Inhibition of coronary thrombosis and local inflammation by a noncarbohydrate selectin inhibitor

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Inhibition of coronary thrombosis and local inflammation by a noncarbohydrate selectin inhibitor. Am J Physiol Heart Circ Physiol 279: H3065–H3075, 2000.—We tested the hypothesis that selectin inhibition with blocking antibodies or a small-molecular-weight inhibitor of L-, P-, and E-selectin, methoxybenzoylpropionic acid (MBPA), prevents thrombus formation in a canine coronary Folts’ model. Cyclic flow variations (CFVs) were induced by crush injury and constriction of the left anterior descending coronary artery in dogs. Systemic infusion of antibodies to P- and L-selectin abolished CFVs, respectively, in 50% and 17% of treated dogs (P = not significant (NS)). The combination of P- and L-selectin antibodies suppressed CFVs in 60% of treated dogs (P = NS). In contrast, systemic selectin blockade by intravenous infusion or local adventitial application of MBPA markedly reduced CFVs and, in addition, reduced myocardial myeloperoxidase (MPO) activity. We conclude that inhibition of L-, P-, and E-selectin binding by a small-molecular-weight, noncarbohydrate compound markedly reduces arterial thrombosis, whereas systemic administration of antibodies to L- and P-selectin fail to reproduce this antithrombotic effect. These results underscore the role of selectins in the pathogenesis of arterial thrombosis under high shear stress and suggest that inhibition of P- and L-selectin may not suffice to prevent thrombus formation in this model. The role of E-selectin in thrombus formation in this model awaits further testing.

selectins; platelet inhibition; P-selectin; antithrombotics

TOGETHER, E-, P-, and L-selectin make up a family of Ca2+-dependent cell adhesion molecules that bind specific carbohydrates that are present on the surfaces of opposing cells (6, 18). It is selectin adherence to monocytes, neutrophils, and eosinophils that is believed to reduce the velocity of these cells, causing them to tether and roll along the endothelial surface prior to firm attachment and subsequent extravasation at sites of tissue damage (6, 12).

L-selectin is constitutively expressed on the surface of leukocytes, binds to inducible ligands on postcapillary venules and endothelial venules of lymph nodes, and is rapidly shed from the cell surface after leukocyte activation (6, 25, 46). Neutrophil L-selectin is a ligand for E- and P-selectin (39, 41) and, cooperatively with P-selectin glycoprotein ligand-1, mediates the initial attachment of neutrophils to P- and E-selectin under flow (27, 41). E-selectin is expressed only by endothelial cells upon activation by cytokines, including interleukin-1, tumor necrosis factor-α, and oxygen-derived free radicals (7, 43). The expression of E-selectin, which binds to ligands on myeloid cells and a subset of lymphocytes, requires de novo transcription and protein synthesis and is detectable 2–3 h after endothelial cell activation (45). In contrast, P-selectin is stored in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells (31) and rapidly translocates to the cell surface in response to agonists, such as thrombin, histamine, and oxygen radicals (9, 40, 48).

The role of the selectins in blood coagulation and blood cell-vessel wall interactions has been recognized only relatively recently (19, 24, 38), as has their involvement in the pathogenesis of myocardial reperfusion injury (28, 34) and allograft vasculopathy (2, 10, 42). However, less is known about their role in thrombus formation at sites of vascular injury. A recent report indicates that the carbohydrate selectin ligand sialyl Lewis-x (sLeα) had no effect on cyclic flow variations (CFVs) when tested in a canine Folts’ model (52). Further analyses by the same authors indicates that treatment with a similar but more potent, synthetic, carbohydrate-based selectin inhibitor, sLeα-oligosaccharide (OS), abolishes CFVs (52).

Here, we report that inhibition of P-selectin with a blocking antibody or a small-molecule selectin inhibi-
tor, 3-(4-methoxybenzoyl)propionic acid (MBPA), abolishes CFVs. Assessment of MPO activity in surrounding tissues indicates that a combination of P- and L-selectin blocking antibodies or MBPA alone effectively reduce leukocyte infiltration into the tissue. Importantly, and clinically potentially relevant, local administration of the selectin inhibitor also abolishes CFVs. These data emphasize and help clarify the role of vascular selectins and local leukocyte infiltration in the pathogenesis of arterial thrombosis at the site of vascular injury.

METHODS AND MATERIALS

Creation of Arterial Injury and Stenosis in a Canine Coronary Folts’ Model

All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Institutional Animal Care and Use Committee at the Texas Heart Institute. Sixty dogs of either sex, weighing between 21 and 32 kg (26 ± 0.4 kg, means ± SD), were studied. Dogs were sedated with acepromazine (5 mg im), anesthetized with pentobarbital sodium (30 mg/kg iv), and ventilated with a mechanical respirator (model 613; Harvard, South Natick, MA). Catheters were placed in the carotid artery for aortic pressure measurements and in a jugular vein for fluid and systemic drug administration. A left thoracotomy was performed, and the heart was suspended in a pericardial cradle as previously described (15). A 2-cm segment of the left anterior descending coronary artery was carefully isolated. An ultrasonic Doppler flow probe (Hartley Instruments, Houston, TX) was placed around the proximal portion of the isolated segment to measure phasic and mean coronary blood flow velocities. Baseline heart rate, systolic and diastolic pressures, and phasic and mean coronary blood flow velocities were recorded on a multichannel recorder (model 3800; Gould, Cleveland, OH).

CFVs to zero flow were induced in the isolated segment of the left anterior descending artery by standardized vascular injury. This was created by externally crushing the isolated vessel segment 40 times with a rubber-tipped DeBakey forceps (20 times per 1-cm segment), followed by placement of an external constrictor at the center of the damaged area. Blood flow through the injured, constricted coronary segment was measured by Doppler flow probe, and the constrictor was adjusted to create a 40–50% reduction in flow. Injury and stenosis in this model lead to severe intermittent flow reductions (CFVs), often to zero flow, as a result of thrombus accumulation and vasoconstriction. Occlusive coronary blood clots may require manual dislodgment to restore flow. After vascular injury and stenosis were induced, all animals were observed for 30 min for the presence of severe CFVs resulting in intermittent complete cessation of coronary flow. Dogs that did not show intermittent flow reductions to zero flow were killed at the end of this control period and were not studied further. The dogs were then assigned to several treatment groups, and the number and severity of CFVs were continuously recorded for 6–8 h (see Treatment Groups in the Coronary Thrombosis Model), followed by death of the animals by pentobarbital and potassium chloride overdose. Hematocrits were measured immediately before administration of the selectin inhibitors and again at death.

Treatment Groups in the Coronary Thrombosis Model

This study was carried out in three phases. In the first phase, we studied the effect of systemically administered MBPA on CFVs induced by severe crush injury and constriction of the left anterior descending coronary artery. In the second phase, we tested the effect of MBPA in pluronic gel, applied locally on the adventitial surface of the injured constricted artery. Finally, we investigated the effect of systemically administered antibodies against L- and P-selectin, or both, on coronary CFVs. Dogs receiving the synthetic selectin inhibitor in the first two phases were observed for 6 h after establishment of CFVs and drug administration (MBPA or placebo administration), whereas animals given antibodies were observed for 8 h.

MBPA. MBPA has been described elsewhere (Bjerke R, Tiitinen R, Hu X, Na P, Sherwood S, Revelle BM, Kogan TP, Dixon RA, Yeh ET, and Beck PJ, unpublished observations; Ref. 32). The material used in this study was purchased from Aldrich (Milwaukee, MI). At pH 7.4, MBPA dissolves in aqueous medium at concentrations up to 125 mM. For its use in the experiments described, MBPA was dissolved in sterile, physiological saline solution (0.9% NaCl) and either administered intravenously at the indicated doses or added to F-127 Pluronic gel (a gift from Dr. Jay Otten, BASF, Wyandotte, MI) to a final concentration of 25 mg/ml of gel. Before the polymer was added to MBPA in normal saline, or to saline alone, all solutions were chilled to 4°C, where the polymer is in the liquid phase.

The relative molecular mass (M_r) of MBPA is 208, and the structure is similar in charge and composition to that of an amino acid. No toxicity resulting from administration of MBPA was observed. Treated animals appeared identical to control animals and displayed no differences in heart rate, blood pressure, or temperature. Furthermore, when >24 g per day of MBPA (0.75 g/kg body wt) were administered to 5 dogs over a 10-day period, the activity and appearance of the animals were normal. Treated animals could not be distinguished from placebo control animals. Complete 1) blood counts, which included platelet count, white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and leukocyte differentials, and 2) blood chemistries, which included glucose, urea nitrogen, creatinine, blood urea nitrogen/creatinine, sodium, potassium, chloride, calcium, phosphorous, total protein, albumin, globulin, A/G ratio, total, direct, and indirect bilirubin, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, aspartate transaminase, alanine transaminase, uric acid, iron, triglycerides, and cholesterol, performed (SmithKline Beecham Clinical Laboratories) at the end of the 10-day regimen were within normal range for both MBPA and placebo control animals (data not shown). Similar results were obtained after administration of MBPA in acute rodent studies (Bjerke et al., unpublished observations). MBPA at concentrations ranging from 1 mM to 0.1 μM had no significant inhibitory activity in each of 15 random receptor binding assays tested (Oceanix Biosciences, Hanover, MD). Likewise, vascular cell adhesion molecule-1 (VCAM-1) binding to α5β1 and Mac-1 binding to intercellular adhesion molecule-1 (ICAM-1) are not inhibited by 10 mM MBPA (Table 1). Together, these results indicate that MBPA is a relatively specific selectin binding inhibitor and that, like other selectin inhibitors including soluble sLeX carbohydrate, MBPA exhibits no toxic or obviously detrimental effects in the dose range tested for the acute studies reported here and elsewhere (Bjerke et al., unpublished observations; Ref. 1).
Table 1. Effect of MBPA on VCAM-1 and Mac-1 receptor binding

<table>
<thead>
<tr>
<th>MBPA, mM</th>
<th>HeLa bound to Mac-1</th>
<th>HeLa bound to Mock</th>
<th>U937 bound to VCAM</th>
<th>U937 bound to Mock</th>
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<tbody>
<tr>
<td>0</td>
<td>102 ± 16</td>
<td>16 ± 6</td>
<td>60 ± 13</td>
<td>20 ± 7</td>
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<tr>
<td>10</td>
<td>212 ± 90</td>
<td>NT</td>
<td>76 ± 15</td>
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COS-1 cells were transfected with empty vector (Mock), vascular cell adhesion molecule (VCAM), or Mac-1 cDNA. Twenty-four hours after transfection, Mac-1 binding to HeLa cells expressing intercellular adhesion molecule-1 (ICAM-1) or VCAM binding to U937 cells expressing α4β1, was assessed in the presence or absence of methoxybenzylpropionic acid (MBPA) using established methods. Results are the means of 2 experiments completed in triplicate; NT, not tested. Results obtained for Mac-1 and VCAM binding in the presence and absence of MBPA are not statistically significant (P < 0.3 for Mac-1 and P ≥ 0.5 for VCAM). Probability values for Mac-1 and VCAM binding in either the presence or absence of MBPA are significant (P < 0.05) compared with binding to mock-transfected cells.

Intravenous administration of MBPA after coronary injury. For group Ia (n = 8), after CFVs were established, MBPA was given as a 50 mg/kg bolus followed by an infusion started at 50 mg·kg⁻¹·h⁻¹. The infusion of MBPA was doubled every 30 min to a maximum of 400 mg·kg⁻¹·h⁻¹ or until CFVs stopped. During the last 45 min of the 6-h observation period, four dogs in this group received intravenous epinephrine, which was started at 0.76 μg/min and increased at 20-min intervals to 1.5, 3.8, 7.6, 15, and 38 μg/min. For group Ib (n = 6), after injury was induced, these animals received initially a bolus and 6-h infusion of physiological saline solution at doses matching the MBPA infusion in group Ia. Severe CFVs were allowed to proceed for 6 h. The animals then received incremental doses of MBPA as described for group Ia. For group Ic (n = 4), after 30 min of CFVs, a bolus and 6-h infusion of isotonic saline solution were given to match the infusion of MBPA.

Local adventitial application of MBPA to the site of coronary injury. For group II, coronary injury and stenosis were created, followed by an initial control period of uninhibited CFVs as described in Intravenous administration of MBPA after coronary injury. MBPA dissolved in Pluronic gel F-127 was then applied to the adventitia of 14 dogs at a concentration of 25 mg/ml. Pluronic gel containing either 200 μl of MBPA (5 mg) in physiological saline solution or an equivalent amount of saline solution alone was applied to the adventitia of the injured, stenosed arterial segment. The time course and severity of CFVs were then monitored for 6 h, during which the gel (with or without MBPA) was reapplied to the artery as needed to ensure that the artery was covered with gel during the entire observation period. In a subgroup of these dogs, epinephrine was administered intravenously at doubling doses from 0.76 to 38 μg/min. Because CFVs sometimes require manual dislodgment of the coronary thrombus, the Pluronic gel (with or without MBPA) was reapplied to the injured site at a maximum of 2 ml/artery (50 mg of MBPA). After the 6-h observation period, the animals were killed by pentobarbital and potassium chloride overdose. Six animals received adventitial Pluronic gel (up to 2 ml total) without MBPA.

Systemic administration of P- and L-selectin antibodies. For group III, injury and stenosis were created as described earlier, and uninhibited CFVs were monitored for 30 min. Monoclonal antibodies to P-selectin (n = 6), L-selectin (n = 6), or both (n = 10), were then administered at a dose of 0.3 mg/kg; administration was repeated once 60 min later if CFVs did not abate. After an 8-h observation period, the animals were killed without epinephrine challenge.

Effect of Selectin Inhibitors on Platelet Aggregation

Ex vivo studies of platelet aggregation were performed in two phases. First, platelet aggregation was performed on citrated blood of animals receiving MBPA in the course of the evaluation of its effects on CFVs. Within 20 min of thoracotomy, but before coronary injury was created, arterial blood was drawn into 1/10 volume of 3.8% sodium citrate and centrifuged at 200 g for 15 min at room temperature to prepare platelet-rich plasma (PRP). An aliquot of PRP was further centrifuged for 15 min at 3,000 g to obtain platelet-poor plasma. Aggregation studies were performed to increasing concentrations (5, 10, and 20 μM) of ADP (Sigma, St. Louis, MO) in a four-channel aggregometer (model PAP-4; BioData, Horsham, PA). A second sample of arterial blood was drawn 2 h after administration of the synthetic selectin inhibitor or the selectin antibodies and was used for aggregation studies to the same concentrations of ADP.

Second, to exclude inhibition of selectin-ligand interaction by heparin or Ca²⁺ removal, the effect of MBPA on the response to platelet agonists was studied in whole blood drawn into 1/10 (vol/vol) of the specific thrombin inhibitor argatroban (1 mg/ml; Texas Biotechnology, Houston, TX). Whole blood aggregation was studied in a Chronolog Lumi-aggregometer (model 560VS). Nine animals were studied. Five dogs received 100 mg/kg of MBPA followed by a maintenance infusion of 262 mg·kg⁻¹·h⁻¹. Four dogs received saline solution alone. After baseline aggregations were performed to collagen, the thromboxane analog U-46619, serotonin (5-HT), and ADP in all animals, CFVs were initiated by vascular injury as described and were allowed to proceed for 30 min. Fifteen minutes after administration of either MBPA or saline alone, blood was collected into argatroban, and platelet aggregation studies were repeated to the agonists at the concentrations used at baseline (see Results). The extent of aggregation is reported as percent decrease (in ohms) from baseline.

Serum Drug Levels

MBPA serum levels were monitored by reverse phase high-performance liquid chromatography using a C-18 column. Serum samples were diluted fivefold in 5% acetonitrile and 0.1% trifluoroacetic acid and injected onto the column. The column was then eluted with a linear gradient of 95% acetonitrile and 0.1% trifluoroacetic acid. Concentrations of MBPA were assessed from absorbance readings at 250 nm against a standard curve generated from serially diluted samples of known concentration.

Assessment of Local Myocardial Inflammation

In the group III dogs, which received systemic P- and L-selectin-specific antibodies, the coronary arteries and immediately adjacent myocardium were flash frozen in liquid nitrogen and stored at −80°C. The activity of MPO, a surrogate measure of leukocyte (monocyte and neutrophil) infiltration, was then assayed in a 1-cm-long myocardium segment immediately adjacent to the center of the injured coronary artery. The assay was performed as previously described (35), and the MPO activity was expressed in units per 100 mg of tissue weight. Myocardial tissues from dogs that had not undergone injury were used to control for possible baseline MPO activity.

To assess the effect of systemic MBPA administration on myocardial MPO activity, we measured MPO activity in 12...
additional dogs treated after 30 min of undisturbed CFVs with either systemic MBPA (100 mg/kg followed by an infusion of 262 mg·kg⁻¹·h⁻¹) or saline (both n = 6). Animals were killed after 8 h, and MPO activity was assayed as described previously(35).

**Statistical Analysis**

The total number of severe CFVs (with flow reductions to zero flow) in each group was expressed as the mean ± SD or, where a nonnormal distribution was noted, as the median plus 25th and 75th percentiles. Fisher’s exact test, with the Bonferroni correction for multiple testing, was used to detect statistically significant (P < 0.05) differences among groups. Identical statistical analysis was performed to explore differences between CFVs in animals treated with selectin antibodies. The differences in the magnitude of MPO activity after administration of the selectin antibodies, MBPA, or saline was tested by ANOVA, followed by Bonferroni testing to locate differences between the groups.

**Antibodies Against P- and L-Selectin**

The hybridoma cell line expressing DU1–29 was purchased from American Type Culture Collection (Rockville, MD). The MD-6 hybridoma was a generous gift from C. Wayne Smith (Baylor College of Medicine, Houston, TX). Each monoclonal antibody was purified from mouse ascites. Purification was performed with a protein G-Sepharose column using the manufacturer’s recommendations (Amerham-Pharmacia Biotech, Piscataway, NJ). The IgG was quantitated by ELISA with the use of a purchased isotype control antibody of known concentration (Cappel). The DU1–29 antibody was tested for binding to canine L-selectin by ELISA and by magnetic cell separation (5). Canine peripheral blood leukocytes were isolated from EDTA-treated whole blood after centrifugation at 600 g. The leukocyte-rich buffy coat was removed from the cell pellet, and leukocytes were recovered after hypotonic lysis of erythrocytes. Leukocytes were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum. When ELISA was performed, 2 × 10⁶ cells were lysed in 0.5 ml of Nonidet P-40 lysis buffer (44). Falcon probind ELISA plates precoated with anti-L-selectin antibody SK11 (10 μg/ml in PBS; Becton-Dickinson, San Jose, CA) were washed and incubated with 5 μl of leukocyte lysate diluted in 45 μl of PBS for 2 h. Alkaline phosphatase-conjugated goat anti-mouse IgG1 antibody (Caltag Laboratories, Burlingame, CA) was the detecting antibody according to the procedures of Harlow and Lane (23). ELISA results indicated that canine L-selectin was readily captured by the SK11 (IgG2A) antibody and specifically recognized by DU1–29 (IgG1), but not by the anti-P-selectin MD-6 (IgG1) antibody (data not shown).

Alternatively, the DU1–29 and MD-6 antibodies were independently adsorbed to goat anti-mouse-conjugated magnetic beads (Dynal, Lake Success, NY). After successive washing, beads were incubated with calcein AM (Molecular Probes, Eugene, OR)-labeled canine leukocytes isolated as described above and labeled according to the manufacturer’s recommendation. After a 10-min incubation at room temperature, bead-bound cells were isolated with the use of a 96-well magnetic separator and quantitated fluorometrically (Cytoflour; Millipore, Bedford, MA). Leukocytes were bound by the DU1–29 antibody, whereas no cells were bound by the MD-6 control.

To investigate the ability of the DU1–29 antibody to block L-selectin binding, canine peripheral blood was treated with heparin to prevent coagulation, and the leukocytes were isolated as described above. The cells were then suspended in RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies, Rockville, MD) and 5% mouse serum (Sigma). The concentration of leukocytes was determined with a hemocytometer and was adjusted to 4 × 10⁶ cells/ml. To perform the ficinoid binding assay, we coated ficinoid (Sigma) onto Falcon probind 96-well plates at a concentration of 10 μg/ml in PBS. Plates were blocked with a 3% solution of BSA in PBS. Canine leukocytes labeled with Syto 17 (15-min incubation on ice in a final concentration of 10 nM Syto 17; Molecular Probes) were treated with a 50 μg/ml final concentration of DU1–29 or a similar, nonspecific mouse antibody and incubated on ice for 30 min. Cells (4 × 10⁵ cells/well) were placed in ficinoid-coated wells and incubated for 10 min at 37°C and then washed twice with 50 μl of PBS. Cell binding was quantitated fluorometrically.

Leukocytes were preblocked with mouse serum (5%), and aggregation experiments were performed in the presence of DU1–29 or a nonspecific mouse antibody. Canine leukocytes were incubated with a 50 μg/ml final concentration of DU1–29 or a nonspecific mouse antibody on ice for 30 min. Cells were transferred to a 37°C water bath for 10 min. Cell aggregation was scored by using phase-contrast light microscopy and a hemocytometer. A total of 200 incidents were counted for each sample and averaged for statistical comparison by using a two-tailed t-test.

**RESULTS**

**Systemic Administration of MBPA Abolishes Coronary CFVs in a Severe Canine Coronary Injury Model**

The ability of selectin blockade to inhibit thrombosis was tested in a coronary Folts’ model with MBPA. After 30 min of consistent CFVs were allowed, group 1a dogs (n = 8) were given MBPA in incremental doses (see METHODS AND MATERIALS). MBPA completely abolished CFVs at a mean effective dose of 262 ± 45 mg·kg⁻¹·h⁻¹ (range 50–400 mg·kg⁻¹·h⁻¹), corresponding to a serum level of 2.1 ± 0.3 mM (range 0.88–4.3 mM). Once abolished, coronary flow remained undisturbed until the end of the 6-h observation period. This was also true of four animals that were given incremental epinephrine infusions at rates of 38 μg/min during the last 45 min of the 6-h observation period.

To determine whether a prolonged phase of severe CFVs following the creation of vascular injury would lessen the antithrombotic efficacy of selectin blockade, we allowed uninhibited CFVs to proceed in group 1b dogs (n = 6) for 6 h before MBPA was administered. Despite this prolonged period of recurrent thrombosis, the average infusion rate to abolish these severe (zero flow) CFVs was 250 ± 60 mg·kg⁻¹·h⁻¹ (range 50–400 mg·kg⁻¹·h⁻¹), yielding a plasma concentration of 2.4 ± 0.4 mM (range 0.8–4.2 mM). This concentration was not significantly different from the MBPA concentration required to abolish CFVs of only 30 min in duration (2.1 ± 0.3 mM), suggesting that the duration of the thrombotic episode, at least between 30 min and 6 h, did not influence the efficacy of this selectin inhibitor. In contrast, severe CFVs did not attenuate in any
of the dogs receiving physiological saline alone (group Ic, n = 4).

Effect on Ex Vivo Systemic Platelet Aggregation

Platelet aggregation studies were performed initially on citrated PRP from blood drawn 2 h after systemic administration of saline, and MBPA showed a mild decrease in aggregation to ADP compared with baseline (Fig. 1). This decrease in platelet aggregation observed 2 h into the infusion of either saline solution or MBPA in saline was not significantly different between the groups.

To exclude the effect of calcium removal on selectin-ligand interaction, we studied the response of platelet aggregation in whole blood (see METHODS AND MATERIALS) to a short-term infusion of MBPA sufficient to abolish CFVs within 15 min in five additional MBPA-treated animals and four additional saline-treated control animals. After coronary CFVs were produced, 100 mg·kg⁻¹·h⁻¹ followed by 262 mg·kg⁻¹·h⁻¹ of MBPA (the mean effective dose in group I dogs) was administered and abolished CFVs within 10 min in all five animals. Control, saline-treated animals received a 15-min infusion of saline after the initial 30 min of CFVs. Platelet aggregation was performed by using several agonists at concentrations shown to produce effective platelet aggregation ex vivo (see METHODS AND MATERIALS). Figure 2 shows that, compared with aggregation with saline alone (A), there was no consistent change in platelet aggregation induced by administration of MBPA (B). A trend toward reduction of aggregation was not statistically significant between MBPA- and saline-treated animals. Comparisons between baseline aggregation and aggregation at 15 min into the infusion of saline or MBPA also did not show statistically significant differences.

Effect of Systemic MBPA on Cell Counts

Complete blood counts, measured at baseline and at death, showed only a mild decrease in platelet counts in both dogs receiving MBPA (178 ± 6 to 158 ± 9 x 10³ platelets/mm³) and dogs receiving saline control (213 ± 13 to 198 ± 15 x 10³ platelets/mm³). There was no significant change in the hematocrit in either group (33.4 ± 1.2 to 34.0 ± 1.4% for the MBPA group and 35.8 ± 1.1 to 35.0 ± 1.4% for the saline control group). Similarly, there was no significant change in leukocyte counts in the two groups (7.2 ± 0.7 to 7.9 ± 0.8 x 10³ cells/mm³ in dogs receiving MBPA and 6.7 ± 0.6 to 7.3 ± 0.8 x 10³ cells/mm³ for saline controls).

Local Application of MBPA Abolishes Coronary CFVs

After establishing that systemic administration of a broad-range selectin inhibition exerts potent antithrombotic effects in this model of coronary thrombosis and vasoconstriction, we tested the hypothesis that a small amount of the inhibitor, topically applied to the injured vascular site, might exert similar antithrombotic effects in the absence of detectable plasma levels of MBPA. Coronary injury and stenosis were created as described earlier, followed by an initial 30-min control period of uninhibited CFVs. Next, 200 µl of Pluronic

Fig. 1. Effect of intravenous methoxybenzoylpropionic acid (MBPA) on ex vivo platelet aggregation in citrated platelet-rich plasma. Platelet aggregation studies performed on citrated platelet-rich plasma from blood drawn 2 h after systemic administration of physiological saline (control) solution or MBPA (in saline) showed a mild decrease in aggregation to ADP compared with baseline. This decrease was not statistically significant. Values are means ± SD.

Fig. 2. Effect of MBPA on whole blood aggregation. Platelet aggregation studies performed on blood drawn into the thrombin inhibitor argatroban after abolition of cyclic flow variations (CFVs; see text) showed no consistent change in the platelet response to the agonist indicated compared with baseline. A: control (saline) group; B: MBPA-treated group. 5-HT, serotonin. Values are means ± SD; n = no. of experiments.
gel containing either MBPA (5 mg) in physiological saline or physiological saline alone were applied to the adventitia of the injured, stenosed arterial segment (see METHODS AND MATERIALS). Of 14 dogs treated topically with the selectin inhibitor, 13 responded with complete abolition of CFVs (mean effective MBPA dose 31.9 ± 35.5 mg, range 5–125 mg). Results are percentages of dogs responding with abolition of CFVs after application of gel alone and gel with added MBPA.

P-Selectin Inhibition Only Partially Reproduces the Antithrombotic Effects of MBPA

To establish whether P- and L-selectin inhibition is involved in maintaining severe thrombosis in this model of vascular injury, we studied CFVs in animals that received monoclonal antibodies against P- and L-selectin, or both. Figure 4 shows the percentage of dogs that showed abolition of CFVs when receiving saline alone or the antibody to P-selectin, L-selectin, or the two antibodies together. Administration of the P-selectin antibody alone abolished CFVs in 3 of 6 animals, and administration of both P- and L-selectin antibodies abolished CFVs in 6 of 10 dogs [P = not significant (NS) compared with the P-selectin antibody alone]. Although administration of the L-selectin antibody alone abolished CFVs in only one of six dogs (17%), the difference to the P-selectin or P- plus L-selectin antibody group did not reach statistical significance.

DU1–29 is an L-Selectin-Blocking Antibody

To demonstrate that the DU1–29 antibody blocks L-selectin on canine leukocytes, a fucoidin binding assay and leukocyte aggregation tests were performed as outlined in METHODS AND MATERIALS. Cell binding to fucoidin is partly dependent on L-selectin (10, 35, 47). When canine peripheral blood leukocyte binding to polystyrene wells coated with fucoidin was studied in the presence of a nonspecific control antibody or DU1–29, binding was reduced by ~30% by DU1–29 in assays performed in quadruplicate (Fig. 5A).

The dependence of homotypic aggregation of leukocytes on L-selectin (47, 49) has been noted by a number of researchers and has been measured in similar fashion for other leukocyte adhesion proteins (50). Compared with leukocyte aggregation in the presence of nonspecific mouse antibody, canine leukocyte cell aggregation in the presence of DU1–29 was significantly reduced (Fig. 5B).
Assessment of Local Inflammatory Cell Infiltrates and Local Neutrophil Activity

To address the effect of systemic MBPA administration on MPO activity, a measure of leukocyte infiltration, we assayed this activity in the myocardium immediately adjacent to the injured artery (see METHODS AND METHODS) after an infusion of MBPA sufficient to abolish CFVs in all six animals studied (Fig. 6A). Compared with six animals given saline for 8 h (none of which showed abolition of CFVs), MPO activity was decreased by 42% in animals treated with MBPA ($P = 0.029$ compared with MPO activity in controls), all of which showed complete abolition of CFVs within 10 min of initiation of the infusion.

In a separate set of experiments, the activity of MPO was assayed in the myocardium of animals that received selectin antibodies for 8 h. The results of MPO activity in these groups are shown in Fig. 6B. As shown, the combination of P- and L-selectin antibodies significantly reduced the MPO activity compared with the administration of either antibody alone. No MPO activity was detected in the myocardium of noninstrumented dogs.

DISCUSSION

In this study, we found that, in a Folts’ canine coronary injury model, MBPA, a noncarbohydrate inhibitor of E-, L-, and P-selectin, abolished severe CFVs and reduced local inflammatory cell infiltrates. Treatment with the synthetic selectin inhibitor effectively abolished CFVs when given systemically or applied topically in Pluronic gel.

CFVs in stenosed arteries have been observed in patients during the course of acute coronary syndromes (3, 14) and reflect recurrent platelet-thrombus deposition and vasoconstriction (16, 30). The nadir of flow during periods of cyclic flow reduction, often to zero flow, coincides with the presence of obstructive platelet-rich thrombi (21, 30). The platelet dependency of CFVs is stressed by their exquisite sensitivity to inhibitors of platelet aggregation and vasoconstriction (3, 17). In our study, only severe CFVs were taken into account.
account. These severe CFVs often resulted, at last transiently, in zero flow.

CFVs after administration of MBPA remained abolished despite systemic administration of epinephrine, previously shown to restore CFVs after their suppression by aspirin (4) or specific receptor inhibitors of thromboxane A2 and serotonin (17, 53). Epinephrine potentiates platelet aggregation induced by platelet agonists, such as ADP, collagen, thrombin, thromboxane A2, and platelet-activating factor. In humans, morning rises of plasma epinephrine levels within the picogram per milliliter range (from a 23 ± 3 pg/ml nadir at 6:00 AM to a 63 ± 8 pg/ml peak at noon) correlate with the morning increase of platelet aggregability and the time of heightened risk of myocardial infarction and sudden death (51). Systemic infusion of epinephrine at a level of 38 μg/min (corresponding to an average infusion rate of 1.4 μg·kg⁻¹·min⁻¹) was shown in previous studies in the same canine injury model to generate plasma epinephrine levels in the low nanogram per milliliter range, three orders of magnitude higher than the circadian levels associated in humans with increased thrombotic risk (51).

No adverse reactions to the inhibitor were noted. The \( M_r \) of MBPA is 208, and the structure is similar in charge and composition to that of an amino acid. Treated animals appeared identical to control animals and displayed no differences in heart rate, blood pressure, or temperature. Furthermore, when >24 g per day of MBPA (0.75 g/kg body wt) were administered to five dogs over a 10-day period, the activity and appearance of the animals was normal. Treated animals could not be distinguished from placebo control animals. Complete blood counts and blood chemistries taken at the end of the 10-day MBPA treatment regimen were within normal range for both MBPA and placebo control animals (data not shown).

In the acute studies reported here, platelet counts fell slightly, but not statistically significantly, compared with controls. The mechanism of this slight decrease in platelet counts is unclear and requires further study. Stable hematocrits and the absence of external bleeding suggest that abolition of CFVs by MBPA occurs without hemorrhagic risk. There was a marginal decrease in ex vivo aggregation of platelets in citrated plasma to ADP after systemic administration of both the selectin inhibitor and saline alone. This may have been due to hemodilution by the saline infusion volume. Because citrate, which removes Ca²⁺ required for selectin-ligand interactions, and heparin also interfere with selectin-ligand binding, we further studied platelet function with whole blood aggregation on blood drawn into a specific thrombin inhibitor. Using these conditions, we did not observe a decrease in the magnitude of ex vivo platelet aggregation.

Whereas an antibody against L-selectin was not effective in terminating CFVs in this model, systemic infusion of a P-selectin antibody (and combined administration of antibodies to P- and L-selectin) appeared to be more effective in abolishing CFVs (17 vs. 50%, respectively). Nonetheless, this difference did not reach statistical significance. In contrast, the small-molecular-weight universal selectin inhibitor MBPA was highly effective in reducing thrombogenesis in this model, even when only small amounts of the inhibitor were administered topically. Whether this reflects a limited role of these P- and L-selectins in thrombogenesis under these conditions or the limited access of the antibodies to the site of thrombus formation (compared to the small-molecular compound MBPA) remains to be determined. However, given the ability of L- and P-selectin antibodies to decrease local inflammation yet to fail to reduce thrombosis, the additional inhibition of E-selectin by MBPA (rather than its better thrombus penetration alone) may play a role in the antithrombotic superiority of this compound. After endothelial cell activation, the selectins are believed to promote the initial rolling of leukocytes along the endothelial surface. This is followed by the firm attachment of leukocytes and, finally, their extravasation across the vessel wall, which is mediated by members of the \( \beta \)- and \( \beta \)-integrin family, VCAM, and ICAM (6, 25, 41, 46). E-selectin is not constitutively expressed on the surface of the resting endothelium and requires de novo transcription. Although induction of E-selectin expression is likely to occur within the time frame of our study, the median time to abolition with locally applied MBPA was 25 min, which, added to the 30-min control period of uninhibited CFVs, would have allowed ~1 h for de novo E-selectin expression to occur. This time frame is relatively close to the minimum time required for E-selectin expression. The development of specific inhibitors of E-selectin, for which we have no blocking antibody, may allow the role of this selectin to be addressed in the future in models similar to those described herein.

The failure of the L-selectin antibody to inhibit thrombosis deserves discussion. First, P-selectin plays a role in both platelet-neutrophil interactions (13) and adhesion of platelets and leukocytes to the activated endothelium (20). In the present thrombus model, however, P-selectin-dependent interactions between endothelium and platelets may be critically involved in thrombus formation, whereas leukocyte-endothelial interactions may be of somewhat lesser importance. Specifically, in constricted arteries generating high shear forces, P-selectin-dependent platelet rolling play a prominent role in initiating platelet thrombus formation and may explain the relatively higher antithrombotic efficacy of the P-selectin antibodies observed in our study and that of Ueyama et al. (52). Platelet-leukocyte aggregation may be of lesser importance under conditions of high shear. The specificity of selectin inhibition of coronary thrombosis is underscored by the study by Ueyama et al. (52), who observed protection against coronary thrombosis in a model similar to ours after infusing an sLeα analog and P-selectin antibodies. Second, the potential importance of L-selectin may have been blunted by the study design itself, where the selectin inhibitors were given 30 min and 6 h after initiation of vascular injury. During this time, cytokine generation, endothelial cell activation, and
vascular inflammatory leukocyte attachment and infiltration may unfold to a level that is unlikely to be rapidly reversed. This model was chosen, however, because of its relative similarity to clinical situations, in which therapy is initiated after plaque rupture has elicited local inflammation and thrombosis (33, 37). Third, although improbable, the affinity of the L-selectin antibody for canine leukocytes may have been too low to prevent leukocyte adherence in vivo. This is unlikely given the strong reaction between canine leukocytes and the L-selectin antibody, as detected by magnetic bead capture experiment (see Antibodies Against P- and L-Selectin), and given that the combined administration of the P- and L-selectin antibodies significantly decreased MPO activity in the tissue surrounding the injured arteries.

While others have used antibodies or oligosaccharides related to the sLe^x antigen (naturally occurring ligands on leukocytes for E-selectin and P-selectin) to study the involvement of selectins in diverse model of vascular injury (11, 26), we studied the effects of a nonprotein, noncarbohydrate compound, MBPA. Our in vitro studies indicate that this inhibitor blocks all three selectin molecules with IC_{50} values of 115, 382, and 489 μM, respectively, for L-selectin, E-selectin, and P-selectin (Bjerke et al., unpublished observations). An important advantage of the nonpeptide inhibitor used in our studies may be its small size, facilitating penetration of the interstices of arterial tears and thrombi. Potentially, the efficacy of all antibodies in abolishing ongoing thrombus formation may be hampered, to some extent, by their relatively large size. We believe that for this very reason, preliminary experiments using local adventitial application of the antibodies in Pluronic gel were ineffective in abolishing CFVs (data not shown), whereas the synthetic inhibitor was highly effective. However, the different pharmacokinetics and pharmacodynamics of antibodies do not allow a direct comparison with a small-molecular-weight synthetic molecule. Potential pitfalls would include the difference in selectin affinity, differences in body distribution and half-life, and differences in access to tissue sites of selectin upregulation. Accordingly, we studied the effects of the P- and L-selectin antibodies only to understand the relative importance of P- vs. L-selectin blockade in this model of thrombosis and inflammation, realizing the experimental limitations involved.

Administration of MBPA and combined administration of P- and L-selectin antibodies in our study decreased local leukocyte infiltration (as reflected by MPO activity) during an 8-h observation period, whereas administration of P-selectin antibody alone did not. Although the magnitude of reduction is in the range of that of animals treated with P- plus L-selectin antibodies (42% for MBPA vs. 50% for P- plus L-selectin), the absolute MPO values in the MBPA- and saline-treated animals are lower. A different operator (J. C. Ober) than in the selectin groups (F. J. Clubb) harvested the myocardium in the added saline control and MBPA groups, harvesting a slightly larger rim of myocardial tissue adjacent to the injured artery. Because MPO is reported in units per 100 mg of tissue, harvesting of tissue more distant from the injured site could have resulted in dilution of the MPO values. Furthermore, it is unclear to what extent the detected MPO activity reflects the intentional vascular insult or manipulation of the myocardium during preparation of the pericardial cradle.

In summary, we have presented the initial biological characterization of a new class of selectin binding inhibitors. These inhibitors are synthetic small molecules that block selectin-sLe^x binding and currently appear to specifically inhibit E-, P-, and L-selectin. Systemic and local administration of a noncarbohydrate selectin inhibitor appears to be a safe and efficient approach to the treatment of established arterial thrombosis and diminishes inflammation in experimental models. The efficacy of the topicaly applied selectin blockade further indicates the potential of linking this novel form of therapy to intraluminal devices, such as stents or possibly grafts to treat sites of vascular injury with local infusions or applications of the inhibitor. Treatment of acute coronary syndromes is also a possibility.

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