Inhibition of P-selectin-mediated cell adhesion by a sulfated derivative of sialic acid

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Abstract

P-selectin, a carbohydrate-binding cell adhesion molecule expressed on activated endothelial cells and platelets, plays a key role in the recruitment of leukocytes to inflammatory and hemorrhagic sites. It simultaneously recognizes a sialic acid-containing carbohydrate chain and the sulfated tyrosine residues of a specific counter-receptor expressed on the leukocyte surface. We examined the inhibitory effects of a synthetic sulfated derivative of sialic acid (NMSO3) on P-selectin-mediated cell adhesion and found the following: (1) P-selectin/IgG chimera bound to immobilized NMSO3. (2) The binding of P-selectin/IgG chimera to purified P-selectin glycoprotein ligand-1 was inhibited by soluble NMSO3. (3) The adhesion of HL60 cells to P-selectin-expressing CHO cells was inhibited by NMSO3. (4) NMSO3 inhibited P-selectin-induced tumor necrosis factor-α production in monocytes and activated platelet-induced generation of reactive oxygen species in neutrophils. In conclusion, NMSO3 acts as a specific inhibitor for P-selectin-mediated cell adhesion and for adhesion-dependent leukocyte activation.

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P-selectin, a member of the selectin family of cell adhesion molecules, is expressed on activated endothelial cells and platelets, and plays a key role in the initial step of extravasation of leukocytes into inflamed or infected tissues in cooperation with two other selectin members, L- and E-selectins [1–6]. P-selectin is also expressed on activated platelets and is thought to mediate the recruitment of leukocytes to hemorrhagic sites to function in the prevention of microbial infection. Upon its expression on endothelial cells and platelets, P-selectin binds to a specific counter-receptor, designated as P-selectin glycoprotein ligand-1 (PSGL-1), on leukocytes [7]. P-selectin simultaneously recognizes carbohydrate chains containing sialylated and fucosylated N-acetyllactosamine structures (e.g., sialyl Lewis X (sLe X) tetrasaccharide unit) and sulfated tyrosine residues in PSGL-1 [8–13]. Studies using blocking antibodies to P-selectin and P-selectin-deficient mice have demonstrated that P-selectin is involved in the pathophysiological process of acute and chronic inflammatory diseases including ischemia/reperfusion injury and atherosclerosis [14–16]. Carbohydrates with sLe X structures and related compounds have been examined for their activity as competitive inhibitors for the selectin-mediated interaction of leukocytes with endothelium and platelets, and as potential anti-inflammation agents [17–22]. Sulfated glycans, such as heparin-derived oligosaccharides [23,24], fucoidin [25], dextran sulfate [25,26], and trestatin A sulfate [27], have also been shown to antagonize P-selectin-dependent cell adhesion.
NMSO3, a sulfated derivative of sialic acid with two alkyl chains, was originally developed as an antiviral agent [28–30]. Because this compound possesses structural elements including sialic acid residue and sulfate groups that are required for the recognition by P-selectin, there is a possibility that it acts as a potential inhibitor of the selectin–carbohydrate interaction. This prompted us to evaluate its effects on selectin-mediated cell adhesion. We also examined its effects on the production of tumor necrosis factor-α (TNF-α) in monocytes and on the reactive oxygen species (ROS) production in neutrophils induced by the adhesion.

Materials and methods

Reagents. N-Acetylneuraminic acid (NeuAc) and colominic acid were purchased from Seikagaku (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Heparin from porcine intestine and dextran sulfate were purchased from Sigma (St. Louis, MO). A sulfated derivative of NeuAc, NMSO3 (sodium [2,2-bis(docosyloxymethyl) propyl-5-acetamido-3,5-dideoxy-4,7,8,9-tetra-O-(sodium oxysulfonyl)-p-glycero-β-D-galacto-2-nonulopyranosido] onate), was supplied by Nissin Central Research Institute [28].

The structure of NMSO3 is shown in Fig. 1. The procedure for the synthesis of this compound will be described elsewhere. Recombinant human P-selectin/IgG chimera was a product of R&D systems (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated protein A and TMB (3,3',5,5'-tetramethylbenzidine) substrate reagent were purchased from Zymed (San Francisco, CA) and BD Bioscience (San Jose, CA), respectively. A monoclonal antibody against human P-selectin (2T60, IgG1) [31] was donated by Dr. K. Tanoue (Tokyo Metropolitan Institute of Medical Science). 2,7'-Bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). A sulfated derivative of NeuAc, NMSO3 (2.5–20 μg/ml in 0.1% BSA/PBS, 0.025 ml) at 4°C for 1.5 h.

The adhesion of HL60 cells to selectin-expressing CHO cells was assayed essentially as described previously [33]. In brief, HL60 cells were labeled with a fluorescent dye, BCECF-AM (3 μM), at 37°C for 30 min. The labeled cell suspension (6 × 10^6 cells/ml, 0.5 ml) in RPMI 1640/1% BSA was placed in a monolayer culture plate. After the plate had been incubated at 4°C for 30 min with gentle shaking, non-adherent cells were removed. The well was gently washed three times with RPMI–PBS. CHO–E cells were added to each well and were incubated at 4°C for 40 min. The wells were washed four times with Tween–PBS, then 0.15 ml of the TMB substrate reagent was added, and the wells were incubated at room temperature for 30 min. Absorbance at 450 nm was measured with a plate reader following the addition of 0.075 ml of 1 M H_3PO_4.

Cell adhesion assay. The adhesion of HL60 cells to selectin-expressing CHO cells was assayed essentially as described previously [35]. In brief, HL60 cells were labeled with a fluorescent dye, BCECF-AM (3 μM), at 37°C for 30 min. The labeled cell suspension (6 × 10^6 cells/ml) in RPMI 1640/1% BSA was placed in a monolayer culture plate. After the plate had been incubated at 4°C for 30 min with gentle shaking, non-adherent cells were removed. The well was gently washed three times with RPMI–PBS. CHO–E cells were added to each well and were incubated at 4°C for 40 min. The wells were washed four times with Tween–PBS. Subsequent procedures, including the incubation with HRP–protein A and the reaction with the TMB substrate reagent, were carried out as described above.

These cells were cultured in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) under a 5% CO_2 atmosphere.

Monocytes and neutrophils were purified from normal human peripheral blood by a combination of dextran sedimentation and Ficoll–Paque gradient centrifugation, as described previously [33,34]. The purity of the monocyte and neutrophil preparations was more than 90%, as evaluated by May–Gruenwald–Giemsa staining. The trypan blue exclusion assay showed that 99% of the cells were viable.

Binding of P-selectin/IgG chimera to immobilized NMSO3. A 96-well ELISA plate was coated with 0.05 ml NMSO3 solution (0.12–30 μM) in 10 mM sodium phosphate buffer (pH 7.5) containing 0.14 M NaCl (PBS) at 4°C for 16 h, blocked with 0.5% bovine serum albumin (BSA)/PBS at 4°C for 4 h, and washed three times with PBS containing 0.05% Tween 20 (TWEEN–PBS). P-selectin/IgG chimera (1 μg/ml, 0.05 ml) in 0.1% BSA/PBS containing 0.2 mM CaCl_2 was then added to each well and wells were incubated at 4°C for 1.5 h. After the wells had been washed three times with Tween–PBS, HRP–conjugated protein A (1:10,000 dilution in 0.1% BSA/PBS, 0.05 ml) was added to each well and the wells were incubated at 4°C for 40 min. The wells were washed four times with Tween–PBS, then 0.15 ml of the TMB substrate reagent was added, and the wells were incubated at room temperature for 30 min. Absorbance at 450 nm was measured with a plate reader following the addition of 0.075 ml of 1 M H_3PO_4.

Cell viability. The viability of the monocyte and neutrophil preparations was more than 90%, as evaluated by May–Gruenwald–Giemsa staining. The trypan blue exclusion assay showed that 99% of the cells were viable.

Fig. 1. Structure of NMSO3, a sulfated derivative of sialic acid. Molecular formula: C_{60}H_{112}NO_{23}S_{4}Na_{5}.

These cells were cultured in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) under a 5% CO_2 atmosphere.
Measurement of reactive oxygen species. Activated platelet-induced ROS production in neutrophils was measured by using a fluorogenic probe, DCFH-DA, as described by Himmelfarb et al. [36]. Human platelet-rich plasma (0.1 ml) was treated with thrombin (0.125 U/ml) in the presence of GPRP peptide (0.5 mM) at 25°C for 30 min, and NMSO3 (100 µM) or PBS was added and incubated at 25°C for 30 min. To the platelet suspension, human neutrophils (1 × 10⁶ cells/ml, 0.1 ml) suspended in PBS and DCFH-DA (10 mM, 2 µl) were added and the mixture was incubated at 37°C for 30 min. The oxidation of the fluorescent probe was analyzed with a flow cytometer (FACScalibur, BD Biosciences).

Results and discussion

Interaction of NMSO3 with P-selectin/IgG chimera

We first examined the interaction between P-selectin/IgG chimera and NMSO3 immobilized on the plate. The P-selectin/IgG chimera was found to bind to NMSO3 and the binding was dependent on the dose of NMSO3 used for the immobilization (Fig. 2A). We next evaluated the inhibitory activity of NMSO3 against the binding of P-selectin/IgG chimera to purified PSGL-1. When P-selectin/IgG chimera was preincubated with NMSO3, the binding of the P-selectin chimera to immobilized PSGL-1 was decreased to the control level (Fig. 2B). The presence of NMSO3 (10 µM) almost completely inhibited the binding and the inhibitory effect was comparable to that of anti-P-selectin antibody (2T60, 20 µg/ml). These results indicated that NMSO3 possessed binding ability for P-selectin/IgG chimera.

Inhibition by NMSO3 of adhesion of HL60 cells to selectin-expressing cells

Effects of NMSO3 on HL60 cell adhesion to a monolayer culture of CHO cells that had been transfected with cDNA encoding P-selectin (CHO-P cells) or E-selectin (CHO-E cells) were then examined. The adhesion of fluorescently labeled HL60 cells to a monolayer of CHO-P cells was inhibited by NMSO3 in a dose-dependent manner and decreased to the control level with 160 µM NMSO3 (Fig. 3A). In contrast, only weak inhibition was observed when HL60 cells were incubated with CHO-E cells in the presence of NMSO3 (Fig. 3B). These results are consistent with the previous observation that the sulfation of tyrosine residues in glycoprotein ligands is required for recognition by P-selectin but not by E-selectin [37–40]. Based on reports that various acidic glycans, including dextran sulfate and heparin, inhibited the P-selectin-mediated cell adhesion, we compared the inhibitory activity of NMSO3 with those of several acidic saccharides. As shown in Fig. 4, NMSO3 inhibited the adhesion more effectively than did heparin, which showed moderate inhibition. None of N-acetyliminonic acid, colominic acid, or dextran sulfate showed significant inhibition at

![Fig. 2. Interaction of NMSO3 with the P-selectin/IgG chimera. (A) Binding of human P-selectin/IgG chimera to immobilized NMSO3 was measured. A 96-well ELISA plate was coated with NMSO3 solution (0.12–30 µM), and P-selectin/IgG chimera (1 µg/ml) was then added to each well, then wells were incubated at 4°C for 1.5 h. The binding in the presence of 5 mM EDTA was also examined (open bar). (B) Inhibitory effect of NMSO3 on the binding of human P-selectin/IgG chimera to immobilized PSGL-1 was examined. To a 96-well ELISA plate that had been coated with PSGL-1 solution (0.36 µg/ml), a mixture of equal volumes of P-selectin/IgG chimera (2 µg/ml) and NMSO3 (2.5–20 µM) was added and wells were incubated at 4°C for 1.5 h. The bound P-selectin/IgG chimera was colorimetrically assayed by using HRP-labeled protein A.](image)

![Fig. 3. Inhibition by NMSO3 of adhesion of HL60 cells to selectin-expressing CHO cells. BCECF-labeled HL60 cells were added to a monolayer culture of CHO cells (6 × 10⁶ cells/ml) expressing P-selectin (A) or E-selectin (B) in a 24-well culture plate and wells were incubated at 4°C for 30 min with gentle shaking in the presence or absence of NMSO3 (0–160 µM). After the removal of non-adherent cells by gentle washing, the fluorescence intensity of adherent cells was measured with a fluorescence spectrophotometer (E_x = 490 nm, E_m = 520 nm).](image)
a concentration up to 1 mg/ml. The IC₅₀ value of NMSO₃ was estimated to be approx. 80 μg/ml (55 μM). This result indicated that the concentration required for inhibition of cell adhesion is higher than that required for inhibition of the binding of P-selectin/IgG chimera to PSGL-1 (Fig. 2B).

**P-selectin-induced TNF-α production in monocytes and activated platelet-induced ROS production in neutrophils**

NMSO₃ dose-dependently inhibited the adhesion of HL60 cells to immobilized P-selectin and the cell adhesion was almost completely blocked by NMSO₃ at 100 μM (Fig. 5). We previously showed that P-selectin induced human monocytes to produce TNF-α [33]. Monocytes from human peripheral blood were cultured on a P-selectin-coated culture plate in the presence or
absence of NMSO3 and the release of TNF-α from monocytes was determined by ELISA. NMSO3 inhibited the production of TNF-α induced by P-selectin in a dose-dependent manner, but had almost no effect on that induced by LPS (Fig. 6). We also reported that the adhesion of activated platelets to leukocytes through P-selectin stimulated the production of superoxide anion [34,41]. We then examined the effects of NMSO3 on activated platelet-induced ROS production in neutrophils. Utilizing a fluorogenic probe, DCFH-DA, for detecting oxidative bursts [36], we measured the intracellular ROS production in neutrophils by flow cytometry. In the presence of thrombin-activated platelets, the fluorescence intensity was increased as compared to that in the presence of untreated platelets, and it was decreased to the control level by the addition of NMSO3 (100 μM) (Fig. 7). These results indicate that the production of ROS in neutrophils was potentiated by activated platelets and inhibited by NMSO3. However, NMSO3 had almost no effect on PMA-induced ROS production.

The data presented in this study demonstrated that a sulfated derivative of sialic acid, NMSO3, acts as a specific inhibitor for P-selectin-mediated cell adhesion and for adhesion-induced leukocyte activation including TNF-α production in monocytes and ROS production in neutrophils. In recent years, a number of P-selectin antagonists have been developed as candidates for anti-inflammatory agents (reviewed in [42]). Most examples are derivatives of carbohydrate ligands such as sLeX and its analogs. However, the sLeX tetrasaccharide structure is not necessarily required for the inhibitory activity against P-selectin-dependent adhesion. Several polysaccharides and low-molecular-weight compounds have been reported to be potent inhibitors; e.g., heparin and its degraded products [23,24], fucoidin [25], dextran sulfate [25,26], inositol phosphate [43], glycyrrhizin [22], trestatin A sulfate [27], and chondroitin/dermatan sulfate [44]. Most of these are sulfated carbohydrates, suggesting that a sulfate group is crucial for the interaction of P-selectin and its glycoprotein ligands. It has been reported that P-selectin recognizes an N-terminal region of PSGL-1 that requires sulfation of at least one of the three clustered tyrosine residues and an adjacent O-glycan expressing the sLeX carbohydrate structure [13]. L-selectin is also known to recognize sialylated and sulfated carbohydrate ligands such as sialyl 6-sulfato Lewis X [45]. A recent report demonstrated that L-selectin had a preference for carbohydrate 6-sulfation rather than tyrosine sulfation of PSGL-1 [46]. More recently, however, L-selectin was reported to interact with sulfated tyrosine residues and O-linked sLeX structures in endoglycan, a member of the CD34 family [47]. Thus, the interaction of NMSO3 with L-selectin will be an important issue in the future studies.

NMSO3 was originally developed as an antiviral agent and was demonstrated to inhibit infection by several viruses including respiratory syncytial virus, adenovirus, and human rotavirus [28–30]. Based on binding assay and kinetic study as well as on temperature shift experiments, NMSO3 was proposed to inhibit the binding to and adsorption of viruses in cells. This inhibitor exhibited minimal cytotoxicity against various normal and tumor cell lines [29], and may be a promising candidate for a P-selectin antagonist and a useful tool for gaining insight into the molecular basis of P-selectin recognition.

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