A). CD41-positive platelets were rarely detected in the plaques. Many inflammatory cells, such as CD68-positive macrophages and monocytes, MPO-positive neutrophils, and especially CD4-positive T cells, were present in atheromatous plaques. An abundance of PSGL-1-expressing CD4 T cells was

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**Figure 3.** PSGL-1-expressing CD4 T cells bind E- and P-selectin. Fresh PBMCs were stimulated with (solid line) and without (broken line) anti-human CD3 antibody for 48h, and examined for (A) CD69 or PSGL-1 expression on CD4 T cells, while (B) CD4 T cell binding to P- or E-selectin was analyzed by flow cytometry. The shaded area in the histograms shows the isotype control. PBMC, peripheral blood mononuclear cell.

**Figure 4.** CD4 T cells from acute coronary syndrome (ACS) patients strongly adhere to endothelial cells (ECs). CD4 T cells from ACS and normal controls were drawn onto human umbilical vein ECs pretreated with TNF-α under laminar flow for 10min. Results are shown as ratios of CD4 T cells to THP-1 cells (control) rolling and adhesion. TNF, tumor necrosis factor.
PSGL-1+ CD4 T Cells Destabilize Plaques in ACS

PSGL-1-expressing CD4 T cells in culprit coronary arteries are cytotoxic to endothelial cells (ECs). Fresh CD4 T cells from acute coronary syndrome (ACS) patients and normal controls were incubated on a human umbilical vein EC (HUVEC) monolayer at an effector:target ratio of 5:1. (A) Fraction of apoptotic HUVECs determined after 3 h; results are shown as box plots. (B) Comparison of fractions of apoptotic HUVECs induced by peripheral or culprit coronary arterial blood CD4 T cells from the same ACS patient. (C) Correlation between PSGL-1 expression on CD4 T cells and HUVEC apoptosis in peripheral or culprit coronary arterial blood. (D) CD4 T cells from peripheral blood pretreated with anti-PSGL-1 antibody or control IgG and cocultured with HUVECs for the apoptosis assay.

**Figure 5.** PSGL-1-expressing CD4 T cells in culprit coronary arteries are cytotoxic to endothelial cells (ECs). Fresh CD4 T cells from acute coronary syndrome (ACS) patients and normal controls were incubated on a human umbilical vein EC (HUVEC) monolayer at an effector:target ratio of 5:1. (A) Fraction of apoptotic HUVECs determined after 3 h; results are shown as box plots. (B) Comparison of fractions of apoptotic HUVECs induced by peripheral or culprit coronary arterial blood CD4 T cells from the same ACS patient. (C) Correlation between PSGL-1 expression on CD4 T cells and HUVEC apoptosis in peripheral or culprit coronary arterial blood. (D) CD4 T cells from peripheral blood pretreated with anti-PSGL-1 antibody or control IgG and cocultured with HUVECs for the apoptosis assay.

To investigate whether PSGL-1 expression by CD4 T cells was associated with plaque instability, their cytotoxicity to ECs was analyzed with an apoptosis assay. PSGL-1-expressing CD4 T cells from culprit coronary arteries induced HUVEC apoptosis more strongly than those from the peripheral blood of ACS patients or NC (P<0.05; Figure 5A). CD4 T cells from culprit coronary arterial blood induced HUVEC apoptosis to a greater degree than those from peripheral blood (P<0.01; Figure 5B). We confirmed HUVEC apoptosis for the expression of PARP, a 113-kDa nuclear chromatin-associated enzyme. Apoptotic ECs cocultured with ACS CD4 T cells, but not with NC CD4 T cells by flow cytometry (Figure S1A). In addition, activated caspase-3 was detected in ECs cocultured with ACS CD4 T cells, but not in ECs cocultured with NC CD4 T cells by flow cytometry (Figure S1B).

The adhesion characteristics of CD4 T cells were analyzed using activated HUVECs under physiological flow conditions. The number of rolling and adhering CD4 T cells on activated HUVECs was higher in ACS patients than in NC (P<0.05; Figure 4), suggesting that activated CD4 T cells in ACS patients adhered to activated ECs via the PSGL-1/E- and P-selectin interaction.

**PSGL-1-Expressing CD4 T Cells Bind E- and P-Selectin and Adhere to ECs**

During atherosclerosis development, the binding of T-cell surface PSGL-1 to selectins on activated vascular ECs controls the rate of leukocyte-EC adhesion. A selectin-binding assay was used to determine whether PSGL-1 on CD4 T cells binds to E- or P-selectin. First, T cells from ACS patients were activated by pretreating PBMCs with anti-CD3 antibody and confirming upregulation of CD69, a marker of T cell activation. PSGL-1 expression on CD4 T cells was increased via triggering of the T-cell receptor (TCR) (Figure 3A), and activated PSGL-1-expressing CD4 T cells from ACS patients bound both E- and P-selectin (Figure 3B). The adhesion characteristics of CD4 T cells were analyzed using activated HUVECs under physiological flow conditions. The number of rolling and adhering CD4 T cells on activated HUVECs was higher in ACS patients than in NC (P<0.05; Figure 4), suggesting that activated CD4 T cells in ACS patients adhered to activated ECs via the PSGL-1/E- and P-selectin interaction.

**PSGL-1-Expressing CD4 T Cells in Culprit Coronary Arteries Induce EC Apoptosis**

To investigate whether PSGL-1 expression by CD4 T cells was associated with plaque instability, their cytotoxicity to ECs was analyzed with an apoptosis assay. PSGL-1-expressing CD4 T cells from culprit coronary arteries induced HUVEC apoptosis more strongly than those from the peripheral blood of ACS patients or NC (P<0.05; Figure 5A). CD4 T cells from culprit coronary arterial blood induced HUVEC apoptosis to a greater degree than those from peripheral blood (P<0.01; Figure 5B). We confirmed HUVEC apoptosis for the expression of PARP, a 113-kDa nuclear chromatin-associated enzyme. Apoptotic ECs cocultured with ACS CD4 T cells expressed a high level of PARP comparable to the TNF-α treated positive control, whereas PARP expression was negligible in ECs cocultured with NC CD4 T cells (Figure S1A). In addition, activated caspase-3 was detected in ECs cocultured with ACS CD4 T cells, but not in ECs cocultured with NC CD4 T cells by flow cytometry (Figure S1B).

Furthermore, PSGL-1 expression correlated with CD4 T cell-induced HUVEC apoptosis in peripheral as well as in culprit coronary arterial blood (R=0.566, P=0.049 and R=0.788, P=0.021, respectively; Figure 5C). To assess whether CD4 T cell-induced HUVEC apoptosis was dependent on PSGL-1, an antibody against PSGL-1 was
Discussion

PSGL-1 is a 240-kDa sialomucin that binds L-, P-, and E-selectins on leukocytes; activated T cells induce the expression of glycosyltransferases, allowing modified PSGL-1 to bind all 3 selectins. Ly6C<sup>hi</sup> monocytes, a major subset of monocytes found in atherosclerotic mice, strongly express PSGL-1, and PSGL-1<sup>−/−</sup>apolipoprotein (Apo) E<sup>−/−</sup>double knockout mice have markedly reduced Ly6C<sup>hi</sup>monocyte infiltration in atherosclerotic lesions and develop atherosclerosis. In addition, PSGL-1<sup>−/−</sup>Apo E<sup>−/−</sup>mice showed reduced leukocyte rolling and firm attachment on ECs as compared with PSGL-1<sup>−/+</sup>Apo E<sup>−/−</sup>mice. Meanwhile, PSGL-1 deficiency rescues the impaired vascular function on ECs as compared with PSGL-1<sup>−/+</sup>Apo E<sup>−/−</sup> mutants. The loss of L-selectin has resulted in a 50% reduction in lymphocyte homing in L-selectin<sup>−/−</sup>double knockout mice. Moreover, P- and E-selectin are expressed in chronically inflamed endothelium, and studies of P-selectin<sup>−/−</sup>ApoE<sup>−/−</sup>and E-selectin<sup>−/−</sup>ApoE<sup>−/−</sup>mutants have revealed that atherosclerotic lesions are decreased in these mice.

Signals transduced by PSGL-1 have been shown to enhance integrin β2 expression on neutrophils and HL60 cells. LFA-1 mediates the binding of helper T (Th)1 cells to ICAM-1. TCR activation and interleukin 12/signal transducer and activator of transcription 4 (STAT4) signaling are important for PSGL-1 synthesis and Th1 cell migration into inflamed tissue. We previously reported that perimenopausal women have increased numbers of PSGL-1-expressing CD4 T cells, which strongly adhere to ECs and induce apoptosis. In this study, we investigated adhesion molecules on peripheral blood CD4 T cells and showed that PSGL-1 and integrin β2 expression were upregulated in ACS patients as compared with NC. In addition, TCR activation triggered the binding of activated PSGL-1-expressing CD4 T cells to E- or P-selectin in ACS patients, and in them these cells showed greater rolling and adhesion to activated ECs under flow conditions. PSGL-1 likely promotes this adhesion by enhancing integrin β2 expression on the ACS CD4 T-cell surface, thereby contributing to atherosclerotic development.

Recently, the prospective Atherosclerosis Risk in Communities study showed that the PSGL-1 M621 polymorphism is associated with a decreased risk of both coronary artery disease (CAD) and stroke in African-Americans. In addition, the expression levels of PSGL-1 on CD14<sup>+</sup>CD16<sup>+</sup>monocytes are significantly higher in patients with plaque rupture or intracoronary thrombi, as assessed by frequency-domain optical coherence tomography. Platelet-monocyte complex formation after percutaneous coronary intervention (PCI) is more effectively inhibited by blocking PSGL-1 than αIIbβ3 (GPIIb/IIIa) or αMβ2 (Mac1). Our observation in this study of numerous PSGL-1-positive cells in plaques from culprit coronary arteries suggests that platelet-monocyte complex formation depends largely on PSGL-1.

Studies of culprit coronary arteries from cardiac sudden death cases have revealed that the primary pathology of vulnerable plaques arises from plaque rupture (60%) and superficial erosion (40%).<sup>34,35</sup> EC apoptosis in the fibrous cap covering stable plaques can cause plaque destabilization and increase ACS risk. In addition to an adhesive function, PSGL-1 contributes to the efficient homing of resting (naive and central memory) T cells to secondary lymphoid organs via PSGL-1-C-C motif chemokine ligand (CCL)21 or -CCL19 interactions and proliferation.<sup>36,37</sup> Recently, it was reported that the inhibition of P-selectin with anti-P-selectin monoclonal antibody reduced myocardial damage during PCI in patients with non-ST-segment elevation myocardial infarction.<sup>38</sup> We reported that interferon-γ and TNFα-producing activated CD4 T cells are attracted to vulnerable plaques by the dendritic cell chemokines CCL19 and CCL21, and therefore we examined culprit coronary arterial blood, thrombi, and plaque samples in the present study. We observed an abundance of PSGL-1-expressing CD4 T cells in culprit coronary arterial plaques. Furthermore, these CD4 T cells potently induced PSGL-1-dependent EC apoptosis. Given that adherent and transmigrating PSGL-1-expressing CD4 T cells in culprit coronary arteries are activated in situ and may cause atherosclerotic plaque instability and damage to tissues containing EC and VSMCs, blocking PSGL-1 is a potential therapeutic approach for preventing cardiovascular events and death in ACS patients.

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Disclosures

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43. Supplementary Files

Figure S1. PSGL-1-expressing CD4 T cells induce EC apoptosis in ACS via caspase-3 activation.

Please find supplementary file(s):