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Sulfatides, L- and P-selectin ligands, exacerbate the intimal hyperplasia occurring after endothelial injury

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Abstract

Leukocytes may be important in the development of intimal hyperplasia, but little is known about the participation of sulfatides (3-sulfated galactosyl ceramides) which are native ligands of L- and P-selectin. This study was designed to determine whether sulfatides affect the development of intimal hyperplasia. ICR mice were randomized to receive vehicle or sulfatides intravenously either at 1, 3, or 10 mg/kg/day for 7 days, or at 10 mg/kg/day for 1, 3, or 7 days. Endothelial damage was inflicted on the femoral artery via the photochemical reaction between rose bengal and green light. Scanning electron and light microscopic observations 3 days after the injury indicated that sulfatides-treated animals had more neutrophils adhering to the injury site than vehicle-treated controls. At 21 days, sulfatides-treated animals had a greater neointimal area than controls. In in vitro studies, sulfatides (i) increased cytosolic free calcium in mouse neutrophils, (ii) caused increases in expression of Mac-1 (CD11b/CD18) on the neutrophil membrane surface in mouse whole blood. These findings suggest that neutrophil accumulation on the subendothelial matrix or adherence of platelets mediated by adhesive interactions between L- or P-selectin and sulfatides may contribute to the development of intimal hyperplasia. The neutrophil accumulation may be mediated by an increase in Mac-1 caused by the agonistic effects of sulfatides on the neutrophil membrane surface, or by an increase in L- and P-selectin ligands resulting from the binding of sulfatides onto the exposed subendothelial matrix.

Keywords: Leukocyte; L-selectin; Platelet; P-selectin; Restenosis; Sulfatide; Thrombosis

1. Introduction

Percutaneous coronary intervention (PCI) is widely used as a therapy for occlusive coronary arterial disease. Despite improvements in technique such as the use of stents with antiplatelet agents or drug-eluting stents, restenosis occurs in ≈8% of patients within 6 months (Montalescot et al., 2001; Kastrati et al., 2005). Previous studies have suggested that platelet adhesion and aggregation on the injured arterial wall may contribute to the migration and proliferation of smooth muscle cells (Wilentz et al., 1987; Ip et al., 1991). Neumann et al. (1996) have reported (i) that the surface expression of Mac-1 (CD11b/CD18) on neutrophils sampled downstream of the dilated coronary artery segments is significantly higher than that in prestenotic coronary artery segments in patients undergoing angioplasty, and (ii) that the expression of L-selectin (CD62L) on neutrophils is concomitantly decreased downstream of the dilated coronary artery segments by comparison with that seen in prestenotic coronary artery segments. These findings strongly suggest that neutrophil activation may occur at the affected vessel.

In an experimental study, it was reported that intercellular adhesion molecule-1 (ICAM-1) is expressed intensely soon after balloon injury in rat carotid arteries, and treatment with

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monoclonal antibodies to ICAM-1 or ICAM-1 plus lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) significantly suppress intimal hyperplasia (Yasukawa et al., 1997). Furthermore, treatment with P-selectin antibody has been reported to reduce the development of neointimal hyperplasia after balloon injury in rats (Hayashi et al., 2000). Thus, leukocyte accumulation on the injured arterial wall, through adhesion molecules such as leukocyte β_2 integrins and selectins (L-, P- and E-selectin), may play an important role in the initiation or development of intimal hyperplasia in animals and humans.

Sulfatides, 3-sulfated galactosyl ceramides, are native ligands for L- and P-selectin, but not for E-selectin (Needham and Schnaar, 1993; Bajorath et al., 1994). Mulligan et al. (1995) showed the preventive effect of sulfatides in selectin-dependent acute lung injury. Sulfatides are expressed on the membrane surface of, and are excreted by, granulocytes (Bajorath et al., 1994). Sulfatides exist in serum lipoproteins and in a variety of tissues including brain, tumors, kidney, liver, spleen, stomach, small intestine, erythrocytes, and leukocytes (Roberts, 1986; Zhu et al., 1991). Sulfatides have also been shown to be released by neutrophils and tumor cells (Bajorath et al., 1994). However, the physiological role played by sulfatides in serum and in the above tissues is not yet clear. Interestingly, sulfatides, which are absent from the aorta of normal rabbits, accumulate in the aorta of the Watanabe hereditable hyperlipidemic rabbit (WHHL rabbit; an animal model for human familial hypercholesterolemia), during the development of atherosclerosis (Hara and Taketomi, 1991). This may suggest that sulfatides have an important role to play in the development of atherosclerosis and restenosis.

However, little is known about the participation of sulfatides in intimal hyperplasia. Given the theoretical and experimental data implicating the selectins in intimal hyperplasia, we sought to determine whether treatment of sulfatides, which presumably attenuates the leukocyte accumulation at the site of endothelial injury, are protected from developing intimal hyperplasia after vascular injury.

2. Materials and methods

2.1. Animals

Male ICR mice (Japan SLC, Shizuoka, Japan) weighing 25 to 30 g were used. The experiments in this study were conducted in accordance with institutional guidelines. The protocols were approved by the Animal Experiments Committee of Hamamatsu University School of Medicine.

2.2. Experiments involving endothelial injury

2.2.1. Infliction of endothelial injury

Ninety-three mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and body temperature was maintained at 37.5 °C throughout the experiment with the aid of a heating pad (K-module Model K-20, American Pharmseal Company, USA). The procedure used to induce a transluminal thrombosis in the femoral artery was the same as that described in detail elsewhere (with minor modifications) (Kikuchi et al., 1998). Briefly, the right femoral artery was exposed and the probe (1 mm diameter, Advance) of a laser Doppler flowmeter (ALF2100, Advance, Tokyo, Japan) was carefully placed onto the surface of the artery for monitoring blood flow, the probe signal being recorded continuously using a recorder (U-228, Nippon Denshi Kagaku, Kyoto, Japan). The jugular vein was cannulated with a polyethylene tube for rose bengal (Wako, Japan) administration. Transillumination with green light (540 nm wavelength) was achieved with a L4487 irradiation apparatus (Hamamatsu Photonics, Japan), the irradiation being directed with an optic fiber positioned 5 mm away from the part of the right femoral artery proximal to the flow probe. After establishing baseline blood flow, irradiation was started and rose bengal at 20 mg/kg (i.v.) was injected over the next 5 min. Photo-irradiation was continued for 15 min with the blood flow being monitored for 15 min from the start of the rose bengal injection. The formation of an occlusive thrombus was indicated by a complete cessation of blood flow. The time to achieve complete occlusion was about 10 min. After removal of the polyethylene tube, the jugular vein was ligated, and the surgical wound was closed. The thrombotic occlusion is followed by spontaneous reperfusion within the first 24 h (Kikuchi et al., 1998).

2.2.2. Effect of sulfatides on intimal hyperplasia

In the first series of experiments (52 mice), sulfatides (1, 3, or 10 mg/kg), cerebrosides (10 mg/kg) or an identical volume of vehicle (5 ml/kg) were administered intravenously 1 h after the initiation of the photochemical reaction, and then once a day for 7 days. In another series of experiments (41 mice), sulfatides (10 mg/kg) were administered i.v. 1 h after the initiation of the photochemical reaction, and then once a day for 1, 3 or 7 days.

2.2.3. Measurement of intimal hyperplasia

Intimal hyperplasia was measured 21 days after the endothelial injury induced by photochemical reaction. Briefly, 93 mice were anesthetized (sodium pentobarbital, 80 mg/kg, i.p.) and perfused at physiological pressure with saline (0.9%) followed by 1% paraformaldehyde and 2% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4). Segments of the irradiated (right) and non-irradiated (left) femoral arteries were removed and kept in the same fixative buffer (pH 7.4) overnight at 4 °C prior to embedding. All arterial segments were embedded in paraffin, sectioned transversely (each section is 3 μm thick) at 0.5 mm intervals, and the sections stained with hematoxylin and eosin. The intimal and medial areas were measured with the aid of a computer analysis system (FLOVEL VIDEOMICROMETER, model VM-30, OLYMPUS, Japan).

2.2.4. Histopathological observations

Changes in the femoral arteries of a separate group of 12 mice that had also received a photochemically induced endothelial injury were analyzed by scanning electron microscopy or light microscopy. At 1 or 3 days after the injury, mice were anesthetized (sodium pentobarbital, 80 mg/kg, i.p.) and perfused at physiological pressure with saline followed by 1% paraformaldehyde and 2% glutaraldehyde in PBS. Segments of the irradiated and non-irradiated arteries were then removed and kept in the same fixative buffer (pH 7.4) overnight at 4 °C. The specimens were then either prepared for scanning electron microscopy (SEM) by the method of Muranaka et al. (1988) and examined with a Hitachi S-800 scanning electron microscope, or stained for light microscopy with HE or May–Grünwald–Giemsa.

Sulfatides (10 mg/kg) or vehicle (5 ml/kg) were administered i.v. 1 h after the initiation of the photochemical reaction, and then once a day for 3 days.

2.3. Thioglycollate-induced acute peritoneal inflammation

Forty-eight intact mice were injected intraperitoneally with 1 ml of 3% thioglycollate broth (Difco, USA) or sterile pyrogen-free saline (sham). Just before this injection, the animals received a slow intravenous infusion of sulfatides (1,3, or 10 mg/kg), cerebrosides (10 mg/kg), $F(ab')_2$ fragments of an anti-mouse-L-selectin monoclonal antibody (clone no. Mel-14, Serotec, USA, 1 mg/kg), rat IgG_{2a} isotype control antibody (clone no. LO-DNP-16, Serotec, USA, 1 mg/kg), or vehicle. The mice were sacrificed 3 h after the peritoneal injection. Cells from the peritoneal cavity were collected by lavage with 9 ml of 0.01 M phosphate-buffered saline (PBS) containing 0.1% BSA, 0.54 mM EDTA, and 10 U/ml heparin to prevent clotting, and counted in a hemocytometer. The percentage of neutrophils was assessed by counting in cytospin preparations stained with Wright–Giemsa stain (Diff-Quik, Baxter, USA).

2.4. Isolation of neutrophils

Neutrophil isolation (>90%) was carried out using dextran sedimentation and density gradient centrifugation of venous blood as previously described (with minor modifications) (Boyum, 1968). Briefly, blood was collected from the inferior vena cava of 80 anesthetized mice (sodium pentobarbital, 80 mg/kg, i.p.) in 3.8% sodium citrate (9:1 v/v). Blood samples were pooled and mixed with 3% dextran–saline (1:1 v/v, M.W.: 200,000, Wako, Japan), then left to stand at 4 °C for 40 min to sediment the erythrocytes. Cells in the supernatant were pelleted by centrifugation and resuspended in 3 ml of PBS. This suspension was layered on 3 ml of NycoPrep 1.077 Animal (Nycomed, Norway) and centrifuged at $600\times g$ for 15 min at 25 °C. Erythrocytes remaining in the cell pellets were lysed with cold water for 30 s. The resulting cells were washed twice with PBS and resuspended in Krebs–HEPES buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM HEPES, and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH) or RPMI-1640-HEPES.

2.5. Measurement of cytosolic free calcium

Neutrophils were loaded by incubation for 30 min at 25 °C in Krebs–HEPES buffer containing 2 μM fura-2/AM (Dojindo, Japan). The cells were washed and resuspended in Krebs–HEPES buffer at 5×10^6 cells/ml. Fura-2 fluorescence was measured in a Hitachi F2000 fluorometer using alternate 340 and 380 nm excitation wavelengths and a 510 nm emission wavelength. The concentration of intracellular calcium ion was calculated from dissociation constant of Fura-2 (K_d: 224 nM) as previously described (Grynkiewicz et al., 1985). Maximal fluorescence was determined following lysis of cells with Triton X-100 while minimum fluorescence was determined following chelation with excess of EGTA.

2.6. Expression of L-selectin and Mac-1 on neutrophils in whole blood in vitro and ex vivo

Blood was collected from the inferior vena cava of 4 anesthetized mice for in vitro and 20 anesthetized mice for ex vivo (sodium pentobarbital, 80 mg/kg, i.p.) in 3.8% sodium citrate (9:1 v/v). The whole blood was stimulated with or without sulfatides for 20 min at 37 °C. Subsequently, the samples were washed in washing buffer (PBS containing 0.1% NaN_3 and 1.0% heat-inactivated fetal bovine serum) by centrifugation at 4 °C. The cells were resuspended to 2×10^7 cells/ml in cold washing buffer containing rat anti-mouse CD32/CD16 monoclonal antibody (clone no. 2.4G2, PharMingen, USA) which block the non-specific adherence of antibodies to murine Fc receptors, then incubated with FITC-conjugated rat anti-mouse Mac-1 monoclonal antibody (clone no. M1/70, PharMingen, USA), rat anti-mouse CD62L monoclonal antibody (clone no. Mel-14, PharMingen, USA), rat IgG_{2a} isotype control antibody (clone no. R35-95, PharMingen, USA), or rat IgG_{2b} isotype control antibody (clone no. R35-38, PharMingen, USA) at 4 °C for 30 min in the dark. Erythrocytes were lysed using an Easy-Lyse™ Whole blood Erythrocyte Lysing Kit (Leinco Technologies, Inc., USA), and the remaining cells were washed with washing buffer. Fluorescent labelling was quantified immediately with a FACScan flow cytometer (EPICS® Profile Analyzer) with a live gate on the neutrophils.

2.7. Reagents

Sulfatides and cerebrosides were purchased from Sigma. These reagents were dissolved in 0.01 M phosphate-buffered saline and administered i.v. in a volume of 5 ml/kg body weight.

2.8. Statistical analysis

Results are expressed as mean±S.E.M. All data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test. Results were considered to show a significant difference at $P<0.05$.

3. Results

3.1. Intimal hyperplasia

A total of 93 mice was used to determine the effects of sulfatides on the development of intimal hyperplasia after endothelial injury within the femoral artery. At 21 days after the endothelial injury, neointimal formation was observed in the subendothelial layers in vehicle-treated animals (Fig. 1A). In the first series of experiments, mice that had received an endothelial injury were injected intravenously with sulfatides (1, 3, or 10 mg/kg/day), cerebrosides (10 mg/kg/day), or an identical volume of vehicle (control) for 7 days. The intimal and medial areas were then quantified using morphometric analysis at 21 days after the injury (Fig. 2). Treatment with sulfatides (1, 3, or 10 mg/kg/day for 7 days, i.v.) produced a dose-dependent increase in the intimal area and intima/media ratio in the injured vessels by comparison with the effects seen in the vehicle-treated control group, the effects being significant at doses of 3 and 10 mg/kg (Figs. 1B and 2). Such effects were not seen in the contralateral (uninjured) artery. In contrast, the medial area was significantly reduced by treatment with sulfatides at 3 and 10 mg/kg (Fig. 2B), while the medial area in the contralateral artery was not affected by such treatment (Fig. 2D). On the other hand, there was no significant difference between cerebrosides-treated and control groups in terms of these measurements. In another series of experiments, different treatment schedules in which sulfatides were administered at a dose of 10 mg/kg/day, i.v. for 1, 3, or 7 days after the injury were used to determine whether a time-dependent biological process was involved (Fig. 3). In fact, such treatment produced increases in intimal area and intima/media ratio and a decrease in medial area at 21 days after the injury all of which were dependent on the duration of the treatment, the effect being significant for 7-day treatment.

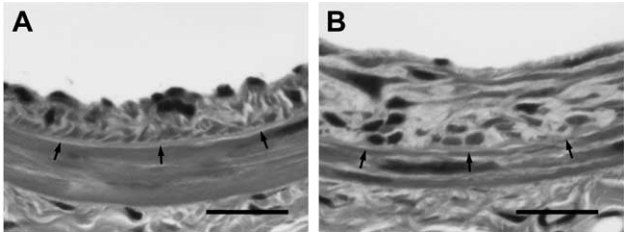

Fig. 1. Photomicrographs of transverse sections of the mouse femoral artery 3 weeks after endothelial injury. (A) Injured vessel from vehicle-treated mouse. (B) Injured vessel from mouse treated with sulfatides at a dose of 10 mg/kg/day (i.v.) 1 h after the initiation of the photochemical reaction and then once a day for 7 days. Arrows indicate the internal elastic lamina. Bar=20 μm.

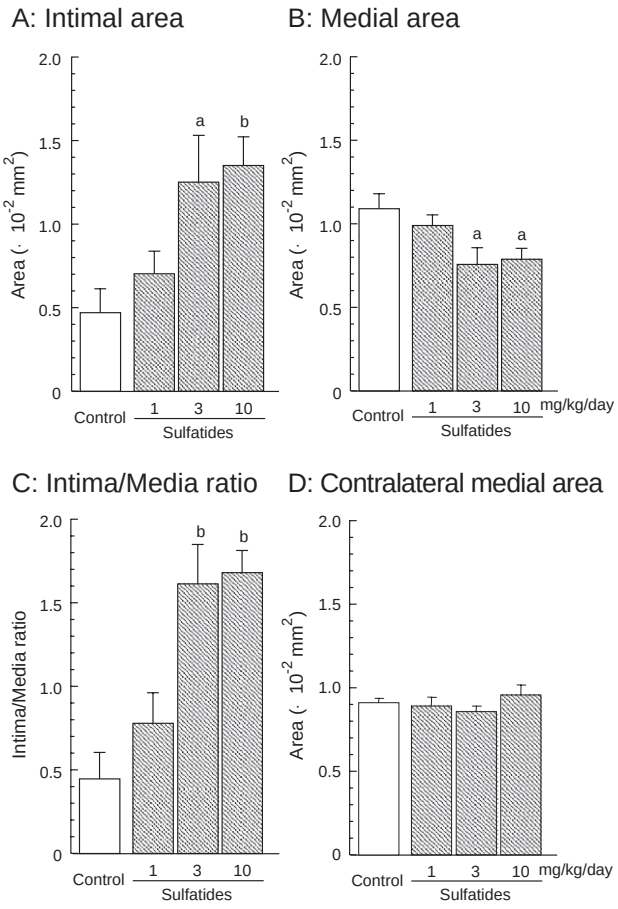

Fig. 2. Effects of sulfatides on development of intimal hyperplasia in the mouse femoral artery. Sulfatides at the doses shown or vehicle were administered intravenously 1 h after the initiation of the photochemical reaction and then once a day for 7 days. Each column represents mean±S.E.M. ($n=8–11$ for each group). $^{a}P<0.05$, $^{b}P<0.01$, indicates significant difference from vehicle-treated (control) group (one-way ANOVA followed by Dunnett's test).

3.2. Leukocyte adhesion to the vessel wall

In vehicle-treated animals, scanning electron microscopy performed 1 day after the injury showed a number of platelets adhering to the vessel wall at the site of the injury, while the wall of the contralateral (uninjured) artery was covered with cobblestone-like endothelial cells (controls, Fig. 4A and B). Three days after the injury, a number of leukocytes could be seen adhering to platelets or to the subendothelial matrix at the site of the injury (Fig. 4C). Scarcely any leukocyte adhesion was found in the injured vessel wall at 7 days after such injury. The leukocyte accumulation observed using scanning electron microscopy and light microscopy at 3 days after the injury was markedly greater in sulfatides-treated animals (10 mg/kg/day, i.v.)

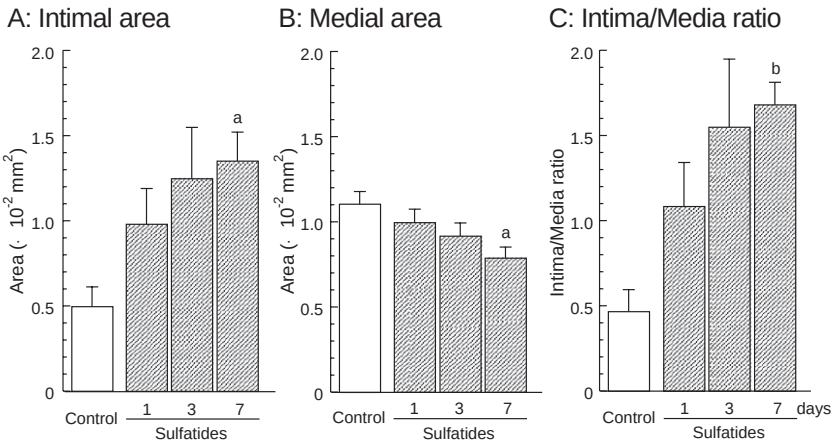

Fig. 3. Effects of sulfatides on development of intimal hyperplasia in the mouse femoral artery. Sulfatides at a dose of 10 mg/kg or vehicle were administered intravenously 1 h after the initiation of the photochemical reaction and then once a day for the number of days shown. Each column represents mean ± S.E.M. (n = 10–11 for each group). [a]P < 0.05, [b]P < 0.01, indicates significant difference from vehicle-treated (control) group (one-way ANOVA followed by Dunnett's test).

than in the controls (Figs. 4D and 5B). Moreover, light microscopy revealed that treatment with sulfatides induced not only leukocyte accumulation in the lumen but also infiltration by leukocytes into the media (Fig. 5B). The cells on the luminal surface at 3 days after the injury were mostly identified as neutrophils by light microscopy (Fig. 5B).

3.3. Thioglycollate-induced acute peritoneal inflammation

In this model, treatment with sulfatides (1, 3, or 10 mg/kg, i.v.) or with $F(ab')_2$ fragments of anti-mouse-L-selectin monoclonal antibody (Mel-14, 1 mg/kg, i.v.) reduced neutrophil accumulation in the peritoneal cavity 3 h after the thioglycollate injection (Table 1). On the other hand, cerebrosides failed to inhibit such neutrophil accumulation (Table 1).

3.4. Cytosolic free calcium in neutrophils

In in vitro studies, sulfatides evoked an increase in cytosolic free calcium in mouse neutrophils (Fig. 6). The effect of sulfatides was detectable at 50 μg/ml, and increased up to 200 μg/ml. On the other hand, cerebrosides (200 μg/ml), non-sulfated galactocerebrosides, had no effect upon cytosolic free calcium in mouse neutrophils. We also tested whether two other ligands of L-selectin were able to increase cytosolic free calcium. Neither fucoidan, a polysaccharide produced by brown algae, nor heparin, a glycosaminoglycan produced by mast cells, triggered an increase in cytosolic free calcium.

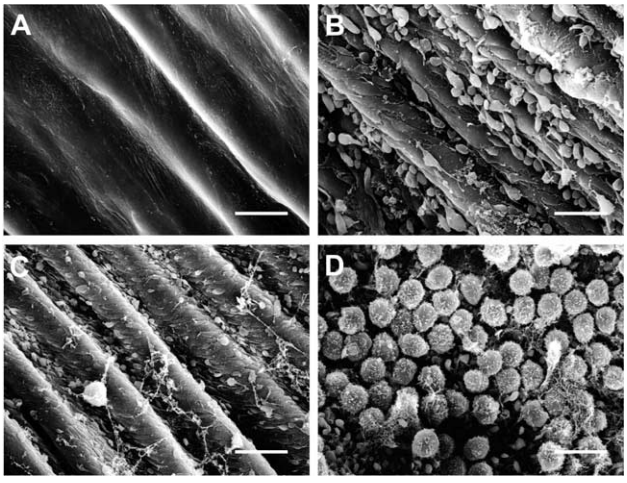

Fig. 4. Scanning electron microscopic observations of irradiated and non-irradiated segments of the mouse femoral artery 1 and 3 days after endothelial injury in sulfatides- or vehicle-treated animals. The luminal surface of a mouse femoral artery is shown in each case. (A) Before the endothelial injury. Intact endothelial cells are seen. (B) One day after the endothelial injury in an animal treated with vehicle. Numerous platelets adhere to the exposed subendothelial matrix. (C) Three days after the endothelial injury in an animal treated with vehicle. (D) Three days after the endothelial injury in an animal treated with sulfatides at a dose of 10 mg/kg, i.v. 1 h after the initiation of the photochemical reaction and then once a day for 3 days. Bar = 10 μm.

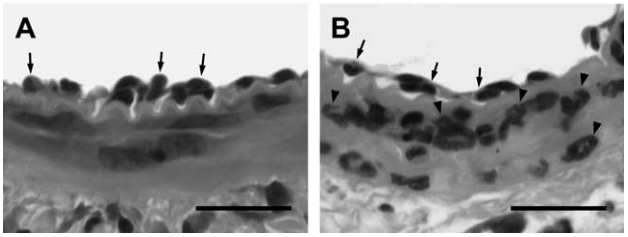

Fig. 5. Light micrographs of cross sections of the mouse femoral artery 3 days after endothelial injury in sulfatides- or vehicle-treated animals. (A) In an animal treated with vehicle. Leukocytes adhere to the exposed subendothelial matrix. Arrows in (A) show unidentified leukocytes. (B) In an animal treated with sulfatides at a dose of 10 mg/kg (i.v.) 1 h after the initiation of the photochemical reaction and then once a day for 3 days. A large number of leukocytes have migrated into the media. Arrows and arrowheads in (B) show unidentified leukocytes and neutrophils, respectively. Bar = 20 μm.

Table 1
Effects of sulfatides and cerebrosides on neutrophil accumulation in the peritoneal cavity in mice with thioglycollate-induced acute peritoneal inflammation

Treatment	Dose (mg/kg, i.v.)	Neutrophils ($\times 10^6$ cells/mm^3)
Sham 1	–	0.38±0.16[a]
Control 1	–	1.96±0.37
Sulfatides	1	0.84±0.19[a]
	3	0.90±0.15[b]
	10	0.35±0.10[a]
Cerebrosides	10	1.98±0.39
Sham 2	–	0.21±0.18[b]
Control 2	–	0.99±0.17
Anti-CD62L F(ab′)$_2$	1	0.41±0.09[b]

Sulfatides, cerebrosides, anti-CD62L F(ab′)$_2$, vehicle (sham 1 and control 1), or isotype-matched control Ab (IgG_{2a}; sham 2 and control 2) was intravenously administered just before the intraperitoneal injection of 3% thioglycollate solution. Sham-treated groups (sham 1 and sham 2) were injected intraperitoneally with saline instead of thioglycollate solution. Thus, "sham 1" animals received vehicle followed by saline, "sham 2" animals received isotype-matched control Ab followed by saline, "control 1" animals received vehicle followed by thioglycollate, and "control 2" animals received isotype-matched control Ab followed by thioglycollate. Peritoneal cells were harvested 3 h after the peritoneal injection and assessed as described in Materials and methods. Data are expressed as mean±S.E.M. $n=4–6$.

[a] $P<0.01$ vs. control (one-way ANOVA followed by Dunnett's test or Student's t-test).

[b] $P<0.05$ vs. control (one-way ANOVA followed by Dunnett's test or Student's t-test).

3.5. Flow cytometry

Neutrophils from mouse peripheral blood were assessed by flow cytometric analysis for the expression of L-selectin and Mac-1 (CD11b/CD18). The addition to mouse whole blood in vitro of sulfatides (100 μg/ml) for 20 min at 37 °C caused a decrease of 13% in the expression of L-selectin and an increase of 57% in the expression of Mac-1 on mouse neutrophils, the effect being significant at only for the expression of Mac-1 (Table 2).

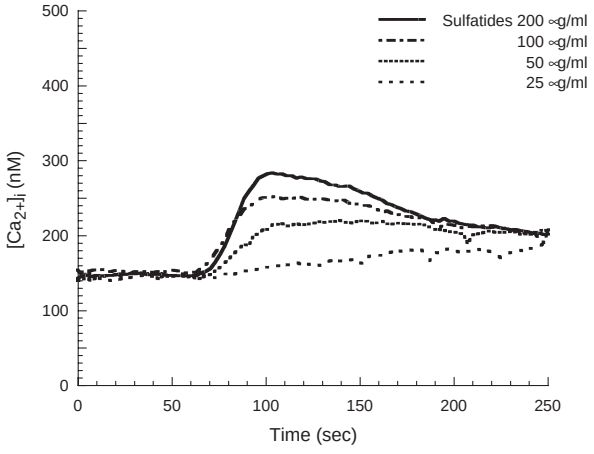

Fig. 6. Effects of sulfatides on intracellular Ca^{2+} concentration, tyrosine phosphorylation of several proteins, and shedding of L-selectin in mouse neutrophils in vitro. Neutrophils were incubated with Fura-2 AM for 30 min at 37 °C. After washing, the cells were resuspended in Ca^{2+}-measuring buffer and stimulated with sulfatides at 25 to 200 μg/ml.

Table 2
Effects of sulfatides on neutrophil surface expression of L-selectin and Mac-1 in murine peripheral blood in vitro

Monoclonal antibody	Mean fluorescence intensity	
	Vehicle	Sulfatides
None	1.89±0.16	1.89±0.06
IgG_{2a}	1.78±1.78	1.78±0.04
IgG_{2b}	1.84±0.09	1.93±0.13
Anti-CD62L	29.11±1.87	25.39±2.56
Anti-CD11b	8.73±0.45	13.67±0.91[a]

Blood was collected from the inferior vena cava of anesthetized mice in 3.8% sodium citrate (9:1 v/v). The whole blood was stimulated with or without sulfatides at 100 μg/ml for 20 min at 37 °C. The expression of L-selectin and Mac-1 on the neutrophil surface was assessed using anti-CD62L and anti-CD11b, respectively, and a flow cytometer, as described in Materials and methods. Data are expressed as mean±S.E.M. $n=4$.

[a] $P<0.05$ vs. vehicle-treated group (Paired t-test).

To ascertain the relevance of the above-described observations, in vivo studies were carried out. As shown in Fig. 7A, the expression of L-selectin on neutrophil surface showed little changes in neutrophils obtained from mice treated with sulfatides at 10 mg/kg/day (i.v.) for 3 days (sulfatides-treated sham group) as compared to those obtained from mice treated with vehicle (vehicle-treated sham group), or those obtained from the endothelial injury-induced group using a photochemical reaction followed by sulfatides treatment (injury plus sulfatides-treated group) as compared to those obtained from injury plus vehicle-treated group. However, there was a tendency for the neutrophils to decrease L-selectin expression in the injury groups as compared with no injury groups. On the other hand, the expression of Mac-1 on neutrophil surface had a tendency to be increased in sulfatides-treated groups as compared with vehicle-treated groups, and showed a significant increase of 32% in injury plus sulfatides-treated group as compared with vehicle-treated sham group (Fig. 7B).

4. Discussion

In this study, intimal hyperplasia of the mouse femoral artery was induced by use of the photochemical reaction between rose bengal and green light to cause endothelial injury. This, unlike the balloon injury model, does not involve any mechanical stimulation of the media (Kikuchi et al., 1998; Hirata et al., 1994). In the present model, peroxidative damage to the endothelial membrane–which is achieved via the photodynamic generation of singlet molecular oxygen from injected rose bengal following irradiation of the artery with green light (540 nm)–provides the initial stimulus for platelet adhesion and their subsequent activation and aggregation, resulting in thrombus formation (Takiguchi et al., 1992). In fact, rose bengal is well known to be one of the most efficient photodynamic generators of

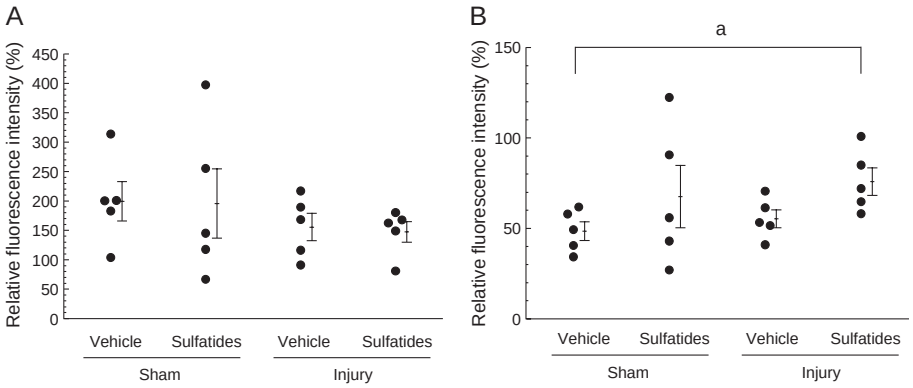

Fig. 7. Changes in expression of L-selectin (A) and Mac-1 (B) on neutrophil surface from mouse peripheral blood 3 days after endothelial injury. Sulfatides at a dose of 10 mg/kg or vehicle were administered intravenously 1 h after the initiation of the photochemical endothelial injury (injury group) or sham operation (sham group) and then once a day for 3 days. Blood was collected from the inferior vena cava of anesthetized mice in 3.8% sodium citrate (9:1 v/v) 3 day after the endothelial injury. The expression of L-selectin and Mac-1 on the neutrophil surface was assessed using anti-CD62L and anti-CD11b, respectively, and a flow cytometer, as described in Materials and methods. Vertical bar are expressed as mean ± S.E.M. $n = 5$. $^{a}P < 0.05$ vs. vehicle-treated sham group (Student's t-test).

singlet molecular oxygen (Gandin et al., 1983). Formation of an occlusive thrombus is followed by naturally produced reperfusion within 24 h in this model, and intimal hyperplasia is observed 7 days after the injury, reaching a plateau within 21 days (Kikuchi et al., 1998).

In the present study, we tested the effect of sulfatides, native ligands of L- and P-selectin, on the development of intimal hyperplasia following a photochemically induced injury of the endothelium of the mouse femoral artery. In contrast to our expectation, treatment with sulfatides dramatically produced an exacerbation of the effect of injury on the intimal area and intima/media ratio both in a dose- and time-dependent manner. On the other hand, administration of galactosyl cerebrosides desulfated sulfatides which have no binding properties to L-, P-, or E-selectins had little effect on the injury-induced increases in intimal area or intima/media ratio.

Sulfatides have been reported to be native ligands of L- and P-selectins (which are members of a family of lectin-like molecules) (Needham and Schnaar, 1993). L-selectin is expressed on the membrane surface of leukocytes (Lewinsohn et al., 1987), and it mediates the rolling of leukocytes along endothelial surfaces and platelets already adhered onto the subendothelial matrix (Finger et al., 1996). P-selectin is stored preformed in the α-granules of platelets and Weibel–Palade bodies of the endothelium (McEver et al., 1989), and it is rapidly expressed at cell surfaces through various stimuli such as ADP, collagen, epinephrine, and thrombin (McEver et al., 1989; Hsu-Lin et al., 1984). In this study, intravenous administration of sulfatides, but not of galactosyl cerebrosides, reduced neutrophil accumulation in the peritoneal cavity of mice after thioglycollate injection. Similarly, treatment with $F(ab')_2$ fragments of an anti-mouse-L-selectin monoclonal antibody (Mel-14) at 1 mg/kg (i.v.) significantly inhibited neutrophil accumulation in the same model. Others have reported that treatment with sulfatides inhibits neutrophil accumulation in two rat models in which acute lung injury is induced by cobra venom factor or an IgG immune complex (Mulligan et al., 1995). From these findings, an inhibition of leukocyte accumulation induced by sulfatides might have been expected to suppress, not exacerbate, the development of intimal hyperplasia in the present model. In fact, Chauvet et al. (1999) showed that other native ligands of selectins, fucoidan, reduced the development of the intimal hyperplasia induced in the porcine carotid artery by balloon injury. We have also confirmed that phytic acid–which inhibits both the binding of L- and P-selectin-Ig-fusion protein to immobilized bovine serum albumin-sialyl $Lewis^X$ and neutrophil accumulation in the peritoneal cavity of mice after thioglycollate injection (Cecconi et al., 1994)–reduces the intimal hyperplasia seen in our mouse model (Shimazawa et al., 1997). Moreover, it has been reported that intimal hyperplasia can be reduced by blocking P- and E-selectin with a sialyl $Lewis^X$ analogue (Barron et al., 1997) and that P-selectin deficient mice are protected from the development of intimal hyperplasia after vascular injury (Kumar et al., 1997; Smyth et al., 2001). These reports strongly suggest that leukocyte accumulation and infiltration in damaged vessels participate in the development of intimal hyperplasia. These findings, however, seemed incompatible with our results showing that sulfatides produced an exacerbation of intimal hyperplasia after endothelial injury in the mouse femoral artery.

To try and clear up these points, we used scanning electron microscopy and light microscopy to observe whether leukocyte adhesion or accumulation to the vessel wall at the site of endothelial injury was affected by treatment with sulfatides. In vehicle-treated animals, some leukocytes were observed adhering to platelets and the subendothelial matrix in the vessel wall at the injury site at 3 days after the infliction of the injury. Treatment with sulfatides apparently caused an increase in this leukocyte accumulation at the injury site. On the other hand, they were not seen in the intact areas covered

with cobblestone-like endothelial cells at sites outside the injured area or in the contralateral (uninjured) artery. Taken collectively, these results suggest that the exacerbation of intimal hyperplasia induced by sulfatides may be mediated, at least in part, by an enhancement of leukocyte accumulation including neutrophils.

In an attempt to identify the differences between the effects of selectin-related compounds mentioned above, we examined mouse neutrophils in vitro for changes in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the expression of adhesion molecules such as L-selectin, Mac-1, LFA-1, and platelet endothelial cellular adhesion molecule-1 (PECAM-1, CD31) on the membrane surface in vitro. In these in vitro studies, sulfatides induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. In contrast, such changes were not observed with galactosyl cerebrosides, fucoidan, or phytic acid. On the other hand, we have found that treatment with sulfatides does not affect $[Ca^{2+}]_i$ in human coronary arterial smooth muscle cells, and so we consider that the exacerbation of intimal hyperplasia induced by sulfatides is probably not due to a direct effect on vascular smooth muscle cells. In previous studies, it has been reported that the addition of sulfatides causes increases in $[Ca^{2+}]_i$ and the tyrosine phosphorylation of several cellular proteins in human neutrophils, and that these effects are mediated by the interaction between L-selectin and sulfatides (Waddell et al., 1995). Unlike other ligands of selectin or compounds related to selectin, sulfatides were found in the present study to have an agonistic effect on mouse neutrophils. The addition of sulfatides to whole blood tended to decrease the expression of L-selectin on the neutrophil membrane surface, though this effect did not reach significance. On the other hand, the expression of Mac-1 on the neutrophil membrane surface was significantly increased by the addition of sulfatides in vitro. Furthermore, the increase of Mac-1 expression on the neutrophil surface was observed in the endothelial injury-induced mice administered sulfatides. Golino et al. (1997) have reported that, in a rabbit model of carotid artery stenosis and endothelial injury, treatment with a monoclonal antibody (R15.7) against the leukocyte CD11/CD18 adhesion complex causes a decrease in myeloperoxidase activity, an indicator of neutrophil accumulation, in the vessel wall at the site of injury, and that it reduces, as a result, the development of intimal hyperplasia. R15.7 antibody inhibits all members of the CD11/CD18 family (known as LFA-1, Mac-1 and p50, 95) by binding to the β-chain of the CD11/CD18 complex (Ma et al., 1991). Furthermore, Mac-1 deficient mice have been reported to reduce neointimal thickening after vascular injury (Simon et al., 2000). Thus, Mac-1 may play an important role in the development of intimal hyperplasia. If this is indeed the case, the exacerbation of intimal hyperplasia induced by sulfatides in our studies may be secondary to an increase in the expression of Mac-1 on the neutrophil membrane surface and the subsequent accumulation of neutrophils in the injured vessel. Recently, Merten et al. (2005) have also found that sulfatides activate platelets through P-selectin and enhance platelet–leukocyte aggregation. Thus, sulfatides may enhance the platelet activation and platelet–leukocyte interaction at the site of injury.

In conclusion, treatment with sulfatides caused an increase in the number of leukocytes adhering to the vessel wall at the site of injury and produced, as a result, an exacerbation of intimal hyperplasia in the mouse femoral artery. These results suggest that leukocyte accumulation including neutrophils on the subendothelial matrix or on the adherent platelets may result from the interaction between L- or P-selectin and sulfatides, and may contribute to the initiation and development of intimal hyperplasia. This neutrophil accumulation may be mediated by increases in $[Ca^{2+}]_i$ and the tyrosine phosphorylation of several cellular proteins through the agonistic effects between sulfatides and L-selectin resulting in an increase in Mac-1 on the neutrophil membrane surface.

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