Expression of sialyltransferase activity on intact human neutrophils

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Abstract: Endogenous polymorphonuclear leukocyte (PMN)-associated sialidase activity enhances PMN adhesion to and migration across the endothelium through the removal of sialylated cell-surface residues. We tested the hypothesis that PMNs also express sialyltransferase (ST) activity that restores sialyl residues to the PMN surface. We developed a highly sensitive fluorometric assay to demonstrate that intact human PMNs can mediate and accept sialyl residue transfer. This ST activity is inhibited by a ST inhibitor, CMP, which also inhibits the transendothelial migration of PMNs in response to IL-8 in vitro and in vivo. We conclude that intact PMNs express sialidase and ST activities that permit rapid modulation of their surface sialylation and their ability to adhere to and migrate across the endothelium. J. Leukoc. Biol. 84: 000–000; 2008.

Key Words: sialic acid cell trafficking · inflammation

INTRODUCTION

Circulating neutrophils or polymorphonuclear leukocytes (PMNs) traffic to sites of inflammation, where they exit in the intravascular space through a multistep process called diapedesis [1]. This process initially requires a carbohydrate-dependent step by which nonactivated PMNs loosely adhere to adhesion molecules expressed on activated endothelial cells (ECs). Following this loose tethering, PMNs are activated by EC-derived mediators, resulting in the tight adherence of PMNs to the endothelium, primarily via the binding of PMN-expressed integrins to ICAMs on the EC surface. This step precedes the migration of PMNs to and through the interendothelial cell junction. During diapedesis, interactions between multiple glycosylated surface molecules and their counterligands regulate PMN adhesion to and migration across the endothelium; these interactions are required for full expression of the acute inflammatory phenotype [1].

Sialic acids or N-acetylneuraminic acids are a family of amino sugars that are coupled to the outermost ends of glycoconjugates on the surface of eukaryotic cells and impart a negative charge to the molecule and the cell. Sialic acid residues on the cell surface reduce cell-to-cell adhesion, prevent the deposition of complement on the cell surface, permit evasion of immune recognition, and regulate the binding of ligands to their cell surface receptors [2, 3].

Enzymatic modification of the sialylation status of these cell-surface glycoconjugates may provide an additional layer of regulation of PMN interactions with other PMNs, other cells, or informational molecules (e.g., cytokines, hormones). Microbial and cellular sialidases, by removing sialyl residues from these surface glycoconjugates, can rapidly modify their affinity for binding partners [3, 4]. Upon activation, human PMNs mobilize endogenous sialidase activity from intracellular compartments to the plasma membrane, where it cleaves sialyl residues from cell-surface glycoconjugates [4]. Such desialylation results in marked PMN functional changes, including increased adhesiveness to and migration across the endothelium.

Our laboratory has focused on host sialic acid modulation and its regulation of PMN and monocyte function [4–7]. We found that stimulation of PMNs in vitro and in vivo is associated with a loss of cell-surface sialic acid, enhanced homotypic and heterotypic intercellular adhesion, and priming of the response to agonistic stimuli [4, 6, 7]. We hypothesize that during a tightly regulated, inflammatory response, resialylation of PMN surface molecules through sialyltransferase (ST) activity is required to return the cell to its resting state (i.e., resialylate). To date, PMN ST activity and its potential roles in the inflammatory process have not been studied. We now demonstrate that intact human PMNs contain endogenous ST activity. Further, prior ST inhibition with CMP impairs transendothelial migration (TEM) of PMNs in response to IL-8 in vitro and in vivo. Such ST activity may insure the availability of cell-surface sialyl residues on resting PMNs for their initial carbohydrate-dependent tethering to EC adhesion molecules. Alternatively, resialylation of the PMN surface may promote the “de-adherence” or release of PMNs from the abluminal endothelial surface during TEM, so they might continue their movement into inflamed tissues. Thus, a highly orchestrated system of sialidases and STs may permit PMNs to respond dynamically to a rapidly changing environment.

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**MATERIALS AND METHODS**

**Cell preparation**

Human RBCs were obtained from the peripheral blood of healthy volunteers by venipuncture under a protocol approved by the Institutional Review Board (IRB). Sterile and defibrinated RBCs were purchased from Watz Farm (Smithsburg, MD, USA) and stored at 4°C. PMNs were processed from the peripheral blood of healthy human donors as described previously [4] under the same IRB-approved protocol.

**Reagents**

Type 5 sialidase (*Clostridium perfringens*), 2,3 deoxy-N-acetylneuraminic acid (2-DN), CMP, and CMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CMP was also obtained from Tokyo Kasei (Japan). CMP-5-FITC-neuraminic acid (CMP-5-FITC-NEU) was purchased from Calbiochem (San Diego, CA, USA) and later prepared by one of the coauthors (R. Brossmer). α2,6-ST was purchased from Genzyme (Cambridge, MA, USA), and protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN, USA). Recombinant human (rhu)IL-8 was obtained from R&D Systems (Minneapolis, MN, USA), and Dulbecco’s PBS (DPBS) was purchased from Invitrogen (Carlsbad, CA, USA).

**ST assay**

For the study of PMN ST activity, we adapted a ST assay described previously in which CMP-5-FITC-NEU served as the labeled sialyl residue donor and sialidase-treated RBCs as the sialyl residue acceptor [8]. Based on preliminary studies, in which we optimized the concentrations of each component of the assay and the duration of treatment, a standard reaction mixture contained a sialyl residue acceptor (RBCs or PMNs), CMP-5-FITC-NEU, and a ST source (Fig. 1). CMP-5-FITC-NEU was added over a dosage range of 12.5–50 μM. As the signal decreased at the 12.5-μM concentration, we routinely used the CMP-5-FITC-NEU at 25 μM. Purified α2,6-ST at a final concentration of 0.5 nU/ml or PMNs as an endogenous source of ST activity were used in a final volume of 100 μl cell incubation buffer (CIB) containing PBS plus 5 mg/ml BSA, pH 6.8. Maximal ST activity was observed with α2,6-ST at a final concentration of 8 × 10^{-10} U/ml, with decreasing activity observed down to 0.5 × 10^{-10} U/ml (40% maximal). We therefore chose 0.5 nU/ml, which was 95% maximal activity. Sialyl residue acceptor SRBCs and PMNs were desialylated with 25 μM/sialidase/1–2 × 10^{10} cells/1 ml CIB for 1 h at 37°C, washed once in CIB, and used at 4 × 10^{6} cells or at 8 × 10^{6} cells, respectively, in a volume of 40 μl. Evaluation of sialidase doses from 0.025 to 100 nU/ml demonstrated that maximal desialylation was achieved at 25 nU/ml. Although the amount of desialylation increased linearly from the earliest time-point, 30 (57% maximal ST activity)–120 min, for convenience, we chose a treatment time of 60 min (75% maximal activity). When used as an endogenous source of ST activity, human PMNs were adjusted to a stock concentration of 1 × 10^{5} PMN/ml, lysed by freeze/thawing, and centrifuged, and the protein concentration of the supernatant was determined (Bio-Rad Laboratories, Hercules, CA, USA). Intact human PMNs at 8 × 10^{5}/0.1 ml assay volume were also used as a source of ST activity as well as an acceptor of sialyl residues.

After gentle mixing, all tubes were incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO₂. There was a progressive increase in resialylation of erythrocytes from 30 to 120 min. To optimize the ST signal, we therefore selected the 2-h assay time. The cells were washed, pelleted, and resuspended in CIB and analyzed fluorocytometrically on a FACScan II (Becton Dickinson, Mountain View, CA, USA). Based on forward- and right-angle, light-scatter properties, an electronic gate was placed around the RBCs or PMNs, which were excited at 488 nM, and emission was measured at 530 nM. At least 10,000 events were recorded per condition. Data were analyzed with a CellQuest analysis program.

For each experiment, controls included omission of each of the components of the assay mixture (i.e., ST source, CMP-5-FITC-NEU, or sialyl residue acceptor) as well as boiled, exogenous α2,6-ST. When indicated, 2-DN (0.04 mM, final concentration), an inhibitor of sialidase activity [9], was introduced to prevent the possible removal of the transferred sialyl residues. ST activity was measured after subtraction of background fluorescence (i.e., complete assay mixture minus the exogenous α2,6-ST or for endogenous ST, in the presence and absence of the ST inhibitor CMP (0.3 mM final concentration)) and was expressed as percent of maximal activity. We observed similar inhibitory effects of CMP on ST activity over a dosage range of 0.125–0.450 mM. For experiments in which we estimated the endogenous ST activity, we constructed a standard curve using serial twofold dilutions of purified α2,6-ST enzyme, from which total ST activity was interpolated and expressed as nU/μg protein.

**Confocal microscopy**

In addition to measuring the ST activity by flow cytometry, we visualized ST activity by confocal microscopy. PMNs were treated with 100 nM/ml bacterial sialidase for 30 min at 37°C, and then the CMP-5-FITC-NEU was added in the absence or presence of CMP (0.3 mM) or a similar concentration of CMP for 37°C for 2 h. PMNs were fixed with 4% paraformaldehyde, and cell nuclei were stained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI). Cells were then cover-slipped with a DABCO-based antifade mounting media. Digital microscopy images were captured by means of a FluoView 500 confocal microscope (Olympus Imaging, San Jose, CA, USA), fitted with standard filters for the visualization of DAPI and CMP-5-FITC-NEU. All images of substrates and controls were captured using identical acquisition parameters (FluoView 500 software) and assembled into panels (CorelDraw 12).

**TEM of PMNs in vitro**

The ability of human PMNs to migrate across human pulmonary artery EC (HPAEC) monolayers in response to a chemotactic stimulus (rhIL-8) was measured as described previously [7]. Only EC monolayers retaining >97% of a 14C-BSA tracer as a measure of barrier integrity were studied. After treatment of HPAECs for 4 h with 25 U/ml TNF-α, calcine-alvesolar macrophage (calcine-AM; Molecular Probes, Eugene, OR, USA)-labeled human PMNs (5×10^{5} cells/well) were introduced into the upper compartment of assay chambers, incubated for 2 h at 37°C, after which time, each lower compartment was sampled and fluorocytometrically assayed. A standard curve was established for each experiment from which PMN numbers could be interpolated from fluorescence units, and PMN TEM was expressed as percent migration.

**Recruitment of murine PMNs in vivo**

C57BL-1 (ICR) BR mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for i.p. recruitment of PMNs. Mice were injected i.p. with 0.2 ml sterile, nonpyrogenic, normal saline (0.9% sodium chloride, Baxter Healthcare Corp., Deerfield, IL, USA) or rhuIL-8 (750 μg/mouse). Mice were i.v.-infused with CMP or GMP (30 μg/ml body weight in 0.1 ml) at 5 min before and 1 h after normal saline or rhuIL-8. Cells were harvested by peritoneal lavage with cold...
DPBS at 4 h, and total cells and PMNs were counted directly in a hemocytometer. In control animals, equivalent volumes of saline were administered at the same time points in lieu of CMP or GMP.

Statistical analysis

Experimental data were analyzed using the GraphPad Prism statistical analysis package (GraphPad Software, Inc., San Diego, CA, USA). Statistical differences were analyzed by one-way ANOVA with comparison among groups analyzed by Tukey’s multiple comparison test or by a two-tailed Mann-Whitney test. A P value < 0.05 was considered statistically significant.

RESULTS

Exogenous α2,6-ST transfers sialyl residues onto SRBCs

In preliminary experiments, we established that sialidase treatment prepared the surface of human RBCs and SRBCs for the comparable acceptance of ST-mediated transfer of CMP-5-FITC-NEU (data not shown). Aliquots of SRBCs kept at 4°C for up to 3 weeks had no decrement in acceptor ability. The complete mixture of SRBCs, CMP-5-FITC-NEU, and exogenous α2,6-ST resulted in a strong signal on flow cytometry (Fig. 2, A–C, dark peaks). Omission of CMP-5-FITC-NEU (i.e., autofluorescence; A, light peak) or exogenous ST (B, light peak) from the reaction mixture or the use of heat-inactivated ST (C, light peak) markedly reduced the signal. CMP-5-FITC-NEU did not bind nonspecifically to cells after washing (data not shown).

Sialidase pretreatment is required for SRBCs but not for PMNs to accept sialyl residues

As RBCs were capable of accepting sialyl residues, we asked whether intact human PMNs might also function as sialyl residue acceptors in the presence of exogenous (α2,6-ST) ST. It was reported previously that in the absence of sialidase treatment, SRBCs were unable to serve as an acceptor for the labeled CMP-5-FITC-NEU. We therefore examined the necessity for sialidase pretreatment of SRBCs and PMNs to serve a similar preparative function in our assay [3]. When exogenous α2,6-ST was added to the reaction mixture, sialidase-treated PMNs and SRBCs were able to serve as a sialyl residue receptor, although SRBCs had <50% of the ST activity compared with similarly treated PMNs (Fig. 3). There was an increase in the ability of PMNs to accept sialyl residues with increasing doses of sialidase pretreatment until a plateau was attained at 30 mU sialidase/ml (data not shown). In the absence of sialidase pretreatment, SRBCs displayed no fluorescent signal, indicating that the “unmasking” of acceptor sites on SRBCs by sialidase is necessary for ST activity. Exogenous α2,6-ST also mediated an easily detectable but diminished CMP-5-FITC-NEU transfer to PMNs without prior sialidase treatment, which suggests that unlike SRBCs, PMNs were able to unmask sialyl acceptor sites.

Intact PMNs are a ST source and sialyl residue acceptors

In assaying endogenous sources for ST activity, we routinely used a specific ST inhibitor [10, 11], CMP, to verify that the assay system was in fact detecting genuine ST activity. As preliminary tests demonstrated that 0.3 mM CMP fully inhibited ST activity in vitro and was not toxic to the PMNs, each assay for endogenous ST activity was performed in the presence and absence of 0.3 mM CMP. The difference in corrected values generated in the presence and absence of CMP was taken as the true ST activity (i.e., CMP-inhibitable activity).

As in preliminary experiments, PMN lysates contained ST activity, we asked whether intact PMNs or SRBCs also could serve as an endogenous source for ST. To optimally expose more sialyl acceptor sites, aliquots of PMNs or SRBCs were pretreated with sialidase and added to the reaction mixture as sialyl residue acceptors, and a second aliquot of untreated PMNs or SRBCs was added in place of the purified α2,6-ST enzyme as a source of ST activity (Fig. 3). In the absence of an exogenous source of ST, α2,6-ST, we observed no ST activity with SRBCs, even with sialidase pretreatment (Fig. 3). Thus, SRBCs had no endogenous ST activity. In contrast, even in the absence of an exogenous source of ST, PMNs exhibited ST activity (Fig. 3). CMP inhibited the ability of sialidase-treated PMNs to serve as sialyl residue acceptors when α2,6-ST was added to the reaction (Fig. 4A). Although PMNs not treated with sialidase had a decreased ability to accept the α2,6-ST-mediated transfer of sialyl residues, it, too, was inhibited by CMP (Fig. 4B). The ability of CMP to inhibit exogenously added ST activity led us to ask whether there might be endogenous ST activity in PMNs. When intact PMNs were added to a second aliquot of PMNs pretreated with sialidase to optimize the likelihood of observing ST activity, CMP-inhibitable ST activity was observed (Fig. 4C).

As PMNs served as acceptor cells in the ST assay in the absence of sialidase pretreatment (Figs. 3 and 4B), we asked whether intact PMNs that were not pretreated with sialidase could be used as the sialyl residue acceptor and endogenous
source of ST activity. After incubation with CMP-5-FITC-NEU, CMP-inhibitable ST activity was evident in intact, untreated PMNs (Fig. 4D). These data suggest that during an inflammatory response, intact PMNs can mediate the autocrine/paracrine transfer of sialyl residues from PMN to PMN.

Direct visualization of ST activity on intact PMNs

The use of a fluorescent instead of a radiolabeled substrate for the ST activity also permitted direct visualization of the ST activity on intact PMNs (Fig. 5). In the absence of the CMP-5-FITC-NEU, there was little background fluorescence, even with sialidase pretreatment of the PMNs (Fig. 5A); however, with the addition of the fluorescent substrate, there was cell-associated fluorescence evident (Fig. 5B). As CMP-5-FITC-NEU does not penetrate the cell [12], the signal most likely represents cell-surface-associated fluorescein. The fluorescence was not distributed uniformly throughout each cell, and although most cells had fluorescence, there were cell-to-cell differences observed. Addition of the ST inhibitor, CMP, to the reaction (Fig. 5D), but not a control molecule of similar molecular weight and charge, GMP (Fig. 5C) reversed the reaction.

Endogenous PMN sialidase activity unmasks sialyl residue acceptors

As RBCs not treated with sialidase cannot serve as sialyl residue acceptors, we reasoned that for PMNs to serve as sialyl residue acceptors in the absence of prior sialidase treatment, some previously unrecognized, constitutive level of endogenous sialidase activity may expose sites on the PMN surface to accept sialyl residues. To test this hypothesis, we first showed that unstimulated, intact PMNs not exposed to sialidase had CMP-inhibitable activity (Fig. 6A). The addition of CMP to the ST assay mixture reduced the MFI from 364 (solid peak) to 222 (open peak). We reported previously that 2-DN inhibits sialidase activity, and a molecule of similar weight and charge, ketodeoxyoctonate, does not [4–7]. Addition of 2-DN to those same PMNs not exposed to sialidase resulted in a small but consistently reproducible decrement in ST activity (decrease in MFI from 364 to 317; Fig. 4B, solid vs. open peak). Therefore, in nonstimulated PMNs, a basal level of sialidase activity appears necessary for the constitutive turnover of surface sialyl residues, perhaps as part of a cell-surface remodeling process. This activity was not detectable with the less-sensitive biochemical assays of sialidase activity used in our earlier reports [4–7] and was insufficient to cause PMN functional changes normally associated with desialylation [4, 7].

ST inhibition reduces TEM of PMNs in vitro

We hypothesized that once migrating PMNs reach the abluminal surface of endothelial cells, a restoration of sialyl residues to the PMN surface would be required to “deadhere” and to continue their migration. There was a robust (greater than threefold increase) migration of calcine-AM-labeled human PMNs across TNF-α-treated HPAEC monolayers in response to IL-8 (Fig. 7). Addition of the ST inhibitor, CMP, inhibited TEM >40% (P < 0.03). This suggests that ST activity plays a critical role in TEM in a static, in vitro assay.
ST inhibition reduces PMN migration in vivo

We asked whether the ability of ST inhibition to diminish TEM of PMNs in vitro could be extended to an in vivo experimental system. At 4 h after the i.p. administration of IL-8 or saline, we measured the appearance of total leukocytes and PMNs in peritoneal fluid (Fig. 8). In selected experiments, mice were i.v.-pretreated with CMP or a noninhibitory control molecule of similar molecular weight and charge, GMP. IL-8-treated mice displayed a two- to threefold increase in the i.p. recruitment of total leukocytes and PMNs compared with saline-treated controls. When mice were pretreated with the ST inhibitor, CMP, there was a marked reduction of total leukocytes and PMNs in the peritoneum. In contrast, when another purine nucleoside, GMP, was administered, no reduction in i.p. cellular migration was evident. Thus, ST activity is required for optimal PMN migration in response to an IL-8 stimulus in vivo.

DISCUSSION

Activated PMNs mobilize endogenous sialidase activity from an intracellular compartment to the plasma membrane [4]. Removal of as little as 20% of total cell-associated sialic acid residues profoundly increases PMN adherence to and migration across endothelial cell monolayers [7]. Inhibition of endogenous sialidase activity in vitro prevented these PMN-endothelial cell interactions and in vivo, reduced PMN recruitment to inflammatory sites [6, 7]. Moreover, we observed that fMLP-activated PMNs directly desialylate the surface of HPAECs, which in turn increases PMN migration across the EC monolayer [7].
As PMNs must respond dynamically to a range of dissimilar environmental stimuli, these cells require the capacity to restore sialyl residues to surface glycoconjugates. We now have demonstrated that intact human PMNs also have ST activity that catalyzes the transfer of sialyl residues onto host cells, including PMNs. Thus, PMNs have the capacity to rapidly alter their (and/or other) cell surface(s) through the removal (sialidase) or restoration (ST) of sialyl residues. We propose this modulation of sialylation as a novel mechanism for the regulation of PMN adhesion and motility.

STs are a family of enzymes that catalyzes the transfer of N-acetylneuraminic acid from CMP-β-N-acetylneuraminic acid onto carbohydrate groups of glycolipids and glycoproteins (i.e., glycoconjugates), usually at the terminal position. More than 10 distinct eukaryotic STs have been identified [13, 14], which differ in substrate specificity for acceptor sugars (e.g., galactose, N-acetylglucosamine, or sialic acid) as well as the type of glycosidic linkages formed (e.g., α2,3, α2,6, α2,8).

Overexpression of ST activity has been associated with oncogenic transformation and increased metastatic potential [11, 15, 16]. ST also plays a pivotal role in the maturation and function of myeloid cells and B and T lymphocytes [15, 17–21]. For example, CD22, a member of the Ig superfamily of adhesion molecules, is a B cell-restricted transmembrane protein required for lymphocyte activation and immune function [22]. ST-mediated sialylation of CD22 abrogates CD22-mediated B and T lymphocyte adhesion essential to lymphocyte function [22, 23]. In the case of CD8+ T lymphocytes, ST activity dictates whether a cell undergoes apoptosis or progresses to become a viable memory T cell [14]. ST activity regulates components of the innate immune system as well. For example, sialylation of the mannose receptor, a pattern recognition receptor, governs its ability to bind to its ligand [3].

STs are preferentially localized to the Golgi apparatus within the cell [13]; however, extensive work by Shur and colleagues [24, 25] has established that glycosyltransferases may be expressed on the cell surface, where they function as cell adhesion molecules, signal transducing receptors for extracellular oligosaccharide ligands, and regulators of cell growth. Ecto-STs have been described in platelets, lymphoblastoid cells, B lymphocytes, and neuronal cells and more recently, on the surface of early-activated CD8 T cells [13, 26, 27]. As there appears to be a connection between the Golgi and the cell surface [28], Golgi-associated STs might be exported to the plasma membrane.

Although it has been inferred that PMNs have ST activity within the Golgi complex that enables PMNs to restore sialyl residues during the recycling of surface receptors [29], to our knowledge, there have been no reports of ST activity on the surface of PMNs. Although we did not localize the ST activity of PMNs to the cell surface, we did find that intact, viable PMNs display ST activity. One could speculate that as PMNs migrate through the endothelial, paracellular pathway without disrupting barrier integrity, sialidase and ST activities might be expressed simultaneously but spatially segregated within distinct, subcellular compartments in polarized cells. Sialidase activity, concentrated on the leading front of a migrating PMN, may remove sialyl residues from glycoconjugates, such as CD31, at the interendothelial cell junction, thereby facilitating their adherence to as well as their ability to squeeze through this junction. In contrast, STs at the PMN “tail” may restore these residues to the cell surface, allowing for physical disengagement from substrates enabling PMNs to continue their migration through tissues. This might explain how PMNs can negotiate the paracellular pathway without compromising the barrier for the movement of macromolecules or fluid. It is also conceivable that PMN ST activity might resialylate endothelial cell surfaces, thereby “resealing” the paracellular pathway. Alternatively, the ability of CMP to decrease recruitment to an inflamed peritoneum in vivo might suggest that ST activity is required to insure the initial carbohydrate-dependent tethering to the activated EC through sialylation of the surface of non-glycosylated surface receptors.
activated PMNs. Our data showing constitutive ST activity on PMNs is consistent with this hypothesis.

The sialidase/ST enzyme system, through its dynamic control of PMN surface sialylation, regulates the cell–cell and likely, the cell–matrix interactions, which are prerequisites to PMN motility. The constitutive ST activity of intact, untreated PMNs demonstrated in these studies may insure that PMNs do not become promiscuously adherent in response to every transient, minimal stimulus and/or that nonstimulated PMNs retain sialyl residues on their surface that facilitates their initial tethering. Finally, the coordinated activities of sialidase and ST activities in the PMN may allow a rapidly reversible, highly localized response to an inflammatory stimulus and may serve as a therapeutic target.

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