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An iminosugar-based heparanase inhibitor heparastatin (SF4) suppresses infiltration of neutrophils and monocytes into inflamed dorsal air pouches☆

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ABSTRACT

Local infiltration of inflammatory cells is regulated by a number of biological steps during which the cells likely penetrate through subendothelial basement membranes that contain heparan sulfate proteoglycans. In the present study, we examined whether administration of heparastatin (SF4), an iminosugar-based inhibitor of heparanase, could suppress local inflammation and degradation of heparan sulfate proteoglycans in basement membranes. In a carrageenan- or formyl peptide-induced dorsal air pouch inflammation model, the number of infiltrated neutrophils and monocytes was significantly lower in mice after topical administration of heparastatin (SF4). The concentration of chemokines MIP-2 and KC in pouch exudates of drug-treated mice was similar to control. In a zymosan-induced peritonitis model, the number of infiltrated cells was not altered in drug-treated mice. To further test how heparastatin (SF4) influences transmigration of inflammatory neutrophils, its suppressive effect on migration and matrix degradation was examined *in vitro*. In the presence of heparastatin (SF4), the number of neutrophils that infiltrated across a Matrigel-coated polycarbonate membrane was significantly lower, while the number of neutrophils passing through an uncoated membrane was not altered. Lysate of bone marrow-derived neutrophils released sulfate-radiolabeled macromolecules from basement membrane-like extracellular matrix, which was suppressed by heparastatin (SF4). Heparan sulfate degradation activity was almost completely abolished after incubation of lysate with protein G-conjugated anti-heparanase monoclonal antibody, strongly suggesting that the activity was due to heparanase-mediated degradation. Taken together, in a dorsal air pouch inflammation model heparastatin (SF4) potentially suppresses extravasation of inflammatory cells by impairing the degradation of basement membrane heparan sulfate.

1. Introduction

Degradation of extracellular matrix (ECM) is involved in the pathogenesis of many diseases, such as cancer metastasis, inflammation, wound healing, and many fibrotic disorders. Basement membranes (BM) that lay beneath the endothelial and epithelial cell sheets form a basal structure for the cells to survive. It is likely that degradation of this ECM accelerates penetration or invasion of malignant cells and inflammatory leukocytes under certain pathogenic conditions. Therefore, it is relevant to understand what changes arise on the BM during pathogenesis that accompanies cell infiltration and to obtain pharmaceutical tools to suppress BM destruction. For the latter purpose, a number of inhibitors against matrix degrading enzymes have been developed. TIMP-1, a physiological inhibitor of MMPs, as well as chemical inhibitors of

Abbreviations: BM, basement membrane; BSA, bovine serum albumin; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; fMLP, formyl-methionyl-leucyl-phenylalanine; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; Hpse, heparanase; HRP, horseradish peroxidase; mAb, monoclonal antibody; SVBCE, SV40-transformed bovine corneal endothelial cells.

☆ The authors have no conflict of interest to declare.

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MMPs and elastase have been tested for their inhibitory effects on cell infiltration resulting from suppressed matrix degradation [1–4]. Considering that the subendothelial BM consists of three main kinds of large molecules, *i.e.*, type IV collagen, laminin, and heparan sulfate proteoglycans, it is desirable to have a series of inhibitory reagents against matrix degrading enzymes each targeting a specific component of BM in order to achieve proper BM protection.

Degradation of heparan sulfate (HS) is induced by heparanase (Hpse), a sole mammalian HS-specific endo-β-glucuronidase. The carbohydrate moiety HS interacts with other matrix components, thereby playing a role in conserving the mechanical integrity of the BM. Hpse has been originally characterized as a matrix degradation enzyme involved in BM degradation and cancer cell invasion. Increased expression level of the enzyme paralleled malignancy and invasiveness of melanoma cells [5]. Further, it is quite likely that Hpse is involved in the invasion process of an experimental metastasis model, because suppression of Hpse gene expression [6] or administration of an Hpse inhibitor [7–9] significantly lowered the efficiency of melanoma cell metastasis into the lung. In this sense, application of Hpse inhibitors such as PI-88 has been intensively studied for pharmacological suppression of cancer metastasis [10,11].

Compared to cancer invasion, roles of Hpse in immune cells still need clarification. It has been known that leukocytes, such as neutrophils, monocytes and dendritic cells, display degradation activity of HS in the BM [12–14]. A neutrophil study identified a chemokine-related molecule CTAP-III as having HS degradation enzyme activity [15], and thereby proposed the concept that Hpse is not a unique molecule responsible for HS degradation [16]. On the other hand, administration of anti-Hpse mAb suppressed the penetration of macrophages through BM [17]. Genetically engineered mice that are deficient in Hpse gene expression have further revealed how Hpse is involved in cell trafficking in several inflammation models. Results indicated that involvement of Hpse in transmigration likely depends on cell types or inflammatory sites [18–21]. Therefore, further study is needed to clarify the actual involvement of Hpse in transmigration.

Heparastatin (SF4), an iminosugar-based inhibitor mimicking a presumptive intermediate structure of Hpse-mediated glycosidase digestion, was shown to suppress lung metastasis of melanoma [8,9] and migration of microglia cells [22]. Therefore, it is easily applicable to *in vivo* migration tests as an Hpse inhibitor. Pharmacological approaches that target the BM penetration process potentially provide a novel way to treat some inflammatory diseases and might also help distinguish which type of inflammation Hpse is involved in. In the present study, we investigated the effect of heparastatin (SF4) in two inflammation models and found that its topical application suppressed infiltration of inflammatory cells in an air pouch model.

2. Materials and methods

2.1. Animal studies

Specific pathogen-free C57BL/6 mice were purchased from CLEA Japan (Kanagawa, Japan) and used after 1 week of primary breeding. Mice were fed and housed under specific-pathogen-free conditions according to the guidelines of the Ministry of Education, Science, Sports and Culture of Japan. The protocols of the animal experiments were approved by the Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo.

2.2. Reagents

Heparin from porcine intestinal mucosa (approx. 15 kDa), lipopolysaccharide, formyl-methionyl-leucyl-phenylalanine (fMLP), bovine serum albumin (BSA) for cell culture, carrageenan (lambda) and zymosan were purchased from Sigma (St. Louis, MO); HRP-conjugated goat anti-rabbit IgG(H + L) from Invitrogen (Carlsbad, CA); anti-CXCL7 mAb (MAB10911) from R&D Systems Inc. (Minneapolis, MN); and normal goat serum from Japan Laboratory Animals, Inc. (Tokyo, Japan). A heterodimeric Hpse protein that mimics mouse active form Hpse and anti-Hpse mAb RIO-1 were prepared as stated elsewhere [14,23]. Heparastatin (SF4) was chemically synthesized as described elsewhere [8,9].

2.3. Air pouch inflammation model

Air pouch inflammation was examined as described elsewhere [24]. C57BL/6 mice (5 week-old, male) were anesthetized and injected with 6 ml of sterile air *s.c.* into the back on day 0, followed by a second injection of 3 ml of sterile air on day 3. On day 6, 1 ml of a suspension of 1 μM fMLP or 1% (w/v) carrageenan solution in saline was injected into the cavity. Heparastatin (SF4) (final concentration: 0.1 mM) was simultaneously administrated. Animals were sacrificed at 4 h post injection. Air pouch exudates were collected by washing the cavity with 3 ml of saline, centrifuged at 15,000 rpm for 20 s, and the total cell number was counted. Cells were attached on slides by Cytospin (Thermo Electron Co., Pittsburgh, PA, 500 rpm, 8 min) and stained with Diff-Quik staining kit (Sysmex, Kobe, Japan). The number of monocytes, neutrophils and mast cells were counted in 5 randomly chosen fields. Supernatants were separately collected and production of chemokines in the exudates was measured using ELISA Development Kits (900-K126, 900-K127, 900-K152; Peprotech, Rocky Hill, NJ) according to the manufacturer's instructions.

2.4. Zymosan-induced peritonitis

Peritoneal inflammation was induced by *i.p.* injection of zymosan A (Sigma-Aldrich, St. Louis, MA) as described previously [2]. Zymosan (2 mg/ml) was freshly prepared in sterile 0.9% (w/v) saline, and 0.5 ml was injected per mouse. Some mice were treated with heparastatin (SF4) as described in Section 2.3. After 4 h, mice were killed by decapitation and their peritoneal cavity was lavaged with 1 ml of saline to collect exudate containing peritoneal fluid and inflammatory leukocytes.

2.5. Preparation of bone-marrow-derived neutrophils

Bone marrow cells obtained from the femur and tibia of C57BL/6 J mice (6–15 week-old, male) were labeled with biotin-conjugated anti-Gr-1 mAb (Biolegend, San Diego, CA) at 4 °C for 15 min and washed with MACS buffer (PBS containing 2 mM EDTA and 3% BSA). Cells were labeled with streptavidin microbeads (Miltenyi Biotec Inc, San Diego, CA), separated with AutoMACS, and used as neutrophils in the present study. For checking the purity, the separated cells were labeled with FITC-conjugated anti-Gr-1 (Biolegend) for 30 min, washed twice with FACS buffer (PBS containing 0.1% BSA and 0.1% NaN_3) and analyzed on a flowcytometer (FACS Aria II, BD Bioscience, San Jose, CA).

2.6. Migration assay

Cell culture inserts (3 μm pore size; KURABO Industries Ltd., Tokyo, Japan) were coated with 10 μg of Matrigel (BD Bioscience) solubilized in 100 μl of PBS at room temperature overnight under aseptic conditions, followed by swelling with 200 μl of serum-free RPMI1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) at room temperature for 1 h, and assembled in a 24-well plate. The lower chambers were filled with 700 μl of RPMI 1640/0.5% FCS with 100 nM fMLP. The upper chamber of each transwell was filled with 200 μl of neutrophils at 1×10^5 cells/well. Heparastatin (SF4) was added both to the lower and upper chambers. The plate was incubated at 37 °C for 0.5 h (uncoated condition, migration test) or 24 h (Matrigel-coated condition, invasion test). Then, cells on the upper surface were scraped gently and removed. After staining with Diff-Quik staining kit, the number of cells