E-Selectin Blockade Decreases Adventitial Inflammation and Attenuates Intimal Hyperplasia in Rat Carotid Arteries After Balloon Injury

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Objective—Inflammation is one of the initial repair processes after vascular injury. E-selectin facilitates adherence of leukocytes to vascular endothelium at the site of inflammation. Because the role of E-selectin in this process is not fully understood, we studied the role of E-selectin in vascular injury with a flow chamber model and a rat model of carotid artery injury.

Methods and Results—We established a rat aortic endothelial cell (RAEC) culture system from the aortas of adult male rats. When rat myelomonocytes were suspended in a flow chamber, rolling and adhesion to lipopolysaccharide (LPS)-stimulated RAECs were observed. Cell rolling and adhesion were greatly reduced by addition of anti–E-selectin monoclonal antibody (mAb). We then induced balloon injury in the left carotid arteries of rats. E-selectin expression was enhanced in endothelial cells at adventitial small vessels 7 days after injury. Rats with balloon injury were injected intraperitoneally with anti–E-selectin mAb for 8 days. Inflammatory cell infiltration was reduced by anti–E-selectin mAb treatment at the adventitia at 7 days after injury. This reduction was associated with attenuation of intimal hyperplasia in the rats treated with the mAb.

Conclusions—These data suggest that E-selectin regulates adventitial inflammation through leukocyte adhesion and contributes to the process of intimal hyperplasia after balloon injury. (Arterioscler Thromb Vasc Biol. 2004; 24:2063-2068.)

Key Words: adhesion molecules ▪ adventitial inflammation ▪ angioplasty ▪ rat aortic endothelial cell ▪ restenosis

Restenosis after percutaneous catheter intervention remains a serious clinical problem despite the development of new catheterization devices.1–5 Neointima formation is caused by vascular smooth muscle cell proliferation. Various factors are involved in this process.1–5 Among them, cell adhesion molecules are essential for the development of atherosclerosis.6–8 A number of soluble factors are expressed by endothelial cells and recruited leukocytes.7,9 P-selectin is one such cell adhesion molecule and is an established indicator of chronic and acute atherosclerotic events.10–13 Elevated plasma levels of soluble P-selectin (sP-selectin) are associated with increased vascular injury in acute and chronic coronary artery disease (CAD).12 We reported previously that anti–P-selectin monoclonal antibody (mAb) attenuates inflammatory responses and inhibits neointima formation in association with platelet accumulation after balloon injury of the carotid artery in rats.13

E-selectin assists in the rolling of leukocytes on activated endothelial cells and is expressed on the endothelium of atherosclerotic lesions.7,9 Several studies have shown elevated plasma levels of soluble E-selectin (sE-selectin) in CAD. In one study, sE-selectin levels did not differ significantly between CAD patients and control subjects,14 whereas other studies indicated that sE-selectin levels increased after angioplasty.15,16 Examination of expression of cell adhesion molecule mRNAs in atherectomy specimens from patients with CAD revealed that expression of E-selectin mRNA was increased in restenosed coronary arteries in comparison with de novo lesions.17 In peripheral arteries, vessel patency after angioplasty is associated with low levels of sE-selectin.18 Despite these clinical findings, the role of E-selectin in acute vascular injury has not been investigated in animals. It is not known if blockade of E-selectin activity can attenuate intimal hyperplasia after vascular injury.

Thus, in the present study, we developed a new anti-rat E-selectin mAb and evaluated roles of E-selectin in the neointimal formation. We used an in vivo rat carotid artery balloon injury model and an in vitro flow chamber leukocyte

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was generated as previously described. Specificities of these blocking mAb was named S1096B. Anti-rat P-selectin mAb (S789G) Cell binding assay was performed as described. The adhesion-hybridomas were screened for the ability to bind to E-CHO cells. (Life Technologies, Inc, Grand Island, NY), and supernatants from cells were fused to mouse myeloma cells from the Japanese BALB/c mice by footpad injection of 2 to 5 /H11003 Anti-rat E-selectin mAbs were generated by immunizing female Rat E-selectin–expressing CHO cells and rat E-selectin–expressing CHO cells were stained with mAbs (bold lines) or isotype control antibodies (thin lines). b, S1096B inhibited the binding of HL-60 cells to rat E-selectin–expressing CHO cells in a concentration-dependent manner. c, S789G inhibited the binding of HL-60 cells to rat P-selectin–expressing CHO cells in a concentration-dependent manner. Data are expressed as mean ± SEM. FIU indicates fluorescence intensity unit; CHO, Chinese hamster ovary; mAb, monoclonal antibody.

adhesion model to test our hypothesis that expression of E-selectin can be a target for therapeutic intervention to reduce restenosis after angioplasty.

Methods

Animals

All animals were obtained from Japan SLC, Inc (Shizuoka, Japan) and were housed and handled as approved by the Institutional Animal Use and Care Committee of Tokyo Medical and Dental University. All experiments were conducted in conformity with the Institutional Guidelines of Tokyo Medical and Dental University.

Preparation of Anti-Rat E-Selectin MAb

Anti-rat E-selectin mAbs were generated by immunizing female BALB/c mice by footpad injection of 2 to 5×10⁷ rat E-selectin–expressing Chinese hamster ovary (E-CHO). Polyclonal lymph node cells were fused to mouse myeloma cells from the Japanese Collection of Research Biosaources (JCRB0113) with PEG4000 (Life Technologies, Inc, Grand Island, NY), and supernatants from the hybridomas were screened for the ability to bind to E-CHO cells. Cell binding assay was performed as described. The adhesion-blocking mAb was named S1096B. Anti-rat P-selectin mAb (S789G) was generated as previously described. Specificities of these mAbs were confirmed by flow cytometry (Figure 1a). Flow cytometry was performed on a fluorescence-activated cell sorter Calibur (BD Bioscience) with these mAbs coupled to fluorescein isothiocyanate.

In Vitro Cell Binding Assay (Static)

E-CHO monolayers were washed with phosphate-buffered saline and incubated with anti–E-selectin mAb at various concentrations (0.1, 0.3, 1, 3, 10 μg/mL) for 30 minutes at 4°C. Cultured HL-60 cells were suspended at a concentration of 1×10³ cells/mL in RPMI1640 (Sigma, St. Louis, Mo) containing 10% fetal bovine serum (FBS) (Sigma). 3′-O-acetyl-2′,7′-bis (carboxyl-ethyl)-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) was added to the cell suspension to a final concentration of 3 μM/L. The cell suspension was incubated at 37°C for 30 minutes, and labeled cells were resuspended at a concentration of 2.5×10⁴ cells/mL in RPMI1640 containing 1% FBS. Cell suspensions were plated at 200 μL/well, incubated for 30 minutes at 4°C, centrifuged at 120 rpm, and washed with phosphate-buffered saline containing 1% FBS. The attached cells were lysed with 1% Triton X-100 (Sigma). The fluorescence intensity unit of the cell lysates was measured with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Rat Aortic Endothelial Cell Culture

Three male Sprague–Dawley rats weighing 300 to 400 grams were killed by an overdose injection of pentobarbital sodium. The thoracic aorta was removed immediately and washed with RPMI1640 containing 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc). The aorta was filled with 1000 U/mL dispase (Godo Shusei, Tokyo, Japan) and 1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and incubated for 30 minutes at 37°C. The solution was then collected and centrifuged for 5 minutes at 1500 rpm. The supernatant was removed, and the cells were resuspended in RPMI 1640 containing 100 U/mL penicillin and 100 μg/mL streptomycin and 1 μg/mL amphotericin B (Sigma) and cultured on a rat collagen–I–coated dish (BD Biosciences). Rat aorta endothelial cells (RAECs) were identified by their characteristic cobblestone appearance, and identification was confirmed by the uptake of acetylated low-density lipoprotein labeled with 1,1′dioctadecyl-1 to 3,3′,3′-tetramethylrhodamine-diacarbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies, Inc., Stoughton, Mass). RAECs at passages 5 through 7 were used for experiments.

Dynamic Flow Assay

Two hundred microliters of RAEC suspension was put on a rat collagen–I–coated coverslip (BD Biosciences) and placed over a 6-well plate at a seeding density of 3×10⁵ cells/mL. Three days later, RAECs were stimulated by 1×10⁶ cells/mL in RPMI1640 containing 1% FBS. Cell suspensions were washed twice with RPMI1640 containing 1% FBS and incubated with or without antibody (10 µg/mL) S1096B or MOPC-21 (non-immunized mouse IgG1; Pharmingen, San Diego, Calif). The flow chamber was constructed as described previously. A plastic heating plate (Tokai Hit Co) was mounted on the stage of an inverted microscope (IX50; Olympus) to maintain the temperature of the chamber at 37°C. The coverslip was attached to the chamber placed on the microscope stage. The system was then filled with 1×10⁶ cells/mL of rat myelomonocytic cell (c-WRT-7-LR, Health Science Research Resources Bank, JCRB0168) suspended in adhesion media (Dulbecco modified phosphate-buffered saline + 0.9 mmol/L CaCl₂, + 0.2% human albumin). This suspension was drawn off at controlled flow rates with a syringe pump (Model 44; Harvard Apparatus) connected to the outlet flow chamber to generate calculated wall shear stresses of 1.0 dyne/cm² for 10 minutes. The perfusion period was videotaped with a digital video recorder containing a time generator. The captured images were transferred to a PC computer for image analysis to determine the number of rolling and adherent c-WRT-7-LR cells in 5 to 10 randomly selected 200× microscopic fields for each experiment. Cells were considered adherent after 10 seconds of stable contact with the monolayer. Rolling leukocytes were easily recognized because their velocities were much slower (up to 80 μm/s) than those of free-flowing cells.

Vascular Injury Model

Male Sprague–Dawley rats were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). The rat carotid
arteries were dilated and denuded of endothelium with a 2-French Fogarty balloon embolectomy catheter (Baxter Health Care) introduced into the left common carotid artery through the external carotid artery.24,25 The catheter was retracted 3 times. After the catheter was removed, the external carotid artery was ligated.

**MAb Treatment Study**

Rats were assigned randomly to 1 of 4 treatment groups: MOPC-21 (nonimmunized IgG) treatment (group C), S1096B (anti–E-selectin mAb) treatment (group E), S789G (anti–P-selectin mAb) treatment (group P), and both S1096B and S789G treatment (group E+P). MAbs were administered intraperitoneally to rats at a dosage of 4 mg/kg 30 minutes before arterial injury and once daily for 7 consecutive days after injury as previously reported.13 Carotid arteries were harvested 14 days (n=8, each group) and 56 days (n=8, each group) after balloon injury.

**Histology and Morphometry**

Treated rats were euthanized by overdose injection of pentobarbital, and the injured carotid artery was then perfusion-fixed at 100 mm Hg and embedded in paraffin. Each artery was stained with van Gieson elastin stain and subjected to blinded morphometric examination under a video microscope (HC-300I; Nikon) equipped with a computerized digital image analysis system (SCION Image, public domain software). The areas of the external elastic lamina (IEL), the internal elastic lamina (IEL), and the lumen were measured. Medial and neointimal areas were calculated as follows: medial area=IEL area – IEL area; neointimal area=IEL area – lumen area; neointima/media (I/M) ratio=neointimal area/media area. The circumferences (lengths) of the EEL and IEL were also measured to determine vascular shrinking.

**Immunohistochemical Study**

Harvested carotid arteries were immediately embedded in optimal cutting temperature compound and frozen at −20°C. Immunohistochemical analysis was performed on the frozen sections. A Vectastain Elite ABC kit (Vector Laboratories) was used on the sections with S1096B (anti–E-selectin), S789G (anti–P-selectin), anti–CD31 antibody (PharMingen), anti–CD45 antibody (PharMingen), anti–ED-1 antibody (Cosmo Bio, Tokyo, Japan), and MOPC-21 (nonimmunized IgG) as primary antibodies. Sections were incubated with diaminobenzidine (Vector Laboratories) at 100 mg/mL for 5 minutes, counterstained with hematoxylin, and then mounted permanently with coverslips.

**Statistical Analysis**

All data are presented as mean±SEM. Experimental groups were compared with 1-way analysis of variance and a post hoc test (Fisher protected least significant difference) for multiple comparisons. Values derived from the dynamic flow assay were compared by Mann–Whitney U test. P<0.05 were considered statistically significant.

**Results**

**Characterization of Anti–E-Selectin Monoclonal Antibody (S1096B)**

S1096B is a mouse IgG1 antibody. The specificity of this anti–E-selectin mAb was confirmed by flow cytometry by binding to E-CHO cells. S1096B bound to E-CHO cells but not to P-selectin–expressing CHO cells (P-CHO). S789G bound to P-CHO cells, but not to E-CHO cells (Figure 1a). HL-60 cells adhered to E-CHO cells untreated with antibody. S1096B blocked adhesion of HL-60 cells to E-CHO cells in a concentration-dependent manner (Figure 1b). S789G blocked adhesion of HL-60 cells to P-CHO cells in a concentration-dependent manner (Figure 1c).

**Expression of E-Selectin After Vascular Injury In Vivo**

Representative photomicrographs of arteries harvested and sectioned 7 days after balloon injury and stained with
antibody to E-selectin or P-selectin are shown in Figure 3. Positive staining was not observed in sections stained with control IgG (data not shown). We used anti-CD31 antibody to visualize endothelial cells. Most CD31-positive cells were present in the adventitia, indicating that the injured lumina had not yet re-endothelialized at 7 days after injury. E-selectin and P-selectin staining of adventitial endothelial cells was increased. In the sham operation group, faint staining of both selectins was noted. Bar=10 μm.

S1096B Blocks E-Selectin–Dependent Leukocyte Accumulation

We examined the effect of S1096B on leukocyte accumulation after vascular injury by immunohistochemistry. We used anti-CD45 antibody to detect leukocytes and anti–ED-1 antibody to detect macrophages (Figure 4a) and analyzed percentages of CD45-positive and ED-1–positive cells over time (Figure 4b). At days 1 and 3 after injury, the intima and media could not be clearly differentiated. In the early period after vascular injury, CD45-positive and ED-1–positive cells accumulated predominantly in the adventitia. With the progression of intimal hyperplasia, these cells transmigrated into the intima. The accumulation of CD45-positive and ED-1–positive cells in the adventitia was inhibited by treatment with S1096B.

Inhibition of E-Selectin–Dependent Adhesion Attenuates Injury-Induced Intimal Hyperplasia and Vascular Remodeling

There was no significant difference in body weight between groups of rats. Morphometric analysis is shown in Figure 5a. The I/M ratio of group E was significantly less than that of group C at 8 weeks after injury (1.49±0.20 versus 2.07±0.14; *P<0.05). The ratios at 2 weeks after injury did not differ statistically (1.40±0.11 versus 1.62±0.15) (Figure 5a, left). The I/M ratio of group P and that of group E+P were significantly lower than that of group C at 2 weeks after injury (1.16±0.17 and 1.10±0.09, respectively, versus 1.62±0.15; *P<0.05). The I/M ratio of group P was not different from that of group E+P at 2 and 8 weeks after injury. Thus, a synergistic effect of dual blockade of E-selectin and P-selectin was not identified. The neointimal areas in group E and group P were reduced compared with that in group C at 8 weeks after injury. The medial area in each of the 4 groups did not differ significantly at 8 weeks after injury. The IEL and EEL lengths are significantly greater in the mAb treatment groups (group E, group P, and group E+P) than group C at 8 weeks after injury indicating suppression of negative remodeling (Figure 5b). A synergistic effect of dual blockade of E-selectin and P-selectin was not identified in this analysis. Representative
E-selectin mediates rolling of leukocytes at the site of inflammation. Our in vivo study showed that anti–E-selectin mAb attenuated intimal hyperplasia after balloon injury, with significantly reduced infiltration of leukocytes in the adventitia.

E-selectin mediates rolling of leukocytes at the site of inflammation, and expression of E-selectin is limited to endothelial cells in inflamed tissue. E-selectin cannot play a role in the earliest phases of acute inflammation because de novo gene transcription is necessary for expression. Our immunohistochemical studies showed that normal vessel walls do not express E-selectin. Similarly, myelomonocytes did not adhere to unstimulated RAECs. We found that injured lumina had not re-endothelialized at 7 days after balloon injury and that E-selectin was not expressed on the luminal side but was expressed on endothelial cells in the adventitia. Most inflammatory cells were identified in the adventitia, particularly in the vasa vasorum, and anti-E-selectin mAb reduced the infiltration of inflammatory cells in this region at this stage. Thus, E-selectin plays a role in leukocyte accumulation in the adventitia in our model of vascular injury.

Mechanical injury of arteries leads to the infiltration of inflammatory cells, release of chemokines, cytokines, and other chemical mediators; transmigration of monocytes and macrophages; and proliferation of vascular smooth muscle cells. Our findings indicate that suppression of inflammatory cell infiltration into the adventitia by anti–E-selectin mAb is associated with reduced intimal hyperplasia after balloon injury. Thus, inflammation in the adventitia could be an important factor in the development of intimal hyperplasia and vascular shrinkage.

The mechanism of adventitial inflammation in intimal hyperplasia is unknown. The intensity of the adventitial inflammatory response correlates with the severity of atherosclerosis and restenosis after balloon angioplasty. Although we did not determine the role of E-selectin in these processes in the present study, there are several possible mechanisms. For example, inflammatory cells that infiltrate in response to enhanced E-selectin expression in the adventitia may release a variety of chemical mediators, including cytokines, chemokines, and growth factors, which promote transmigration and proliferation of vascular smooth muscle cells in the media and lead to hyperplasia of the intima. In addition, our data show improvement of vascular shrinkage after mAb treatment, indicating that inflammation of the adventitia leads to negative remodeling of the injured vessels. This remodeling could be reduced by anti-selectin treatment.

Our previous study showed that an anti–P-selectin mAb reduces the accumulation of leukocytes in the adventitia and neointima and prevents neointimal formation at 2 weeks after balloon injury. Similar results were obtained in the present study. According to our previous study, expression of P-selectin on platelets is important for suppression of inflammation and for reduction of intimal formation. In the present study, anti–E-selectin mAb significantly attenuates intimal hyperplasia at 8 weeks after injury. However, it did not attenuate intimal hyperplasia at 2 weeks after injury. Unlike E-selectin, P-selectin expression by endothelial cells and platelets does not require de novo gene transcription. Therefore, P-selectin responds immediately to acute inflammation that is caused by arterial injury. These differences between the 2 selectins may account for their different roles in intimal thickening. We suggest that adventitial inflammation mediates late-stage intimal hyperplasia via expression of E-selectin. This is supported by our analysis of inflammatory cell infiltration overtime, as shown in Figure 4. Cell infiltration in the adventitia was observed initially at 3 days after induction of injury. This was followed by inflammation in the intima at 7 to 14 days after injury and by intimal hyperplasia at later stages. Anti–E–selectin mAb suppressed early-stage adventitial infiltration. Therefore, it is reasonable to speculate that inhibition of adventitial inflammation by blockade of

**Discussion**

In the present study, we found that anti–E-selectin mAb S1096B blocked rolling and adhesion of myelomonocytes on aortic endothelial cells in an in vitro flow chamber model. Our previous study showed that anti–E-selectin mAb attenuated intimal hyperplasia after balloon injury, with significantly reduced infiltration of leukocytes in the adventitia.

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E-selectin–dependent cell adhesion leads to the attenuation of intimal hyperplasia observed at 8 weeks after injury. Because P-selectin and E-selectin play different roles in the recruitment of leukocytes into the site of inflammation, a synergistic action of dual blockade of the 2 selectins was expected. However, we did not observe such synergism in the present study. Tendencies toward decreased I/M ratios and intimal areas but without statistical significance were detected. Also, the IEL and EEL lengths of group P and group E were not different from those of group E+P at 8 weeks after injury. Possible synergism of these selectins will be studied in future experiments.

In conclusion, these data suggest that E-selectin controls adventitial inflammation through leukocyte adhesion and contributes to the process of intimal hyperplasia in the late stage after balloon injury. Blockade of E-selectin may be a new strategy to control restenosis after coronary balloon angioplasty.

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