Endothelial Injuries of Coronary Arteries Distal to Thrombotic Sites
Role of Adhesive Interaction Between Endothelial P-Selectin and Leukocyte Sialyl LewisX

Hiroyuki Eguchi, Hisao Ikeda, Toyoaki Murohara, Hideo Yasukawa, Nobuya Haramaki, Shotaro Sakisaka, Tsutomu Imaizumi

Abstract—Intracoronary thrombus formation is associated with epicardial vasoconstriction distal to the thrombotic site. To investigate the mechanisms of abnormal vasomotor function of the artery distal to the thrombotic site, we studied coronary vessels in dogs with cyclic flow variations (CFVs) of the left anterior descending coronary artery (LAD) stenosis with endothelial injury. Coronary rings isolated from the LAD (proximal, stenotic, and distal sites) and control circumflex coronary arteries were tested for responsiveness to endothelium-dependent (acetylcholine and A23187) and endothelium-independent vasodilators (NaNO2). Endothelium-independent relaxation was intact in all 4 sites. Endothelium-dependent relaxation was intact in the control and proximal sites and impaired in the stenotic sites. Relaxations not only to acetylcholine and A23187 but also to serotonin, ADP, and thrombin were impaired in the distal sites after observing CFVs for 80 minutes. Electron microscopy revealed the loss of endothelial integrity with leukocyte adherence to the endothelium in the distal sites. Immunohistochemical expression of P-selectin on the endothelial cells was more upregulated in the distal site than in the proximal site, and P-selectin mRNA expression was significantly greater in the ischemic region distal to the thrombotic site than in the proximal nonischemic region. PB1.3, a neutralizing monoclonal antibody against P-selectin, and sialyl LewisX (SLeX)—containing oligosaccharide SLeX, a carbohydrate analogue of selectin ligand, preserved endothelial function without affecting CFVs. SLeX-containing oligosaccharide preserved endothelial integrity of the distal site and inhibited P-selectin expression of the distal site. Thus, the adhesive interaction between endothelial P-selectin and leukocyte SLeX may play an important role in endothelial injuries of the coronary artery distal to the thrombotic site. (Circ Res. 1999;84:525-535.)

Key Words: thrombosis ■ P-selectin ■ sialyl LewisX ■ cell adhesion ■ endothelial injury

Platelet aggregation and thrombus formation at the site of fissured atheromatous plaques are implicated as major pathogenic mechanisms underlying acute coronary syndromes.1,2 Studies in animals3 and humans4,5 have demonstrated that intracoronary thrombus formation is associated with abnormal vasomotor tone of epicardial coronary arteries distal to the thrombotic site, which may exacerbate myocardial ischemia in those syndromes. However, the precise mechanisms of abnormal vasomotor tone of coronary arteries distal to the thrombotic site remain unclear. The endothelium plays an important role in the control of vascular tone. It is then possible that the abnormal vasomotor tone distal to the thrombotic site is due to endothelial dysfunction. There is a canine model of intermittent coronary thrombus formation (the Folts coronary thrombosis model) produced by coronary artery stenosis and endothelial injury.6 We have reported that this model mimics the clinical situations of the acute coronary syndromes observed in humans.7 Accordingly, the first aim of the present study was to examine ex vivo endothelial function of the excised epicardial coronary artery distal to the thrombotic site in this canine model of the acute coronary syndromes.

Intracoronary infusion of various vasoactive substances such as serotonin causes vasoconstriction, especially in patients with coronary artery disease.8,9 It is also known that vasoactive substances are formed at the thrombotic site and released into the coronary circulation. Thus, it is considered that vasoactive substances produced at the thrombotic site contribute to the control of vasomotor tone of the distal artery and to the pathogenesis of acute coronary syndromes. Accordingly, the second aim was to examine vasoactivity of the excised distal coronary artery to vasoactive substances such as thrombin and serotonin to further elucidate the role of thrombus formation in the pathogenesis of these syndromes.

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Activated leukocytes adhere to the endothelium and impair its function in acute ischemic events. In fact, in models of ischemia/reperfusion injury, leukocytes adhere to the endothelium, and inhibition of the leukocyte-endothelial interaction protects against reperfusion-induced myocardial and endothelial injuries. The initial process of this cellular interaction is mediated by adhesion molecules such as P-selectin, which is stored in both α-granules of platelets, and the Weibel-Palade bodies of endothelial cells. When these cells are activated by thrombin, oxygen free radicals, or ischemia/reperfusion, P-selectin is rapidly translocated to cell surfaces and adheres to a sialylated fucosylated carbohydrate structure, such as sialyl Lewis X (SLEx), on leukocytes. The functional significance of P-selectin binding to SLEx is not completely understood. However, several lines of evidence have indicated that activated platelets enhance extracellular oxygen free radical generation by leukocytes through P-selectin and that P-selectin mediates “rolling” of leukocytes. In animal models of myocardial reperfusion injury, either monoclonal antibody to P-selectin or soluble SLEx-containing oligosaccharide (SLEx–OS) protected against reperfusion-induced endothelial and myocardial injuries. Thus, both in vitro and in vivo experimental studies have illustrated that the adhesive interaction between P-selectin and SLEx may have a critical function in modulating vascular and tissue injuries. In patients with acute coronary syndromes, significant increases in P-selectin expression on platelets and a soluble form of P-selectin have been demonstrated by our laboratory and others. However, it is unknown whether this adhesive interaction between the leukocyte and endothelium contributes to the control of endothelial function of the coronary artery distal to the thrombotic site in acute coronary syndromes. Accordingly, the third aim was to examine the effects of leukocyte-endothelial interaction on endothelial function of coronary arteries distal to the thrombotic site. For this purpose, we examined the effects of PB1.3, a neutralizing monoclonal antibody against P-selectin, and SLEx–OS, a unique carbohydrate analogue of selectin ligand on the endothelial function distal to the thrombotic site. Using electron microscopy, we also examined leukocyte adherence to the coronary endothelium and examined the expression of P-selectin by immunohistochemical staining and with a confocal laser scanning microscope system. We also examined P-selectin mRNA expression in cardiac tissues of the nonischemic regions proximal to the thrombotic site and ischemic regions distal to it.

Materials and Methods

Surgical Preparation

All animal experiments were conducted in accordance with the guidelines issued from the Animal Research Committee of the Kurume University School of Medicine. Healthy mongrel dogs weighing 18 to 25 kg were anesthetized with sodium pentobarbital (30 mg/kg). They were ventilated artificially with 100% oxygen. A thoracotomy was performed in the fifth left intercostal space, and the heart was suspended in the pericardial cradle. Polyethylene catheters were placed in the right atrium for drug infusion. ECG was used to determine heart rate. A segment of the left anterior descending coronary artery (LAD) was gently dissected free from the surrounding tissue, and a pulse Doppler flow probe (Hartley Instruments) was placed proximal to the constricting cylinder. Coronary blood flow (CBF) velocity was measured with a pulsed Doppler flow system (VF-1, Crystal Biotech). Arterial blood gases and body temperature were maintained within normal physiological ranges. Aortic pressure was monitored with a micromanometer placed in the femoral artery. Heart rate, systolic and diastolic aortic blood pressure, and phasic and mean CBF velocities were continuously recorded throughout the study. An animal model of intermittent intracoronary thrombus formation, ie, cyclic flow variations (CFVs) caused by recurrent platelet aggregation and subsequent dislodgment at the coronary stenotic site with endothelial injury, were produced according to the method originally described by Fols et al. Briefly, after a 30-minute stabilization period, the endothelium of the exposed LAD was injured by gently squeezing the artery with cushioned forceps. Then, a cylindrical constrictor was placed around the injured coronary artery distal to the flow probe to reduce the phasic CBF velocity to about 40% of the baseline level, eliminating reactive hyperemia after 15 seconds of temporary coronary occlusion. Subsequently, CFVs developed in 75 of 101 dogs. The remaining 26 dogs were excluded from this study.

The severity of CFVs was evaluated by monitoring mean CBF (mL/min), phasic and mean nadir CBF velocities (percentage of baseline), and the frequency (cycles per hour) for the observation period. CBF was determined according to a method described previously. Briefly, CBF velocity near the center of the vessel was recorded by using the pulsed Doppler principle, and CBF velocity was calculated by a digital planimeter. The cross-sectional area of the vessel was approximated to an inside diameter of the Doppler flow probe, ranging in size from 2.0 to 2.5 mm. Then, mean CBF was derived by multiplying mean CBF velocity by the cross-sectional area. Nadir CBF velocity was calculated by averaging the 3 lowest flow velocities and was expressed as a percentage of the uncontracted CBF velocity (baseline) according to the previous method. In dogs that exhibited only 2 flow restorations, nadir CBF velocity was calculated by averaging the 2.

Organ Chamber Experiments

After the observation period of CFVs, the heart was quickly removed and immersed in cold, oxygenated modified Krebs-Henseleit (K-H) solution of the following composition (in mmol/L): NaCl 118.3, KCl 4.7, KH2PO4 1.2, MgSO4 1.2 CaCl2 2.5, NaHCO3 25.0, and glucose 11.1 at pH 7.35 to 7.45. Both the LAD and left circumflex coronary artery (LCXs) segments were carefully removed and placed into cold K-H solution. Isolated coronary vessels were cut into rings of 2 to 3 mm in length. The LAD rings were obtained from the stenotic site and the proximal (10 mm apart) and distal sites (10 mm apart) of stenosis. The LCX ring of the same dog was used as a control vessel. Then, rings were mounted on stainless steel hooks, suspended in 2 mL tissue baths, and connected to force-displacement transducers (UC-2, Kishimoto Medical and Chemical Industry) to record changes in isometric force on an 8-channel recorder (Rechi-Horiz-8K, San-ei). The baths were filled with 2 mL of K-H solution and aerated at 37°C with a gas mixture of 95% O2–5% CO2. Rings were initially stretched to give a preload of 2.0g force and were equilibrated for 60 to 90 minutes. During this period, the K-H solution in the tissue bath was replaced every 15 minutes. After equilibration, relaxations were examined during a contraction caused by 10−7 mol/L U46619 (9,11-dideoxy-9α,11α-methanoxyprostaglandin F2α), a thromboxane A2 analogue. The LAD rings and control ring from the same dog were examined in parallel in the 6 groups discussed below (Figure 1).

Group 1

After CFVs were observed for 20 minutes, coronary rings were isolated from dogs (n=6) that intravenously received a bolus of saline followed by a continuous infusion of saline (1 mL per hour). Once a stable contraction with U46619 (10−7 mol/L) was obtained, an endothelium-dependent vasodilator, acetylcholine (ACH), was added to the bath in cumulative concentrations at 10−7 to 10−4 mol/L. After the responses were stabilized, rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with...
Morphological Studies

To assess morphological changes of the coronary arteries, additional dogs were subjected to 20- or 80-minute CFVs (n=8). After the hearts were quickly removed, a catheter was placed into the left coronary ostium. Then, 2% glutaraldehyde in PBS was perfused through the catheter at 100 mm Hg pressure for 10 minutes. The LAD and LCx were carefully dissected from the heart, and the constrictor was removed. The segments were longitudinally dissected and visualized under a dissecting microscope. The specimens were incubated in the same fixation for 2 hours and rinsed with 0.1 mol/L cacodylate buffer containing 0.1 mol/L sucrose for 12 hours. Furthermore, the specimens were immersed in 1% osmium tetroxide for 1 hour, dehydrated in a series of graded concentrations of cold ethanol, dried by the critical-point drying method, mounted on silver blocks, coated with ~10 nm of gold, and observed under a scanning electron microscope (S-800, Hitachi) operated at 20 kV.

Immunohistochemical and Immunofluorescent Studies

For immunohistochemical study of the coronary arteries using a monoclonal antibody against P-selectin, additional dogs were subjected to 20- or 80-minute CFVs (n=9). After the hearts were quickly removed, the LAD and LCx were carefully dissected from the heart. The isolated coronary arteries were embedded in OCT compound and frozen in liquid nitrogen. Serial 4-μm-thick frozen sections were adhered to poly-L-lysine–coated slides and then fixed in cold acetone for 10 minutes. The labeled streptavidin-biotin method was used for immunohistochemical staining as described previously31 (DAKO LSAB kit). Briefly, specimens were treated with 3% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase and then incubated with 1% BSA. Subsequently, they were incubated with 10 μg/mL of CRC81, which is a specific monoclonal antibody against P-selectin and does not recognize the functional binding site of P-selectin to SLeX (Biodesign International), or with a similar amount of nonimmune mouse IgG for 2 hours at 4°C. After washing 3 times in PBS (pH 7.4), biotinylated anti-mouse IgG secondary antibodies were applied, followed by peroxidase-labeled streptavidin. Peroxidase activity was visualized with 3-amin-9-ethylcarbazole, and the sections were faintly counterstained with Mayer’s hematoxylin.

To quantify the cellular expression of P-selectin on the endothelium of the coronary arteries, additional dogs were subjected to 20- or 80-minute CFVs (n=9). In the same manner and procedure as described above, specimens were incubated with 1% BSA. Subsequently, they were incubated with 5 μg/mL of CRC81 or with a similar amount of nonimmune mouse IgG for 2 hours at 4°C. After washing 3 times in PBS (pH 7.4), they were incubated with FITC-labeled goat anti-mouse IgG antibody (Cappel Laboratories) at a 1:200 dilution with PBS at room temperature for 1 hour. They were washed with PBS and then were mounted using Vectashield (Vector Laboratories). Immunostained specimens were observed using a confocal laser scanning microscope system (LSM-GB2000, Olympus) at an excitation wavelength of 488 nm and emission wavelength of 530 nm, as previously described.32 Then, the confocal images were continuously digitized into a 1024×768–pixel matrix image with 256 gray-scale levels/pixel, and the 7 rectangle regions of interest were set on the different regions of the confocal image by an independent observer (S.S.), who had no knowledge of the study protocol. In each region of interest, immunofluorescent intensity was measured by a computer-aided technique, and the averaged intensity was calculated. To correct for background intensity, the intensity at the use of a nonimmune mouse IgG was subtracted from the intensity at the use of a monoclonal antibody against P-selectin.

Northern Analysis for Canine P-Selectin mRNA Expression in Cardiac Tissue

Digoxigenin (DIG)–labeled canine P-selectin RNA probe was prepared by in vitro transcription from a linearized template according to the Genius RNA probe labeling kit (Boehringer Mannheim). Canine P-selectin cDNA cloned into pBluescript II SK+ (kindly
Thrombosis-Mediated Endothelial Injuries

### Hemodynamic Variables

<table>
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<tr>
<th></th>
<th>Heart Rate, bpm</th>
<th>Aortic Pressure, mm Hg</th>
<th>Phasic Flow Velocity, % of Control</th>
<th>Mean Flow Velocity, % of Control</th>
<th>Mean Coronary Flow, mL/min</th>
<th>Frequency, CFVs per Hour</th>
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<td>63±4*</td>
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</table>

Values are expressed as mean±SEM. Phasic flow indicates phasic coronary blood flow as percentage of control; mean flow, mean coronary blood flow as percentage of control; peak, average peak flow during CFVs; nadir, average lowest flow during CFVs; control, time period before constrictor placement; stenosis, time period after constrictor placement and before development of CFVs; and 20- and 80-minute CFVs, 20-minute and 80-minute observations of CFVs.

*P<0.05 compared with control.

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Materials

The following reagents were used: ACh, A23187, NaNO3, ADP, serotonin, thrombin, indomethacin (Sigma), and U46619 (Cayman Chemical). All drugs were prepared daily with distilled water except for indomethacin, which was dissolved in Na2CO3 (10⁻⁵ mol/L) after sonication, and A23187, which was dissolved in DMSO, with further dilutions obtained in distilled water. The concentrations are expressed as the final molar concentration in the organ bath.

Statistical Analysis

All values are presented as mean±SE; n refers to the number of dogs from which the coronary artery was taken. The paired t test was used for comparison of 2 means. Repeated-measures ANOVA with the Scheffé test was applied for multiple comparisons. Differences were considered statistically significant when the probability was <0.05.

Results

Hemodynamic Variables Before and During CFVs

Before developing CFVs (stenosis in the Table), endothelium injury and coronary constriction decreased the averaged peak phasic CBF velocity to 38% to 42% of baseline and mean peak CBF velocity to 47% to 51% of baseline. Heart rate,
aortic pressure, and peak phasic and mean CBF velocities were similar among the 6 groups. After developing CFVs (20- and 80-minute CFVs in the Table), no significant changes were observed in heart rates and in systolic and diastolic aortic blood pressures. The peak phasic and mean CBF velocities were similarly decreased among the 6 groups. The phasic nadir CBF velocity was decreased to 7% to 8% of control and mean nadir CBF velocity to 12% to 14% of control, not significantly different among the 6 groups. The mean CBF and frequency was 6.5 to 6.8 mL/min and 8.3 to 8.8 cycles per hour, respectively. These values were also similar among the 6 groups. Thus, the PB1.3, SLeX-OS, and PNB1.6 administration did not affect CFVs in groups 3, 4, and 5, respectively.

**Organ Chamber Experiments**

In group 1 (Figure 2A), in dogs with 20-minute CFVs, both ACh and A23187 caused comparable endothelium-dependent, concentration-dependent relaxations in the control, proximal, and distal sites. However, endothelium-dependent, concentration-dependent relaxations in the stenotic sites were significantly impaired. NaNO₂ caused comparable concentration-dependent relaxations among the 4 sites, indicating normal endothelium-independent relaxation. In group 2 (Figure 2B), in dogs with 80-minute CFVs, endothelium-dependent relaxations to ACh and A23187 were impaired not only in the stenotic sites but also in the distal sites. Treatment with PB1.3 or SLeX-OS did not affect 80-minute CFVs in groups 3, 4, and 5, respectively.

**Figure 2.** Cumulative concentration-response curves to ACh, A23187, and acidified NaNO₂ in coronary rings obtained after 20-minute CFVs (A) and obtained after 80-minute CFVs (B) from the proximal (●), stenotic (■), and distal sites (□) of the LAD and from the LCx as a control vessel (○). In dogs with 20-minute CFVs, the endothelial function is impaired only in the stenotic sites. In dogs with 80-minute CFVs, the endothelium-dependent relaxations are significantly impaired not only in the stenotic sites but also in the distal sites. *P<0.05 as compared with controls.

**Figure 3.** Cumulative concentration-response curves to ACh, A23187, and acidified NaNO₂ in coronary rings obtained after 80-minute CFVs treated with PB1.3 (A), SLeX-OS (B), and PNB1.6 (C) from the proximal (●), stenotic (■), and distal sites (□) of the LAD and from the LCx as a control vessel (○). Note that PB1.3 and SLeX-OS exhibit protective effects on the endothelium in the distal sites. *P<0.05 as compared with controls.
PNB1.6 did not affect 80-minute CFVs. Treatment with PNB1.6 did not reverse the impaired endothelium-dependent relaxation of the distal arteries. In group 6 (data not shown), endothelium-dependent relaxations to serotonin, ADP, or thrombin were also significantly impaired in the stenotic and distal sites in dogs of 80-minute CFVs. These changes were similar to those for ACh and A23187 observed in group 2.

Figure 4. Scanning electron microscopy of the control site of the LCx (A) and the proximal site of the LAD stenosis (B) shows the intact endothelium. The stenotic site of LAD (C) shows the endothelial disruption (ED), where the LAD was mechanically injured, and numerous adhered platelets (P) with leukocytes (L). The distal site of LAD to stenosis after 20-minute CFVs (D) showed intact endothelium, whereas that after 80-minute CFVs (E) showed loss of endothelial integrity with leukocytes (L) adhered to the endothelium. Endothelial integrity at the distal site was preserved after treatment with SLe\(^{\alpha}\)-OS (F). Magnification, \(\times 1000\).

Morphology of Coronary Arteries
Representative morphological data by scanning electron photomicrography are illustrated in Figure 4. In coronary arteries after 80-minute CFVs, the control (Figure 4A) and proximal (Figure 4B) sites showed the intact endothelium. The stenotic site showed mechanically damaged endothelium with numerous platelets and leukocytes (Figure 4C). The distal site after 20-minute CFVs (Figure 4D) showed the intact endothelium, whereas that after 80-minute CFVs showed the loss of endothelial integrity with adhered leukocytes on the luminal surface (Figure 4E). The endothelial integrity at the distal site was preserved after treatment with SLe\(^{\alpha}\)-OS (Figure 4F).

Immunohistochemical Localization and Immunofluorescent Expression of P-Selectin
Representative immunohistochemical stainings are illustrated in Figure 5. There was only faint patchy expression of P-selectin by endothelial cells in the control (Figure 5A) and proximal (Figure 5B) sites after 80-minute CFVs. In contrast, the endothelial cell staining patterns for the distal site after 20-minute (Figure 5C) and 80-minute (Figure 5D) CFVs were more intense and continuous than that in the control site. The endothelial cell staining in the distal site after treatment with SLe\(^{\alpha}\)-OS (Figure 5E) was less intense than that with saline after 80-minute CFVs (Figure 5D). No such staining was observed with a nonimmune mouse IgG (Figure 5F). When the immunostained specimens were observed using a confocal laser scanning microscope (Figure 6), the expression of P-selectin by the endothelial cells in the distal site of the LAD after 80-minute CFVs (Figure 6B) was more strongly demonstrated than that after 20-minute CFVs (Figure 6A) or after treatment with SLe\(^{\alpha}\)-OS (Figure 6C). The quantitative analysis of the extent of immunofluorescent expression of P-selectin is shown in Figure 7. The localization of P-selectin was observed on the endothelium of the control, proximal, and distal sites in the epicardial coronary arteries. The averaged intensity of the distal site after 20-minute CFVs was significantly higher than that of the control or proximal sites after 80-minute CFVs (\(P<0.05\)). Furthermore, the intensity of the distal site after 80-minute CFVs was significantly decreased after treatment with SLe\(^{\alpha}\)-OS (\(P<0.05\)).
Northern Analysis for P-Selectin mRNA Expression in Cardiac Tissue

P-selectin mRNA expression was greater in the ischemic distal region as compared with the nonischemic proximal region in all 6 animals (Figure 8). By the densitometric analysis, the degree of P-selectin mRNA expression was significantly greater in the ischemic distal region than in the nonischemic proximal region (1.56 ± 0.25 versus 0.88 ± 0.17 arbitrary units, \( P < 0.01 \)).

Discussion

It is clinically well recognized that vasomotor tone of the coronary artery distal to the thrombotic site is abnormal in patients with coronary artery disease. However, the mechanisms remain unknown. The Folts coronary thrombosis model used in this study is characterized by CFVs. We have previously demonstrated that this model produces pathophysiological manifestations similar to those of acute coronary syndromes in humans, such as ST-segment elevation, depending on the fluctuation of CBF during the episode of CFVs. Indeed, the phenomenon of CFVs can be observed in patients with unstable angina. Thus, the Folts model is well established as an in vivo model of coronary arterial thrombosis. In the present study, we therefore focused on the mechanisms of abnormal vasomotor tone of coronary arteries distal to the thrombotic site observed in patients with acute coronary syndromes and investigated this issue by use of a well-established canine coronary artery thrombosis model of CFVs.

In the present study, the coronary arteries distal to the thrombotic site exhibited not only impaired endothelial function but also morphological damages of endothelium with leukocyte adherence. Moreover, the immunostainings revealed the increased expression of P-selectin by endothelial cells of coronary artery distal to the thrombotic site after developing CFVs. The administration of PB1.3, a neutralizing monoclonal antibody against P-selectin, or SLe\(^{\alpha}\)-OS, a unique carbohydrate analogue of selectin ligand on leukocytes, which did not affect CFVs, preserved the endothelial function in the distal sites of LAD coronary artery. The SLe\(^{\alpha}\)-OS prevented morphological abnormalities and inhibited the expression of P-selectin. Finally, P-selectin mRNA expression was significantly greater in the ischemic region distal to the thrombotic site as compared with the proximal nonischemic region. These findings suggest that thrombosis per se or substances released from the thrombi may cause
endothelial injury of arteries distal to the thrombotic site through the adhesive interaction between endothelial P-selectin and leukocyte SLeX. Our findings may give deep new insight into the pathogenesis of unstable angina and provide in vivo evidence for vasculoprotective efficacy of PB1.3 and SLeX-OS.

**Endothelial Injury Distal to Thrombotic Site**

In control dogs with 80-minute CFVs, we demonstrated that the coronary arteries distal to the thrombotic site had weaker vasodilator responses to ACh and A23187 than did the proximal or control LCx arteries, whereas the vasodilator responses to NaNO2 were similar among these 3 sites, indicating functional impairment of the endothelium and normal vascular smooth muscle dilator function. The impaired endothelium-dependent relaxation of the stenotic site was apparently due to a surgical trauma. Endothelium-dependent relaxations of the distal arteries to ACh and A23187 were comparably impaired. Since A23187 activates the synthesis and release of nitric oxide while bypassing cell membrane receptors,36 the endothelial dysfunction of the distal artery may not be specifically receptor mediated. This functional impairment of the endothelium of the distal artery was associated with loss of the morphological integrity of the endothelium with adhered leukocytes. This impairment was not due to the surgical trauma to the endothelium, because the distal site was at least 10 mm away from the stenotic site and because no such damages were observed in dogs with 20-minute CFVs in which the same surgical procedure was performed. This endothelial damage was not caused by CFVs per se, because the endothelium of the distal artery of 20-minute CFVs was intact. Endothelium-dependent relaxation was impaired in response not only to ACh but also to other vasoactive substances, such as serotonin, ADP, and thrombin. Because these substances are generally produced by thrombi and released into the circulation,2 it may be assumed that, in the presence of abnormal endothelial function of the distal artery, vasoactive substances produced by thrombi cause more vasoconstriction and further aggravate myocardial ischemia.

**Role of Adhesive Interaction Between Endothelial P-Selectin and Leukocyte SLeX**

Leukocyte adhesion to the endothelium plays an important role in the sequelae of myocardial ischemia/reperfusion injury. Adhered and activated leukocytes release a variety of cytotoxic mediators including oxygen free radicals, inflammatory cytokines, platelet-activating factor, leukotriene B4, and proteolytic enzymes.37,39 These mediators aggravate endothelial dysfunction, resulting in increased leukocyte adhesion to the endothelium and myocardial injury.10 The inhibition11,12 or depletion40,41 of leukocytes has been demonstrated to result in a reduction in reperfusion injury. Because ischemia/reperfusion results in elaboration of a variety of humoral mediators, P-selectin is expressed within minutes after reperfusion.17 On activation of the endothelium, P-selectin is rapidly translocated onto the endothelial surface, where it tethers leukocytes and activates them. In the present study, we demonstrated the functional and morphological injuries of the endothelium with adhered leukocytes and the upregulated P-selectin expression on the endothelium of coronary arteries distal to the thrombotic site after developing CFVs. In contrast, P-selectin was only faintly expressed on the endothelial surface of the control and proximal sites as shown by immunohistochemistry. To further elucidate the localization and extent of P-selectin expression in the coronary arteries, we quantitatively assessed the immunofluorescent expression of P-selectin by the confocal laser scanning system (Figure 7). The immunofluorescent localization of P-selectin was observed on the endothelium of the epicardial coronary arteries. These findings are consistent with the results of previous studies demonstrating that P-selectin is located on the endothelium of large arteries.33,42,43 The P-selectin expression of the distal site after 80-minute CFVs was significantly upregulated by 5-fold as compared with that of the control and proximal sites after 80-minute CFVs and by 2.5-fold as compared with that after 20-minute CFVs. Thus, our findings indicate that P-selectin exists in the endothelium of the epicardial coronary arteries and that the expression of P-selectin distal to the thrombotic site progressively increases depending on the duration of CFVs.

In the present study, the functional and morphological injuries to the endothelial distal to the thrombotic site after 20-minute CFVs were not observed despite the increased expression of P-selectin at this time point. There may be several possible reasons. First, there may exist a time difference between the expression of P-selectin and endothelial dysfunction. Firm attachment of leukocytes to the endothelium is necessary to induce endothelial injuries.10,12 P-selectin supports leukocyte tethering and rolling at the early phase of leukocyte-endothelial interaction,21,22 and then endothelial intercellular adhesion molecule-1 interacts with B2 integrin on the leukocytes to further strengthen the adhesive interaction.44 Thus, even when P-selectin is significantly upregulated at 20 minutes after developing CFVs, endothelial dysfunction may not be necessarily induced at this time point. Another possibility is that endothelial dysfunction may be quantitatively related to the extent of P-selectin expression on the endothelial cells. More enhanced P-selectin expression could recruit more leukocytes to the coronary endothelial cells distal to the thrombotic site. In the present study, the extent of P-selectin expression was 2.5-fold more at 80 minutes than at 20 minutes after developing CFVs. Therefore, the severity of endothelial dysfunction may be related to the magnitude of P-selectin expression.

In the present study, several possible mechanisms of the expression of P-selectin distal to the thrombotic site are considered. Our total experimental period is 80 minutes. Hence, it is more likely that upregulated P-selectin on coronary endothelium at the distal site is related to externalization of P-selectin from the Weibel-Palade bodies, because de novo protein synthesis generally requires 3 to 6 hours.45,46 We examined whether P-selectin mRNA in the cardiac tissues distal to the thrombotic site might be stimulated within the observation period of 80-minute CFVs. Consequently, the degree of P-selectin mRNA expression was significantly greater in the ischemic distal region by 1.8-fold as compared with that of the nonischemic proximal region. Thus, these
findings suggest that de novo transcription and protein synthesis of P-selectin may modulate the disease process of thrombus formation in the later phase of CFVs, although this possibility is less likely in our 80-minute CFVs model. If CFVs were abolished during PB1.3 or SLeX-OS administration, the improvement of coronary endothelial dysfunction could be attributed to the disappearance of CFVs by the treatment per se rather than by the direct effect of these agents on the endothelial dysfunction. To exclude this possibility, we chose a dose of 1 mg/kg as a bolus injection of PB1.3 and a dose of 5 mg/kg as a bolus injection followed by a continuous infusion at 5 mg/kg per hour of SLeX-OS, because we have previously confirmed that these treatments did not reduce CFVs.29 In this study, it should be noted that the administration of PB1.3 and SLeX-OS significantly restored endothelial function without affecting CFVs, whereas the administration of PNB1.6 did not. Furthermore, the administration of SLeX-OS inhibited the expression of P-selectin and prevented morphological damages of the endothelium. These findings suggest that P-selectin of the endothelium of the artery distal to the thrombotic site is upregulated and traffics leukocytes that further damage the endothelium. Although our animal model is intermittent ischemia/reperfusion, the endothelial damages were not attributed to ischemia/reperfusion per se, because both PB1.3 and SLeX-OS protected the endothelium without affecting CFVs. Thus, the endothelial damage in this model was caused by mechanisms related to thrombosis rather than by ischemia/reperfusion itself. In this regard, our findings are different and new as compared with previous studies using ischemia/reperfusion models.

We think that thrombus formation, or certain substances released from thrombi, might have caused the expression of P-selectin and endothelial damages. Numerous leukocytes with platelet thrombi have been shown to be present at the stenotic site in dogs with CFVs.47 It has been shown that activated platelets induce the production of oxygen free radicals by leukocytes through platelet P-selectin.20 and oxygen free radicals have an active role in initiating or sustaining CFVs.48–50 As discussed above, oxidative stress upregulates the expression of endothelial P-selectin.14 Thus, it is possible that platelet P-selectin–mediated, neutrophil-induced oxygen free radicals might have induced endothelial expression of P-selectin and that effects of PB1.3 and SLeX-OS may have been mediated by interruption of the platelet-leukocyte interaction. However, the contribution of platelets is unknown from our study, although platelets were present with leukocytes on the damaged endothelium of the stenotic site. Another candidate is thrombin, which causes platelet aggregation and induces the expression of P-selectin by the endothelial cells.15 Because thrombin is an important mediator of CFVs51 and its levels appear to be quite high in the present model, thrombin might have induced endothelial expression of P-selectin.

The underlying mechanisms by which SLeX-OS decreases P-selectin expression and improves endothelial dysfunction are still unknown. The following explanation is considered. The protective effect of SLeX-OS against endothelial dysfunction may be explained by the inhibition of P-selectin–mediated leukocyte adherence to the endothelium by attenuating native SLeX binding to P-selectin.24 Because adhered and activated leukocytes release a number of cytotoxic substances, such as oxygen free radicals and inflammatory cytokines, these substances stimulate endothelial P-selectin expression.16,52 In the present study, SLeX-OS treatment clearly inhibited leukocyte adherence to the endothelium distal to the thrombotic site in the canine coronary artery, as presented by our electron micrography (Figure 4), which could lead to the decrease in P-selectin expression of the endothelium.

Limitations
There are 2 limitations to this study. First, because SLeX-OS blocks the interaction not only between P-selectin and SLeX but also between other selectin families present on the endothelium or platelets and SLeX on leukocytes, its vasculoprotective effect may not have been specific for P-selectin. However, the neutralizing action of PB1.3 is specific for P-selectin, because it does not cross-react with other selectin families.53 Furthermore, PB1.3 used in this study is of IgG1 isotype and reacts with P-selectin on the activated platelet surface of not only humans but also other mammals.23,53 Thus, vasculoprotective effects in this study are likely due to the inhibition of interaction between endothelial P-selectin and leukocyte SLeX. Second, although SLeX-OS does not perform leukopenic actions,24 this mechanism was not excluded in this study because we did not count the number of leukocytes.

Clinical Implications
Several previous studies have demonstrated in dogs with CFVs that vasoactive substances including serotonin,54 ADP,55 and thrombin51 are important mediators of CFVs and that serotonin concentration increases by ≈18-fold at the stenotic site during the episode of CFVs.54 These substances, which induce platelet aggregation, exert vasoconstriction when the endothelium is injured. In this study, the coronary arteries distal to the thrombotic site showed impaired endothelium-dependent relaxation to serotonin, ADP, and thrombin. These findings may explain the results of previous studies demonstrating that the coronary arterial diameter distal to the stenosis decreases during CFVs and increases after treatments with a serotonin-receptor antagonist.3 We have recently shown that a high dose of SLeX-OS abolished CFVs in a canine model of coronary thrombosis.29 Furthermore, a small sugar moiety such as SLeX has low antigenicity when used in vivo and has a potential efficacy after oral administration.56 Thus, SLeX-OS may be an important therapeutic candidate for unstable angina from the point of view of not only antithrombotic but also vasculoprotective effects.

Conclusions
We have demonstrated that the time-dependent impairment of endothelium-dependent relaxation occurs in the coronary arteries distal to the thrombotic site and that this impairment is possibly caused by the adhesive interaction between endothelial P-selectin and leukocyte SLeX. To our knowledge, this is the first in vivo study investigating the mechanisms of coronary thrombosis-mediated endothelial dysfunction. An
analogue of SLeX, SLeX-OS, may be useful for prevention of vascular injuries distal to the thrombolytic site in patients with acute coronary syndromes. This issue should be further studied in humans.

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