A sulfatide receptor distinct from L-selectin is involved in lymphocyte activation

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Received 29 September 1997; revised version received 28 October 1997

Abstract Sulfatide induces leukocyte activation, which is thought to be mediated via L-selectin. Here we demonstrate that lymphocytes express a receptor for sulfatide distinct from L-selectin and that this receptor is involved in sulfatide-induced cell activation. While cell surface L-selectin expression was abolished by phorbol 12-myristate 13-acetate (PMA), lymphocytes retained the ability to bind sulfatide in liquid phase as well as in immobilized solid phase. The novel sulfatide receptor obtained from PMA-treated lymphocytes showed a molecular size of 65 kDa. Stimulation through this receptor triggered cytosolic free Ca\textsuperscript{2+} elevation and intercellular aggregation.

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Key words: L-Selectin; Glycolipid; Cell adhesion molecule; Phorbol 12-myristate 13-acetate; Cell aggregation; Lymphocyte

1. Introduction

Sulfatide, 3-sulfated galactosyl-ceramide, is distributed widely in the brain, kidney, spleen, gastrointestinal tract, granulocytes, platelets, some cancer cells and serum [1–3]. Sulfatide has anticoagulant activity [4], but its biological significance remains unknown. Sulfatide also is involved in cell adhesion processes necessary for infection by some bacteria, malaria and viruses [5–8]. For instance, Helicobacter pylori apparently uses sulfatide on gastric mucosa as an adhesion receptor for the establishment of infection [5]. Other studies have reported the interaction of sulfatide with extracellular matrix proteins such as thrombospondin and laminin, which may play a role in the regulation of cell adhesion and spreading (reviewed in [2]). Recently, sulfatide has received much attention, since it was shown to bind to L- and P-selectins [3,9], and its administration prevented leukocyte accumulation and tissue injury in some models of inflammatory diseases [10,11].

Sulfatide induces signaling and metabolic changes in neutrophils, which are thought to be mediated via L-selectin [12]. However, the following observations raise the question of whether L-selectin is the sole functional receptor for sulfatide or not. In a series of preliminary experiments, we observed that PMA-treated lymphocytes and neutrophils lacking surface expression of L-selectin adhered well to immobilized sulfatide (H. Kawashima, unpublished observation). Laudanna et al. [13] showed that fucoidan (a sulfated polysaccharide from marine algae rich in L-fucose) and PPME (mannose-6-phosphate rich polyphosphomann ester from yeast), both of which are reactive with L-selectin, did not induce Ca\textsuperscript{2+} influx signal in neutrophils, although sulfatide did. In the study reported by Waddell et al. [12], sulfatide induced larger increases in tyrosine phosphorylation and MAP kinase activity than dioligation of L-selectin with the monoclonal antibodies (mAb) DREG 56 or DREG 200. Together, these observations suggest that a novel sulfatide receptor distinct from L-selectin is expressed on the leukocyte surface.

In the present study, we used phorbol 12-myristate 13-acetate (PMA) to abrogate cell surface L-selectin and studied the capacity of PMA-treated cells to bind sulfatide. We report that a sulfatide receptor other than L-selectin, with a molecular mass of 65 kDa, is expressed on lymphocytes. This molecule can mediate increases in sulfatide-triggered cytosolic free Ca\textsuperscript{2+} and lymphocyte aggregation, and may play an important role in lymphocyte activation and recruitment into specific tissues.

2. Materials and methods

2.1. Animals

Eight- to 12-week-old male specific-pathogen-free Wistar rats were provided by the Shionogi Pharmaceutical Co., Osaka, Japan, and used according to the guidelines of the Animal Ethics Committee of Osaka University Medical School.

2.2. Antibodies and reagents

HRL2 and HRL3 are hamster anti-rat L-selectin mAbs (both IgG) prepared in our laboratory [14]. HRL2 is a non-blocking mAb, directed to the complement-binding protein homology domain [15], whereas HRL3 is a blocking mAb directed to the lectin domain of L-selectin. GS5 [16], a mouse anti-sulfatide mAb (IgM), was used as culture supernatant. FITC-labeled goat anti-hamster IgG was purchased from Organon Teknika Co. (Durham, NC). FITC-labeled donkey anti-mouse IgG (IgG+IgM) was a product of Southern Biotechnology Associates, Inc. (Birmingham, AL). PMA, A23187, and sulfatide were all obtained from Sigma (St. Louis, MO). Galactosylceramide was from Funakoshi (Tokyo, Japan). Fluo 3-AM and 2',7'-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were from Dojindo (Osaka, Japan). Eight- to 12-week-old male specific-pathogen-free Wistar rats were provided by the Shionogi Pharmaceutical Co., Osaka, Japan, and used according to the guidelines of the Animal Ethics Committee of Osaka University Medical School.

2.3. Flow cytometry

Lymphocytes were either unstimulated or stimulated with 50 ng/ml PMA at 37°C for 5, 10, and 30 min. Cells were washed twice with PBS, incubated with 300 μg/ml of sulfatide (dissolved in DMSO at 50 mg/ml and diluted with PBS) on ice for 30 min. Then cells were again washed twice with PBS. Cell-bound sulfatide was detected by GS5, using a 1:50 dilution of culture supernatant and FITC-labeled donkey anti-mouse IgM. L-Selectin expression was detected by incubating the cells with HRL3 at 10 μg/ml for 30 min on ice, and then washed twice with washing buffer (PBS containing 0.1% BSA and 5% FCS) and stained with FITC-labeled donkey anti-rat IgG.
and 0.05% sodium azide). Cells were then stained with FITC-labeled anti-hamster IgG at 5 μg/ml for 30 min on ice. Finally, the cells were washed twice and resuspended in PBS for flow cytometric analysis in an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL).

2.7. Affinity purification of sulfatide binding protein
Sulfatide dissolved in methanol was immobilized on 96-well ELISA plates (SUMILON, H type, Japan) by drying at 37°C for 1.5 h. Plates were then blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) overnight at 4°C. The wells were washed three times with 0.05% Tween 20 in PBS before cells were plated. Lymphocytes were isolated from mesenteric lymph nodes (MLN) of male Wistar rats. After washing twice with RPMI 1640 medium, cells were labeled for 40 min at 37°C with BCECF-AM at a final concentration of 5 μM. A portion of cells was exposed to 50 ng/ml PMA in the last 10 min of the labeling process. The cells were then washed once with RPMI medium containing 10% FCS and twice with RPMI only. Aliquots of the cells were preincubated with 100-300 μg/ml sulfatide for 30 min on ice bath, washed and resuspended in RPMI at 5 × 10⁶ cells/ml. In the next step, 50 μl of cell suspension was added in quadruplicate to wells coated with sulfatide. After 40 min incubation at 7°C, the cells were filled with RPMI medium and sealed with parafilm. The plates were inverted for 40 min at 7°C to allow non-adherent cells to detach from the bottom surface of the wells, after which the medium containing unbound cells was removed by suction. The remaining bound cells were lysed in 50 μl of 1% NP-40, and fluorescence intensity was read in a fluoroscan II microplate fluorometer (Labsystem, Tokyo, Japan) at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The results are expressed as mean ± S.D. of quadruplicate determinations.

2.5. Measurement of cytosolic free calcium changes
Lymphocytes suspended in RPMI were incubated with fluo-3 AM (final concentration of 2.5 μM) for 40 min at 37°C. Part of the cells was treated with 50 ng/ml PMA in the final 10 min of incubation. Cells were washed and resuspended in Hanks’ balanced salt solution (HBSS) with 10 mM HEPES. Fluorescence was measured in a spectrophotometer (model F3000, Hitachi, Tokyo, Japan), equipped with a magnetic stirrer and a thermostatic cuvette chamber. Excitation was set at 488 nm and emission at 535 nm. After a stable baseline was obtained, DMSO was added to assess the effect of solvent control. The cells were then stimulated with 400 μg/ml sulfatide. Calcium ionophore A23187 (5 μM) was finally applied to assess the reliability of the cellular responses.

2.6. Aggregation assay
Lymphocytes treated with or without PMA (50 ng/ml, 37°C, 10 min) were resuspended at 5 × 10⁶ cells/ml in RPMI medium containing 10% FCS. Next, 100 μl of cells were added to duplicate wells of 96-well culture plates (IWAKI, Japan). WT.1, mouse anti-rat LFA-1 mAb [17], was added to PMA-treated lymphocytes at 20 ng/ml. Sulfatide (300 μg/ml) and HRL3 (1 μg/ml) were added to control and PMA-treated cells respectively. The plates were incubated at 37°C for 2 h. Photographs were taken under a phase contrast microscope (Olympus, Japan).

2.7. Affinity purification of sulfatide binding protein
Sulfatide was hydrophobically conjugated to octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as reported previously [18]. To block non-specific binding, the beads were incubated with 1% BSA in PBS at 4°C for 1 h at room temperature, washed with PBS and packed into columns. MLN lymphocytes pretreated with 50 ng/ml PMA for 10 min at 37°C were surface-biotinylated with 50 μg/ml NHS-LC-biotin (Pierce, IL) and lysed with 0.5% NP-40 in PBS supplemented with 1 mM PMSF. The cell lysates were then incubated with sulfatide-conjugated octyl-Sepharose. Precipitated proteins were eluted in SDS sample buffer, separated by SDS-PAGE transferred to IPVH membrane (Millipore, Japan), and detected by a horseradish peroxidase-conjugated avidin-biotin complex (ABC kit, Vector, Burlingame, CA) and enhanced chemiluminescence (ECL, Amersham) as described in the attached specifications.

3. Results
3.1. Lymphocytes express a sulfatide receptor distinct from L-selectin
PMA-treated lymphocytes expressed no surface L-selectin but retained the ability to bind sulfatide, although levels of sulfatide binding were obviously decreased as compared to those by control lymphocytes (Fig. 1). The net decrease is probably due to the shedding of L-selectin that also binds sulfatide [9]. Sulfatide binding to lymphocytes devoid of L-selectin expression was independent of divalent cations (data not shown), and increased significantly by prolonging the incubation time with PMA. The L-selectin-independent binding of sulfatide by lymphocytes was further confirmed by cell adhesion to plastic-immobilized sulfatide (Fig. 2). PMA-treated lymphocytes lacking L-selectin expression adhered well to sulfatide, albeit with slightly lower efficiency than that of untreated cells (Fig. 2A). The adhesive interaction was apparently specific, because the adhesion did not occur to immobilized non-sulfated analogous glycolipid, galactosylceramide. In addition, the adhesion correlated well with the quantity of immobilized sulfatide, and did not increase further when immobilized sulfatide was present at over 1 μg per well (data not shown). Furthermore, preincubation of cells with soluble sulfatide dose-dependently decreased cell adhesion (Fig. 2B). These results suggested that sulfatide binds to a receptor(s) distinct from L-selectin.

3.2. Sulfatide induces a rapid increase in intracellular Ca²⁺ concentration and lymphocyte aggregation via L-selectin-dependent and -independent pathways
To examine whether the sulfatide receptor(s) can transduce

Fig. 1. PMA-treated lymphocytes lack surface L-selectin but retain the ability to bind sulfatide. Lymphocytes were either untreated or treated with 50 ng/ml PMA at 37°C for 5, 10, and 30 min. Cells were washed, incubated with 300 μg/ml of sulfatide for 30 min on ice. Then cell-bound sulfatide was detected by GS5 and FITC-labeled donkey anti-mouse IgM in a flow cytometer. L-selectin expression was detected by HRL3 and FITC-labeled goat anti-hamster IgG. The dashed lines represent the background level obtained with FITC-labeled secondary antibodies only.
3.3. The new sulfatide receptor is a 65 kDa protein

In order to identify the non-L-selectin type binding protein for sulfatide on lymphocytes, lysates of PMA-treated and surface-biotinylated lymphocytes were applied to an affinity column loaded with sulfatide-conjugated octyl-Sepharose CL-4B. After washing, the bound materials were eluted with 3 M NaSCN, dialyzed and then precipitated consecutively with galactosylceramide-conjugated octyl-Sepharose and sulfatide-conjugated octyl-Sepharose. The precipitates obtained by the use of sulfatide-conjugated Sepharose yielded a reproducible, prominent band of 65 kDa in SDS-PAGE analysis under reducing conditions (Fig. 5, lane 2). The band was absent in the precipitate obtained with galactosylceramide-conjugated octyl-Sepharose (lane 1), and disappeared when the NaSCN eluate was incubated with sulfatide prior to precipitation (data not shown).

4. Discussion

In this study, we have demonstrated for the first time that a sulfatide binding site distinct from L-selectin is expressed on lymphocytes and is involved in sulfatide-induced lymphocyte activation. We tentatively call this binding site a receptor for sulfatide or sulfatide receptor, since it specifically bound sulfatide in liquid phase as well as in solid phase in a saturable manner. In sharp contrast to L-selectin, which is sensitive to proteolytic cleavage by PMA-activatable protease [20,21], the sulfatide receptor appears to be resistant to PMA-induced galactosylceramide-conjugated octyl-Sepharose and sulfatide-conjugated octyl-Sepharose.
Fig. 5. The sulfatide receptor has an $M_r$ of 65 kDa. Lysates of PMA-treated and biotinylated lymphocytes were first preclarified by an octyl-Sepharose CL-4B column. The pass-through solution was then loaded on a column with sulfatide-bound octyl-Sepharose. After washing the column with PBS and 1 M NaCl, the bound proteins were eluted with 3 M NaSCN, dialyzed and then precipitated sequentially with galactosylceramide-conjugated Sepharose and sulfatide-conjugated octyl-Sepharose. The precipitates were eluted in SDS sample buffer, separated by SDS-PAGE under reducing conditions and blotted onto an IPVH membrane. Biotinylated proteins were detected by using an ABC kit and ECL. Lanes 1 and 2 represent precipitates with galactosylceramide-conjugated Sepharose and sulfatide-conjugated Sepharose, respectively.

shedding. PMA-treated lymphocytes adhered well to immobilized sulfatide, although adhesion was less efficient than that of untreated cells. The non-L-selectin-mediated adhesion to sulfatide is unlikely to be due to a non-specific hydrophobic interaction, because adhesion did not occur to a desulfated analogous glycolipid, galactosylceramide, and was blocked by preincubation of cells with sulfatide.

Since sulfatide is known to bind to L- and P-selectin [12,18], the effects of sulfatide on leukocytes have been generally considered to be mediated by selectins. Sulfatide is expressed not only in some hemopoietic cells (granulocytes, platelets and myeloid tumor cells) but also in non-vascular cells and tissues. If extravascular sulfatide serves as an adhesion ligand for leukocytes, L-selectin would not be the cognate receptor, since leukocytes recruited into tissues express extremely low levels, if any, of L-selectin [22]. This notion prompted us to investigate the existence of a sulfatide receptor other than L-selectin. A number of previous observations are also consistent with the presence of a non-L-selectin type sulfatide receptor. For example, in the Stamper-Woodruff assay, mouse lymphocytes adhered to the myelinated regions of the central nervous system rich in sulfatide. Lymphocyte adherence was only partially inhibited (30%) by MEL-14, a blocking mAb to mouse L-selectin [23]. In vitro studies with human neutrophils showed that sulfatide stimulation induced more prominent tyrosine phosphorylation, MAP kinase activation and superoxide production than did stimulation of L-selectin alone with mAbs [12,24]. In this study, we prepared surface-biotinylated lysates of PMA-treated lymphocytes lacking L-selectin expression and isolated a protein of ~65 kDa by affinity column chromatography and precipitation with sulfatide-conjugated octyl-Sepharose beads. The prominent band of 65 kDa was not observed when galactosylceramide-conjugated beads were used or cell lysates were preincubated with sulfatide, indicating that the 65 kDa protein is a specific molecule for sulfatide binding. We also observed strong and constitutive expression of a 65 kDa sulfatide binding-protein in a L-selectin-negative lymphoma cell line (Z. Ding et al., manuscript in preparation). We are currently making attempts to isolate this protein to homogeneity for N-terminal amino acid sequencing.

To examine the signaling function and physiological significance of this new sulfatide receptor, we investigated whether sulfatide induces any changes in lymphocytes. Sulfatide induced a rapid and sustained elevation of intracellular free Ca$^{2+}$ concentration in normal lymphocytes. The response was moderately decreased in magnitude and duration in PMA-treated lymphocytes. This may be because the sulfatide receptor confers additive or synergistic activation signals to the cells along with L-selectin-mediated signal(s). In support of this argument, sulfatide-induced aggregation in normal lymphocytes was more intense than in PMA-treated L-selectin-negative lymphocytes. The mechanism for homotypic lymphocyte aggregation triggered by sulfatide stimulation is not clear. Antibodies to LFA-1, CD44, and $\alpha_4$ integrins did not block sulfatide-induced cell aggregation, indicating that these adhesion molecules are not important in this phenomenon (data not shown). Passive crosslinking of receptors on separate cells by sulfatide is apparently not responsible either, because aggregate formation did not occur at 4°C and required metabolic energy as well as an intact cytoskeleton (Z. Ding et al., manuscript in preparation).

In summary, the current study demonstrates for the first time the expression of a sulfatide receptor of 65 kDa on lymphocytes. This receptor is distinguishable from L-selectin by showing resistance to PMA-induced shedding. The new sulfatide receptor apparently mediates intracellular Ca$^{2+}$ changes and intercellular aggregation, suggesting its potential role in activation and functional changes of lymphocytes during inflammation.

Acknowledgements: This work is supported in part by a grant-in-aid for Scientific Research from the Ministry of Science, and Culture of Japan, and a grant from the Ministry of Science and Technology of Japan. We thank Dr. Toshiyuki Tanaka for stimulating discussions.

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