Cerebrosidesulfotransferasedeficiencyameliorates
L-selectin-dependentmonocyteinfiltrationinthekidneyafterureteralobstruction*

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Mononuclearcellsinfiltratingtheinterstitiumareinvolvedinrenaltubulointerstitialinjury.Theunilateral
ureteralobstruction(UUO)isanestablishedexperimental
modelofrenalinterstitialinflammation.Inourpre-
vious study, we postulated that L-selectin on monocytes
is involved in their infiltration into the interstitium by
UUO and that a sulfated glycolipid, sulfatide, is the
physiological L-selectin ligand in the kidney. Here we
tested the above hypothesis using sulfatide- and L-select-
in-deficient mice. Sulfatide-deficient mice were gener-
ated by gene targeting of the cerebrosidesulfotrans-
ferase(Cst)gene.AlthoughtheL-selectin-IgGchimera
protein specifically bound to sulfatide fraction in acidic
lipids from wild-type kidney, it did not show such bind-
ing in fractions of Cst−/−mice, indicating that sulfatide is the major L-selectin-binding glycolipid in the
kidney. The distribution of L-selectin ligand in wild-
type mice changed after UUO; sulfatide was relocated
from the distal tubules to the peritubular capillaries
where monocytes infiltrate, suggesting that sulfatide re-
located to the endothelium after UUO interacted with
L-selectin on monocytes. In contrast, L-selectin ligand
was not detected in Cst−/−mice irrespective of UUO
treatment. Compared with wild-type mice, Cst−/−mice
showed a considerable reduction in the number of monocytes/macrophages that infiltrated the intersti-
tium after UUO. The number of monocytes/macrophages
was also reduced to a similar extent in L-selectin−/−
mice. Our results suggest that sulfatide is a major L-
selectin-binding molecule in the kidney and that the
interaction between L-selectin and sulfatide plays a crit-
ical role in monocyte infiltration into the kidney interstitial.

Blood monocytes that extravasate to sites of inflammation
differentiate into macrophages and induce inflammatory re-
sponse and apoptosis of tubular epithelial cells leading to tu-
bulointerstitial injury in renal inflammation (1, 2). In the
experimental model of kidney interstitial inflammation (3, 4),
unilateral ureteral obstruction (UUO)3 leads to interstitial
inflammation, interstitial fibrosis, and tubular atrophy (5). Al-
though infiltration of monocytes/macrophages into the intersti-
tium following UUO is well documented, the mechanism
involved in this process remains elusive.

Monocyte infiltration is a multistep process in which chemo-
kines and adhesion molecules play key roles. The initial step
of this process involves the binding of monocytes to the endothel-
ium of venules, mediated by various adhesion molecules such
as selectins (6). The selectin family consists of three members,
designated P-, E-, and L-selectin. The P- and E-selectins are
present on activated endothelial cells, whereas L-selectin is
expressed on leukocytes. Results of our previous study in rats
with UUO (7) suggested that L-selectin on monocytes mediates
their infiltration into the interstitium, based on the observa-
tion that a neutralizing antibody against L-selectin inhibited
monocyte infiltration.

L-selectin was originally identified as a homing receptor to
capture lymphocytes in the flowing bloodstream in lymph
nodes (8–10). However, L-selectin ligands are expressed not
only in lymphoid organs but also in other tissues (11–13). The
main L-selectin-binding molecules are sialylated, fucosylated,
and sulfated glycans on mucin-like molecules such as Gly-
CAM-I and CD34, which are located on lymph node high endo-
theelial venules and are involved in the homing (14). L-selectin
also binds to a sulfated glycolipid, sulfatide (15–17), and chon-
droitin sulfate and heparin/heparan sulfate proteoglycans (18–
20). We have recently shown that collagen XVIII interacts with
L-selectin (21). Furthermore, chemically synthesized 3′-sulfo
Lewis^a^ and 3′-sulfo Lewis^a^ oligosaccharides were found to be
potent L-selectin ligands as are sialyl Lewis^a^Lewis^a^ determi-
nants or sulfatide (22). Results of our previous study (7) sug-
gested that sulfatide is an L-selectin ligand in the rat kidney

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1 The abbreviations used are: UUO, unilateral ureteral obstruction;
CST, cerebrosidesulfotransferase; LEC-IgG, L-selectin-IgG chimera.

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and contributes to the interstitial monocyte infiltration following UUO. These conclusions were based on the histochemical distribution of sulfatide, demonstrated by an anti-sulfatide monoclonal antibody that was very close to that of L-selectin ligand detected by an L-selectin-IgG chimera protein before and after ureteric ligation and that exogenously added anti-L-selectin monoclonal antibody or sulfatide considerably inhibited monocyte infiltration (7).

Sulfoglycolipids are comprised of acidic glycolipids containing sulfate esters on their oligosaccharides. They have been implicated in a variety of physiological functions through their interactions with extracellular matrix proteins, cellular adhesion receptors, blood coagulation systems, complement activation systems, cation transporter systems, and microorganisms (23). The distribution of sulfoglycolipids is tissue-specific and they are abundant in myelin sheaths, spermatozoa, renal tubular cells, and epithelial cells of the gastrointestinal tract (23).

Sulfation of glycolipids is catalyzed by cerebroside sulfotransferase (CST, EC 2.8.2.11), which is located in the Golgi apparatus (24). Recently, Cst-null mice were generated by gene targeting to elucidate the physiological functions of sulfoglycolipids (25). The Cst-/- mice completely lack sulfatide in the brain and seminolipid in the testis and manifest some neurological disorders because of myelin dysfunction as well as spermatogenesis arrest (25, 26). These observations emphasize the critical roles of sulfoglycolipids in the intercellular interactions during myelin formation and spermatogenesis. Because sulfatide is rich in renal tubular cells, its involvement in renal function such as ion transport has been suggested (23). However, the Cst-null mice do not exhibit signs of renal failure or apparent histological abnormality in the kidney (25). A detailed analysis with some stress would be desirable to elucidate the biological function of sulfatide in the kidney.

Here we tested the hypothesis that interaction between sulfatide and L-selectin mediates infiltration of monocytes into the kidney interstitium, using sulfatide- and L-selectin-deficient mice. The present results established the critical role of their interaction in monocyte infiltration that occurs in the inflamed kidney.

**EXPERIMENTAL PROCEDURES**

**Animals—**Cst-/- mice and L-selectin-/- mice were generated as described previously (25, 27). All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry.

**Lipid Extraction and Analysis—**Pooled kidneys (1.4-2 g) from 8- to 12-week-old mice of each genotype and sex were extracted with 19 volumes of chloroform/methanol (2:1 by volume) and then with 10 volumes of chloroform/methanol/water (60:120:9 by volume). The pooled extract (the total lipid extract) were analyzed by two-dimensional thin-layer chromatography (TLC), using the solvent system chloroform/methanol/water containing 0.2% CaCl2 (65:35:8 by volume) (first direction) and chloroform/methanol/acetic acid/water (7:2:4:2.1 by volume) (second direction) (28). Glycolipids were visualized with the orcinol-sulfuric acid reagent.

**Thin-layer Chromatogram Blotting—**Total lipid was extracted from kidney with chloroform/methanol/water (30:60:5 by volume) followed by chloroform/methanol (2:1 by volume). The extracts were combined and applied to a DEAE-Sephadex A-25 (Amersham Biosciences) column. After washing with chloroform/methanol/water (30:60:5 by volume) and methanol, acidic glycolipids were eluted with 200 mM ammonium acetate in methanol and desalted using a SepPak C18 cartridge (Waters, Milford, MA). The desalted glycolipids were applied to a TLC plate (Silica Gel 60; Merck) and developed with chloroform/methanol/0.2% CaCl2 (60:30:7 by volume). After chromatography, glycolipids were transferred to a polyvinylidine difluoride membrane (Immobilon; Millipore) and incubated with L-selectin-IgG chimera (LEC-IgG) (11), followed by peroxidase-conjugated sheep anti-human IgG (Cappel, Durham, NC) as described previously (29).

**Unilateral Ureteral Obstruction—**Cst-/- mice (n = 5, female) were subjected to complete UUO as described previously (3). Briefly, the right ureter was ligated with a silk ligature under anesthesia at the junction of the upper third and lower two-thirds. Wild-type mice (n = 5, female) from the same litter were used as control. At day 2, the mice were sacrificed, and the obstructed and non-obstructed kidneys were removed. Portions of the tissue were processed for TLC blotting and immunohistochemistry. L-selectin-/- mice (n = 5, female) and wild-type mice (n = 5, female) were operated on in the same way as Cst-/- mice and were sacrificed and the kidneys dissected out for immunohistochemistry.

**Histopathological Examination—**Each kidney specimen was divided into two parts. One portion was fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer-thick sections were stained with periodic acid Schiff and periodic acid-methanol-schiff at neutral pH. The other portion of each specimen was rapidly frozen in liquid nitrogen and cut with a cryostat. Four-micrometer-thick acetone-fixed frozen sections were used for immunohistochemical evaluation. Ligands for L-selectin and sulfatide were detected by the indirect immunofluorescence method as described previously (7). Briefly, the frozen sections were fixed with cold acetone for 3 min and stained with LEC-IgG or anti-sulfatide monoclonal antibody (GS5) (7, 30) for 24 h at 4 °C. Recombinant Ig chimera or mouse IgM was used as a control for nonspecific staining (7, 11). Then, the sections were stained with fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG antibody (ICN Pharmaceuticals, Inc., Aurora, OH) or FITC-labeled goat anti-mouse IgM antibody (ICN Pharmaceuticals, Inc.) for 30 min at room temperature. The sections were treated in phosphate-buffered saline, washed with Perifluor (Shandon, Pittsburgh, PA), and examined under a fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany).

Monocyte/macrophage infiltration into the renal interstitium was estimated by immunostaining, using a specific rat monoclonal antibody against mouse monocyte/macrophage (F4/80; Serotec, Oxford, UK). In brief, the frozen sections were fixed with cold acetone for 3 min, and nonspecific protein binding was blocked by incubation with normal goat serum and avidin for 20 min. The sections were first incubated with F4/80 for 60 min at room temperature. Rat IgG was used as a control for nonspecific staining. Then the sections were incubated with biotin-labeled goat anti-rat IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at room temperature. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% H2O2 for 30 min. After that, the sections were stained with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were then counterstained with Mayer’s hematoxylin.

Cell Counts—The nuclei of F4/80-positive cells were counted by examining 10 randomly selected areas near the borderline of the cortex and medulla under high magnification (×400). Cell number per mm2 was expressed according to the method described by Saito and Atkins (31).

**Statistical Analysis—**All values are expressed as mean ± S.E. Differences between groups were examined for statistical significance using one-way analysis of variance followed by Scheffe’s test. A p value less than 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**Sulfatide Is Absent in Cst-null Mice—**Because the profiles of the kidney glycolipids were noticeably different between normal male and female mice (Fig. 1, a and c) as described before (28), total glycolipids from male and female kidneys of Cst-null mice were separately investigated. As shown in Fig. 1, spots corresponding to authentic sulfatide SM4s were absent in both cases. Instead, spots corresponding to GalCer was used as a control for nonspecific binding (7, 11). Then, the sections were incubated with biotin-labeled goat anti-rat IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at room temperature. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% H2O2 for 30 min. After that, the sections were stained with a aVectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were then counterstained with Mayer’s hematoxylin.

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**Sulfatide Is a Ligand for L-selectin in Mouse Kidney—**To investigate biochemically the ligands for L-selectin in mouse kidney, acidic lipids were extracted from the kidney and their reactivity to L-selectin was examined by TLC blotting analyses. As shown in Fig. 2B, the LEC-IgG bound to a material comigrating with sulfatide in the acidic lipid fraction from the wild-type kidney as well as the authentic bovine brain sulfatide, whereas it did not react to ganglioside GM3, consistent with the results of Suzuki et al. (15). In Cst-/- kidney, sulfatide...
disappeared from the acidic lipid fraction (Fig. 2A), consistent with the results in Fig. 1, and the reactivity to LEC-IgG was also completely abolished (Fig. 2B). These results clearly indicate that the L-selectin ligand in lipid fraction of mouse kidney is sulfatide. Interstitial Changes in UUO—No abnormalities were detected in Cst<sup>−/−</sup> or wild-type mice before UUO (Fig. 3, A and B). UUO resulted in tubular dilation and atrophic changes in the interstitium of the obstructed kidney (Fig. 3, C and D). In contrast, the non-obstructed kidney showed no signs of tubular dilation or atrophy (Fig. 3, E and F). The non-obstructed kidneys of L-selectin<sup>−/−</sup> and wild-type mice were also similar to the normal control kidneys (data not shown).

Distribution of L-selectin Ligands and Sulfatide in Mouse Kidney—LEC-IgG reacted exclusively with the distal tubules of wild-type mice (Figs. 4C and 5C). This reactivity was almost absent in the kidney of Cst<sup>−/−</sup> mice (Figs. 4B and 5B), supporting the results of biochemical experiments showing that the major L-selectin ligand in the kidney is sulfatide. Following ureteric ligation, the reactivity of LEC-IgG in the distal tubules disappeared and, instead, a new reactivity emerged in the interstitium and peritubular capillaries (arrowheads) after UUO. Inset, a high magnification of the dashed line area. B and D, the reactivity with LEC-IgG was almost abolished in Cst<sup>−/−</sup> kidney irrespective of UUO treatment.
UUO was compared between groups. Reduced in our ureteral obstruction model, the extent of increased monocyte/macrophage infiltration into the renal interstitium in wild-type mice was suppressed to almost 50% of that before UUO in Cst knockout mice (Fig. 7). However, UUO resulted in a 4-fold increase in the number of these cells compared with pre-UUO in wild-type mice. Although there was no difference in the number of monocytes/macrophages between Cst knockout mice and wild-type mice before UUO, the increased number of these cells induced by UUO was significantly reduced in Cst knockout mice. This suggests that sulfatide is highly responsible for the infiltration of macrophages into the renal interstitium.

**Monocyte/Macrophage Infiltration in the Renal Interstitium of Cst knockout Mice**—To elucidate whether sulfatide is responsible for the monocyte infiltration into the renal interstitium induced in our ureteral obstruction model, the extent of increased number of monocytes/macrophages in the interstitium after UUO was compared between Cst-null and wild-type mice. Because monocyte infiltration commences as early as 4–12 h after UUO, it was evaluated at 48 h after UUO in the present study. Monocytes/macrophages were stained with a specific anti-monocyte/macrophage monoclonal antibody, F4/80 (Fig. 6). No staining was observed in the kidney when irrelevant immunoglobulin was used as a negative control. There was no difference in the number of monocytes/macrophages between Cst knockout mice and wild-type mice before UUO (Fig. 7). After UUO, there was a 4-fold increase in the number of these cells compared with pre-UUO in wild-type mice (Fig. 7). However, the increased number of monocytes/macrophages induced by UUO was suppressed to almost 50% of that before UUO in Cst knockout mice (Figs. 6 and 7), suggesting that sulfatide is highly responsible for the infiltration of macrophages into the renal interstitium.

**Monocyte/Macrophage Infiltration in the Renal Intersstitium of L-selectin knockout Mice**—To determine the contribution of L-selectin to the monocyte infiltration into the interstitium, the number of monocytes/macrophages in the interstitium after UUO was compared between L-selectin knockout and wild-type mice. Although there was no difference in the number of monocytes/macrophages between these mice before UUO, the increased number of these cells induced by UUO was significantly reduced in L-selectin-deficient mice (Fig. 8). The suppressed rate of the increase in infiltrated macrophages/macrophages induced by UUO was almost the same between Cst knockout and L-selectin knockout mice, suggesting that sulfatide on the endothelial cells of peritubular capillaries is the major ligand for L-selectin on the monocytes when they enter the interstitium. The residual elevation in the number of monocytes/macrophages after UUO in Cst knockout and L-selectin knockout mice indicates the contribution of other as yet unknown factors.

**DISCUSSION**

Our previous results indicated that interaction between sulfatide in the kidney and L-selectin on monocytes mediates...
monocyte infiltration into the kidney interstitium (7). This conclusion was based on the following results: blockade of L-selectin function by a neutralizing antibody protects against monocyte infiltration after UUO in rats (7); sulfatide binds specifically to L-selectin through the sulfated sugar chain (15); L-selectin ligand relocates from the distal tubules to the interstitium and peritubular capillaries by UUO as sulfatide does (7); and exogenously administrated sulfatide inhibits monocyte infiltration after UUO (7). The present study further strengthens the above results and shows that L-selectin ligand is absent in sulfatide-deficient mice and that monocyte infiltration after UUO is reduced in sulfatide-deficient mice to the same extent as in L-selectin-deficient mice. During the preparation of this report, Lange-Sperandio et al. (32) reported that inhibition of macrophage recruitment to the obstructed kidneys with L-selectin deficiency is associated with a reduction of apoptosis of tubular epithelial cells and interstitial fibrosis. This finding adds strong support to our conclusion.

Where does L-selectin on the monocytes contact with sulfatide in the kidney? Sulfatide is expressed in the distal tubules in normal kidney, where no leukocyte traffic is seen. In addition, leukocytes rarely enter the renal interstitium under normal conditions. It is speculated that sulfatide mediates rolling and/or migration after UUO. First, after UUO, sulfatide relocates from the distal tubules to the interstitium and peritubular capillaries, where monocytes are considered to extravasate into the interstitium. Thus, L-selectin and sulfatide seem to make contact on the peritubular capillary walls. Second, sulfatide, which relocates from the distal tubules to the interstitium, mediates extravasation and migration of monocytes into the kidney interstitium. Recently, it was demonstrated that the number of emigrated leukocytes and the distance of extravascular migration was significantly reduced in L-selectin-deficient mice (33). The same phenomenon might occur in sulfatide-deficient mice. Then why does the distribution of sulfatide change following UUO? There are also two possible mechanisms. One is that sulfatide is shed from injured tubular epithelial cells and moves into the interstitium and peritubular capillary walls. A similar event has been observed in Tamm Horsfall protein (4). The other is that sulfatide is newly synthesized in the interstitium and peritubular capillary walls following ureteric ligation.

In addition to obstructive nephropathy, interstitial infiltration of mononuclear cells is also observed in tubulo-interstitial nephritis, severe glomerulonephritis, and rejection of transplanted kidney. Selectins and their ligands have been shown to mediate monocyte infiltration in a variety of kidney inflammation. For instance, induced E-selectin on the peritubular capillary is implicated in monocyte infiltration in diabetic nephropathy (34). Induction of L-selectin ligands on peritubular and venous endothelium is suggested to be involved in acute kidney allograft rejection (35). E- and P-selectin have been shown to mediate leukocyte infiltration during ischemia/reperfusion-induced acute renal failure (36, 37). Thus, all members of the selectin family may contribute to monocyte infiltration in the kidney. In the present study, monocyte infiltration induced by UUO was considerably, but not completely, suppressed in L-selectin-deficient mice. The residual recruitment activity might be attributed to E- and P-selectins (38). However, there was no significant difference in the number of infiltrated monocytes/macrophages induced by UUO between triple selectin-deficient mice and L-selectin-deficient mice (32). This finding strongly suggests that E- and P-selectins scarcely contribute to monocyte infiltration in obstructive nephropathy.

Sulfatide has been demonstrated to bind specifically to L-selectin and P-selectin (15–17, 39). In fact, sulfatide appears to regulate inflammation and tumor metastasis mediated by P-selectin. Mulligan et al. (40) showed the preventive effect of sulfatide in P-selectin-dependent lung injury. On the other hand, Borsig et al. (41) reported that sulfatide on tumor cells serves as a P-selectin ligand and facilitates tumor metastasis by interaction with platelets leading to tumor microemboli. The present study provided in vivo evidence that sulfatide plays a critical role as an L-selectin-binding molecule during the process of inflammation.

In conclusion, we demonstrated that sulfatide is a major L-selectin-binding molecule in the kidney and mediates monocyte infiltration in obstructive nephropathy. Our results suggest that blockade of the interaction of sulfatide and L-selectin could be a potentially useful strategy for the treatment of interstitial nephritis.
Sulfatide Mediates Monocyte Infiltration into the Kidney

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