Roles of P-Selectin in Inflammation, Neointimal Formation, and Vascular Remodeling in Balloon-Injured Rat Carotid Arteries

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Background—P-selectin plays key roles in mediating inflammation through promoting adherence of leukocytes to activated platelets and endothelium. This process is one of the initial events in atherosclerosis and restenosis after coronary angioplasty.

Methods and Results—Using a rat balloon-injury model, we examined the role of P-selectin in vascular inflammatory processes. In the acute phase, immunohistochemistry revealed that P-selectin was intensely expressed on both activated platelets covering the denuded segment and endothelial cells of the inflamed adventitial small vessels. Treatment with an anti–P-selectin monoclonal antibody (MAb) for 8 consecutive days significantly inhibited neointimal formation at day 14 (42% inhibition; \( P<0.05 \)), and this effect persisted at day 56 (40% inhibition; \( P<0.01 \)) compared with the control group. Vascular shrinking accompanying adventitial fibrosis was also attenuated at day 56. Inhibition of both neointimal formation and vascular shrinking resulted in the lumen area of the anti–P-selectin treatment group being \( \approx 3 \) times larger at day 56 than that of the control group. Accumulation of CD45-positive leukocytes in the developing neointima, media, and adventitia at day 8 was significantly inhibited by treatment with the anti–P-selectin MAb. Scanning electron microscopy demonstrated that anti–P-selectin treatment resulted in a less thrombogenic surface of the arterial intima, which featured a pseudoendothelial appearance at day 14 after injury.

Conclusions—These results suggest that inhibition of P-selectin–mediated leukocyte recruitment prevents the development of neointimal formation, adventitial inflammation, and vascular shrinking and promotes pseudoendothelialization by luminal smooth muscle cells. This treatment thus beneficially affects vascular remodeling after balloon injury in rats. (Circulation. 2000;102:1710-1717.)

Key Words: adhesion molecules • leukocytes • platelets • restenosis • remodeling

Late luminal renarrowing after coronary angioplasty remains a major problem, because it occurs in \( \approx 30\% \) to 50\% of treated arteries within 6 months after the procedure.\(^1\) Neointimal formation is considered essential for restenosis to occur after angioplasty. However, restenosis has recently been recognized as a combination of neointimal formation and vascular remodeling.\(^2\) Although the exact mechanism of arterial remodeling remains unclear, it has been suggested that adventitial reaction and inflammation are involved in the development of atherosclerosis and restenosis after balloon angioplasty.\(^3\)

Inflammatory processes in atherosclerosis acquired growing importance in the 1990s.\(^4\) Immune system–mediated inflammatory reactions were related to the expression of cell adhesion molecules, migration of leukocytes, cytokine release, and secretion of growth factors at the site of injury. Cell adhesion molecules have emerged as essential for the development of atherosclerosis and restenosis after angioplasty.\(^5\)\(^6\) In particular, the selectin family (L, E, and P) is crucial for the earliest events in the inflammatory response, leading to the “rolling” phenomenon followed by leukocyte migration. P-selectin is stored in the \( \alpha \)-granules of platelets and the Weibel-Palade bodies of endothelial cells,\(^7\) and it appears in the outer membrane soon after activation of both platelets and endothelial cells. It mediates the adherence of activated platelets to monocytes and neutrophils\(^8\) and the rapid transient interactions between activated endothelial cells and leukocytes\(^9\) via its carbohydrate ligands sialyl Lewis\(^1\) and P-selectin glycoprotein (GP) ligand-1.\(^10\)

Previous studies have indicated that plasma P-selectin levels are higher after coronary angioplasty in patients who develop restenosis.\(^11\) In addition, atherectomy specimens from patients with unstable angina showed significantly more intense P-selectin expression than did those from patients with stable angina.\(^12\) Recently, Kumar et al,\(^13\) using a cessation flow model without endothelial denudation, demonstrated that neointimal formation was attenuated in P-selec-
tin–deficient mice. However, the inhibition of negative vascular remodeling, a major cause of restenosis after angioplasty, has not been clearly defined. For this reason, we tested the hypothesis that treatment with anti–P-selectin monoclonal antibody (MAb) can attenuate inflammatory responses and inhibit the development of neointimal formation and negative remodeling. We used a rat balloon-injury model, which is widely accepted as an animal model of restenosis after balloon angioplasty with regard to the similarity in endothelial denudation and arterial wall stretch.

Methods

Arterial Injury Model

Male Sprague-Dawley rats weighing ~300 g were obtained from Japan SLC Inc (Hamamatsu, Japan). All animals were provided with care guided by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals; NIH publication No. 86-23, revised 1985). Rats were anesthetized with pentobarbital sodium (50 mg/kg IP). Their left carotid arteries were injured as previously described14 by 3 passages of an inflated 2F Fogarty embolectomy catheter (Baxter Healthcare). To attain a constant degree of vessel wall injury for each of the animals, we kept the diameter of the balloon and the resistance during withdrawal constant and the same for each of the animals. The sham operation involved simple ligation of the left external carotid arteries without balloon injury. At various time intervals, the animals were killed with a lethal dose of pentobarbital sodium. The injured and contralateral uninjured carotids were perfused with cold 0.1 mol/L PBS (pH 7.4) under physiological pressure followed by careful excision of the carotid arteries. The existence of endothelial denudation was evaluated by staining with Evans blue dye (60 mg/kg IV) injected 30 minutes before death at 2 and 8 weeks after balloon injury in preliminary experiments (n = 5 for each period). The central portion of the injured left carotids stained blue and was regarded as the nonreendothelialized area.

Preparation of Anti–Rat P-Selectin MAb (S789G)

Anti–rat P-selectin MAb was prepared by immunizing female BALB/c mice every seventh day with a footpad injection of 2 × 10^6 to 5 × 10^6 rat P-selectin cDNA transfectants. The booster injection was made into the footpad 2 days before fusion. Papolitope lymph node cells were fused with myeloma cells, PAL using PEG 4000 (Life Technologies, Inc), and the resulting hybridomas were screened according to their ability to stain P-selectin transfectants. The MAb called S789G (mouse IgG2b) reacted with rat P-selectin transfectants and also blocked the binding of HL-60 cells to the transfectants.

In Vitro Cell-Binding Assay

HL-60 cells were labeled with BCECF-AM (Molecular Probes) for 30 minutes at 37°C, washed, and resuspended in RPMI 1640 medium containing 10% FBS. P-selectin–transfected Chinese hamster ovary (CHO) cells were cultured in 24-well culture plates for 48 to 72 hours. Wells were washed with RPMI 1640 medium. Labeled HL-60 cells (1 × 10^5 to 5 × 10^5 cells/well) were added to the wells and incubated for 1 hour at 4°C in the absence or presence of S789G. Isotype-matched antibody (Ab) (mouse IgG2b) was also used as a negative control. Unbound cells were removed by 2 gentle washings with the wells with RPMI 1640 medium, and the bound cells were lysed with 100 mL of 1% Nonidet P-40. The relative number of cells per well was calculated from fluorescence at 485/538 nm, obtained with a Fluoroskan II microplate fluorometer (Laboratory Systems).

Time Course of P-Selectin Expression and Leukocyte Recruitment

To evaluate the time course of P-selectin expression and leukocyte recruitment in the balloon-injured arteries (n = 5) and in the uninjured arteries from sham-operated animals (n = 5), rats were killed at 1, 3, and 5 days and 1, 2, 4, and 8 weeks after balloon injury. The excised carotid arteries were immediately embedded in OCT compound and rapidly frozen in liquid nitrogen. Immunohistochemical studies were performed by previously described methods16 on the frozen sections. Briefly, a Vectastain Elite ABC kit (Vector Laboratories) was used with the following primary MAbs: MAb S789G, and MAb OX-1 (mouse IgG1, PharMingen) which reacts with leukocyte common antigen CD45 on all hematopoietic cells except erythrocytes.16 To confirm the identification of platelets, an anti–platelet GP Ib/IIa Ab (sheep IgG, Enzyme Research Laboratories Inc) was also used. Isotype control Abs were used for negative controls.

Anti–Rat P-Selectin MAb (S789G) Treatment Study

Rats in the anti–P-selectin treatment group (P group) received 4 mg/kg IP of S789G, and those in the control group (C group) received the same dose of nonimmune mouse IgG (Seikagaku Kogyo), 30 minutes before arterial injury and for 7 consecutive days after balloon injury. Fourteen days (n = 7, each group) and 56 days (n = 5, each group) after balloon injury, the injured and uninjured contralateral carotids were harvested as described above, and the middle parts of both arteries were divided into 2 rings. One of them was fixed in 4% paraformaldehyde and embedded in paraffin for light microscopy; the other was prepared for scanning electron microscopy (SEM).

Morphometry

Five sections from each carotid artery were stained with von Gieson’s elastin stain and examined morphometrically by videomicroscopy (HC-300i, Nikon) with a computerized digital image analysis system (NIH Image) in a blind manner. The areas within the external elastic lamina (EEL area), the internal elastic lamina (IEL area), and the lumen area were measured. Other areas were calculated as follows: medial area = EEL area – IEL area; neointimal area = IEL area – lumen area; neointima-to-media ratio (I/M) = neointimal area/media. The circumferences (length) of the EEL, IEL, and lumen were also measured to determine vascular shrinking.

Accumulation of Leukocytes

All CD45-positive leukocytes were counted in the neointima, media, and adventitia at day 8 after balloon injury in both groups (n = 6, each). The percentage of CD45-positive cells was determined as the number of CD45-positive cells divided by the total number of cells in the 5 sections obtained from each injured carotid.17

Thrombogenicity of the Injured Luminal Surface

Thrombogenicity of the injured arterial luminal surface at day 14 after injury was evaluated by SEM. The injured and contralateral uninjured arterial rings, were fixed, prepared as described above, were immersed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sliced into longitudinal strips, and then postfixed with 1% osmium tetroxide. They were dehydrated through a graded alcohol series, critical-point–dried with CO2, and splatter-coated with platinum-palladium. They were then examined with a JSM-6000 (JEOL) SEM at 5 kV. Thrombogenicity was quantitatively assessed by counting the number of platelets adhering to the injured luminal surface in both groups. SEM pictures of 15 randomly selected visual fields were taken at a magnification of ×2500. The total number of platelets adhering to the injured surface was then counted in each of the pictures in a blind manner. In addition, immunohistochemistry was performed using adjacent sections embedded in paraffin to confirm that the luminal surface was covered with luminal smooth muscle cells. MAb 1C-10 (mouse IgG2b, Seikagaku Kogyo) against smooth muscle myosin heavy chain isoform (SM1)18 was used to identify smooth muscle cells and polyclonal Ab against factor VIII–related antigen (rabbit IgG, DAKO Laboratories) to identify endothelial cells.18

Statistical Analysis

All data are presented as mean±SEM. Statistical analysis for multiple comparisons between groups used a post hoc test (Fisher’s
Inhibitory Effect of S789G on Binding of HL-60 Cells to Rat P-Selectin–Transfected CHO Cells

<table>
<thead>
<tr>
<th>CHO cells</th>
<th>Antibody</th>
<th>Cell Binding, FIU</th>
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<tr>
<td>CHO parent cells</td>
<td>...</td>
<td>139.2±6.9</td>
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<tr>
<td>P-selectin–transfected CHO cells</td>
<td>...</td>
<td>463.7±1.5</td>
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Isotype control
- 10 μg/mL: 502.0±0.5
- 3 μg/mL: 75.9±14.8
- 1 μg/mL: 80.9±2.4
- 0.3 μg/mL: 69.4±22.8
- 0.1 μg/mL: 138.8±24.8
- 0.03 μg/mL: 409.9±24.5

FIU indicates fluorescence intensity units. Data are expressed as mean±SEM.

Results

Inhibitory Effect of MAb S789G on Binding of HL-60 Cells to Rat P-Selectin–Transfected CHO Cells

The binding of HL-60 cells to CHO parent cells (nontransfected), the negative control cell line, was clearly lower than the binding to P-selectin–transfected CHO cells (Table). This suggests that HL-60 cells specifically bind to P-selectin–transfected CHO cells. S789G strongly inhibited the binding of HL-60 cells to P-selectin–transfected CHO cells (Table). A negative control experiment using an isotype-matched control revealed that the binding of HL-60 cells to P-selectin–transfected CHO cells was hardly suppressed compared with results for S789G (Table). These results indicate that S789G functionally and specifically reacts with rat P-selectin.

Time Course of P-Selectin Expression and Leukocyte Recruitment

In the uninjured artery, the endothelial monolayer remained intact and P-selectin expression was absent (Figure 1A). From days 1 to 3 after injury, P-selectin was intensely expressed in adhering platelets and platelet aggregates on the exposed subendothelium with adherent leukocytes (Figure 1, B and C). No positive staining for P-selectin was observed in the serial section used for negative control staining (inset, Figure 1B). Intense positive staining for GP IIb/IIIa, corresponding to positive staining for P-selectin, was observed on platelets adhering to the injured luminal surface (inset, Figure 1C). At day 5, P-selectin expression became weaker (Figure 1D); at day 14, it became faint on platelets adhering to the neointima (Figure 1E); and it finally disappeared at day 28 (Figure 1F). P-selectin was also intensely expressed on the endothelium of the inflamed adventitial small vessels at day 3 after injury (Figure 2A) and persisted up to day 7, accompanied by severe CD45-positive leukocyte infiltration at the adventitia (Figure 2B). No positive staining for P-selectin was observed in the serial section used for negative control staining on the endothelium of the inflamed adventitia small vessels at day 3 after injury (Figure 2A) and persisted up to day 7, accompanied by severe CD45-positive leukocyte infiltration at the adventitia (Figure 2B). No positive staining for P-selectin was observed in the serial section used for negative control staining.

Figure 1. Immunohistochemical analysis showing time course of P-selectin expression in rat carotid arteries after balloon injury. A, Uninjured carotid artery without P-selectin expression. B through F, Injured carotid arteries after injury, from day 1 (B) to day 3 (C), in which high levels of P-selectin expression are observed on activated platelets covering denuded segment with an adherent leukocyte (large arrow). B, Inset, Negative control staining produces no P-selectin staining. C, Inset, Intense positive staining for GP IIb/IIIa, corresponding to positive staining for P-selectin, is observed on platelets adhering to injured luminal surface. At day 5 (D), P-selectin staining has become weaker; at day 14 (E), it is faint on platelets adhering to neointima (small arrows); and at day 28, it has disappeared (F). Bars=50 μm.
control staining (inset, Figure 2A). In the sham-operated animals, however, P-selectin expression at the adventitial small vessels was faint (Figure 2C), with few inflammatory cells (Figure 2D). P-selectin expression in the inflamed adventitia and inflammatory response became undetectable 4 weeks after injury (data not shown).

**Effects of P-Selectin Blockade on the Development of Neointimal Formation and Vascular Remodeling**

There was no significant difference in body weight between the C group and P group 2 and 8 weeks after injury. Representative sections of the vessels from the C group and P group harvested at day 56 are shown in Figure 3. The control vessel developed a marked neointima with adventitial fibrosis seen in green on the Masson trichrome–stained sections (Figure 3, A and C). The vessel from the P group revealed less neointimal formation, less adventitial fibrosis, and a larger lumen area (Figure 3, B and D).

Morphometric analysis showed that the neointimal area in the P group was reduced by 41.8% and I/M by 38.8% (Figure 4, A and B) compared with the C group 2 weeks after injury. The inhibitory effects on neointimal formation were still apparent 8 weeks after injury in the P group, with respective

**Figure 2.** Immunohistochemical analysis showing P-selectin expression and CD45-positive leukocytes on adventitial side of carotid arteries. A, At day 3 after injury, P-selectin is intensely expressed on walls of adventitial small vessels. Inset, Negative control staining produces no P-selectin staining. B, Many CD45-positive cells are infiltrating adventitial side. C and D, In sham-operated animals, very little P-selectin expression (C) and few CD45-positive cells (D) are observed. Bars=50 μm.

**Figure 3.** Photomicrographs of representative cross sections showing injured arteries from C group (A and C) and P group (B and D) at day 56 after balloon injury. Vessels from P group reveal less neointimal formation and adventitial fibrosis with less shrinking than those of C group. EEL and IEL are indicated by large and small arrows, respectively. Masson trichrome stain. Bars=100 μm.
reductions of 40.1% and 43.6% (Figure 4, A and B). The medial area of the 2 groups did not differ significantly either 2 or 8 weeks after injury.

There was no significant difference in vascular constriction between the 2 groups 2 weeks after injury (Figure 4, C and D). However, the P group revealed significantly less shrinking of the IEL and EEL than did the C group; in fact, the IEL length had increased by 9.2% (Figure 4C) and the EEL length by 7.3% (Figure 4D), respectively, 8 weeks after injury. As a result of the inhibition of both neointimal formation and vascular shrinking, late luminal narrowing was prevented. The lumen area of the P group remained ≈3 times larger than that of the C group 8 weeks after injury (Figure 4E).

Accumulation of Leukocytes
The administration of MAb S789G significantly attenuated the recruitment of CD45-positive leukocytes to the injured vessel walls of the P group (Figure 5B) than of the C group (Figure 5A). The percentage of CD45-positive cells was significantly lower in the developing neointima (85.0% reduction; \( P < 0.01 \)), media (78.1% reduction; \( P < 0.01 \)), and adventitia (64.9% reduction; \( P < 0.01 \)) of the P group than of the C group (Figure 5C).

Thrombogenicity of Injured Arterial Luminal Surface
Figure 6A shows an SEM of the luminal surface of an uninjured right carotid artery with fusiform and elongated endothelial cells. Figure 6, B and C, indicates that treatment with S789G resulted in an improvement of the morphology of the luminal surface at day 14 after balloon injury. The P group showed an arterial intima with a less thrombogenic surface featuring flat and polygonal cells with complex interdigitation with neighboring cells (Figure 6C). The number of platelets adhering to the luminal surface in the P group as determined by SEM decreased significantly to 79.4% in comparison with the C group (C group, \( 29.6 \pm 2.1 \times 10^3 /\text{mm}^2 \) versus P group, \( 6.1 \pm 2.8 \times 10^3 /\text{mm}^2 ; P < 0.01 \)). Immunohistochemistry showed linear positive staining for factor VIII–related antigen (Figure 7A) and negative staining for SM1 (Figure 7B) in the endothelial cells of the uninjured artery. Conversely, factor VIII–related antigen was absent (Figure 4).
SM1 staining was positive in cells at the injured luminal surface of the P group (Figure 7D).

**Discussion**

**Time Course of P-Selectin Expression in Injured Carotid Artery**

To the best of our knowledge, this investigation is the first to identify the time course of P-selectin expression and its early and intense expression in both activated platelets and adventitial small vessels with balloon-induced injury. Barron et al. demonstrated the early expression of both E-selectin in the retained endothelium and L-selectin on circulating leukocytes after balloon angioplasty of rabbit iliac arteries. However, lack of a P-selectin Ab to rabbit tissue prevented examination of P-selectin expression. Kumar et al. demonstrated that P-selectin was expressed in developing neointima and media as well as endothelial cells and adhering platelets after ligation of mouse carotid arteries. However, expression of P-selectin in the inflamed adventitia was not identified. In our study, P-selectin immunostaining showed that inflammatory infiltrates are involved not only from the injured lumen side but also from the adventitial side. A possible mechanism for P-selectin expression on the adventitial side would be adventitial reaction to overstretch by the inflated balloon catheter, which could also occur during balloon angioplasty in the clinical setting.

**Effects of Anti–P-Selectin MAb on Neointimal Formation and Vascular Remodeling**

The treatment with MAb S789G significantly suppressed neointimal formation 14 days after injury, and this effect persisted up to day 56. This demonstrates that the effects of early anti–P-selectin treatment not only delayed the development of neointimal formation but also influenced the severity of neointimal hyperplasia in the chronic stage. Moreover, late negative remodeling with marked adventitial fibrosis was also attenuated by anti–P-selectin treatment. Accordingly, P-selectin is involved in the progression of inflammation and unfavorable fibrotic changes in the adventitia in this model. Inhibition by S789G of neointimal formation and vascular shrinking resulted in a larger lumen area.

Kumar et al. recently used a cessation flow model of P-selectin–deficient mice to study the role of P-selectin in early vascular remodeling and neointimal formation. Our results agree with theirs in terms of a significant reduction in neointimal formation in P-selectin–deficient mice. However, no change in vascular shrinking was observed in their study, whereas ours demonstrated that administration of anti–P-selectin MAb inhibits both neointimal formation and chronic vascular shrinking in a balloon-injury model. We assume that the absence of endothelial denudation and overstretch injury in their model contributes to the difference in the results. Endothelial denudation accompanied by platelet aggregation is a crucial initial event, and balloon overstretch...
injury could result in vascular remodeling through an adventitial response during postangioplasty restenosis.\textsuperscript{21}

**Possible Mechanisms of Attenuation of Lesion Formation**

Accumulation of leukocytes to the injured vessel wall occurred, in order of decreasing frequency, in the adventitia, neointima, and media, suggesting the potential role of adventitial inflammation in the development of vascular lesion formation. Wilcox and Scott\textsuperscript{3} reported that the intensity of the adventitial inflammatory response correlated with the severity of atherosclerosis. The treatment with MAb S789G in our study reduced the accumulation of leukocytes to the adventitia as well as the neointima and media. As for a possible mechanism of inhibitory effect on vessel shrinking, it should be noted that this suppression of adventitial inflammatory response may contribute to a reduction in adventitial fibrosis after balloon injury. Furthermore, adventitial fibrosis physically limits vessel expansion, which may result in unfavorable remodeling.\textsuperscript{22}

Short-term perioperative treatment with S789G resulted in an improvement in the histological appearance of and a reduction of platelet accumulation on the injured luminal surface 14 days after injury. Immunohistochemical analysis verified that the less thrombogenic luminal surface in the P group was composed of smooth muscle cells (pseudoendothelium) 14 days after balloon injury.\textsuperscript{23} Because platelets are a source of mitogen for smooth muscle cells,\textsuperscript{4} the reduction in

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**Figure 6.** SEMs of luminal surface of rat carotid artery. A, Intact endothelium of uninjured carotid artery. B, Injured luminal surface from C group with some platelets adhering to injured surface (arrows). C, Surface morphology improves in P group, indicating less thrombogenic surface with flat and polygonal cells. Bar=10 μm.

**Figure 7.** Immunohistochemical analysis of luminal cells of uninjured or injured carotid arteries. A, Uninjured artery stained with factor VIII–related antigen. B, Uninjured artery stained with SM1. Intact endothelial cells are linearly positive for factor VIII–related antigen and negative for SM1. C, Luminal cells of injured artery at day 14 from P group are stained with factor VIII–related antigen. D, Same cells stained with SM1. Factor VIII–related antigen is absent, whereas SM1 staining is positive, in luminal cells. Bar=50 μm.
platelet accumulation on the injured luminal surface may limit their contribution to neointimal formation after injury. Akers et al.\(^2\) showed that treatment with an MAb against P-selectin (PB 1.3, Cytel Corporation) accelerated reendothelialization in balloon-injured rabbit thoracic aorta.

To summarize, we demonstrated that P-selectin was involved in vascular inflammatory processes on the adventitial side as well as on the injured lumen side. Furthermore, the inhibition of P-selectin–mediated leukocyte recruitment prevented negative remodeling with adventitial fibrosis and neointimal formation in an arterial balloon-injury model. Our results suggest that P-selectin could play a key role in the restenotic processes after balloon injury and that restenosis after angioplasty could be modified by the attenuation of harmful inflammatory factors through anti-P-selectin treatment. Further investigations need to focus on the roles of P-selectin and adventitial inflammation in postangioplasty restenosis.

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