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Cell Surface Localization of Heparanase on Macrophages Regulates Degradation of Extracellular Matrix Heparan Sulfate

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Extravasation of peripheral blood monocytes through vascular basement membranes requires degradation of extracellular matrix components including heparan sulfate proteoglycans (HSPGs). Heparanase, the heparan sulfate-specific endo-β-glucuronidase, has previously been shown to be a key enzyme in melanoma invasion, yet its involvement in monocyte extravasation has not been elucidated. We examined a potential regulatory mechanism of heparanase in HSPG degradation and transmigration through basement membranes in leukocyte trafficking using human promonocytic leukemia U937 and THP-1 cells. PMA-treated cells were shown to degrade 3S-sulfated HSPG in endothelial extracellular matrix into fragments of an approximate molecular mass of 5 kDa. This was not found with untreated cells. The gene expression levels of heparanase or the enzyme activity of the amount of cell lysates were no different between untreated and treated cells. Immunocytochemical staining with anti-heparanase mAb revealed pericellular distribution of heparanase in PMA-treated cells but not in untreated cells. Cell surface heparanase capped into a restricted area on PMA-treated cells when they were allowed to adhere. Addition of a chemoattractant fMLP induced polarization of the PMA-treated cells and heparanase redistribution at the leading edge of migration. Therefore a major regulatory process of heparanase activity in the cells seems to be surface expression and capping of the enzyme. Addition of the anti-heparanase Ab significantly inhibited enzymatic activity and transmigration of the PMA-treated cells, suggesting that the cell surface redistribution of heparanase is involved in monocyte extravasation through basement membranes.


Macrophages and related cells play essential roles in the immune system, such as inflammation, defense against microbial infection, immunity to foreign substances, wound healing, and angiogenesis. Monocytes circulate throughout the body, extravasate through the endothelial lining of the blood vessel wall, and enter the underlying tissue in response to local inflammation. During the process, monocytes should pass through the vascular basement membrane that supports the structure and survival of endothelial cells and also prevents the vessels from mechanical destruction. The basement membrane mainly consists of type IV collagen, laminin, and heparan sulfate (HS) proteoglycans (HSPGs). Degradation of these basement membrane components results in disintegration of the structure and it is conceivable that such processes are a regulatory step for the extravasation. HSPGs are ubiquitous in extracellular matrices (ECMs) including basement membranes, and consist of diverse core polypeptides and HS. The HS moieties play essential roles in the interaction of HSPG with a wide range of molecules including ECM components (collagen, laminin, fibronectin, and others), cytokines (basic fibroblast growth factor, platelet-derived growth factor, hepatocyte growth factor, and others), and enzymes (lipoprotein lipase and others) (1–3). Degradation of HS causes loss of mechanical integrity of basement membrane and release of soluble mediators.

The first indication that HS maintains the mechanical integrity of basement membrane came from a work in which heparitinase digestion of glomerular basement membranes resulted in a loss of function (4). The ability of tumor cells to degrade basement membrane was shown to be due to a HS-specific endo-β-glucuronidase (5, 6). cDNA cloning and expression of human heparanase have recently been reported by four groups (7–10). The cDNA encodes a unique protein of 543 amino acids that contains a potential signal peptide sequence and six putative N-linked glycosylation sites. It was predicted that the 543-aa polypeptide formed a proenzyme that was processed to be a mature 50 kDa active enzyme after removal of 157 N-terminal amino acids. The active enzyme has been claimed to be a heterodimer that comprises the 50-kDa polypeptide and a short fragment of 8-kDa peptide derived from the N terminus of heparanase proenzyme (11). The active enzyme shows at least 100-fold HS degradation activity in comparison to the proenzyme (8), therefore this processing could be one of the critical regulatory steps of heparanase. Although it was assumed that secreted or membrane-associated heparanase is responsible for the degradation of ECM, the mechanisms involved in translocation of the enzyme have not been elucidated.

Heparanase activity was reported in platelets, neutrophils, monocytes, macrophages, Langerhans cells, astrocytes, activated (but not resting) rat T lymphocytes, and umbilical vein endothelial cells or smooth-muscle cells (12–19). It is likely that heparanase is required for extravasation of the cells in the immune system, and...
the activity should be under tight regulation to avoid tissue damage. In the present study, we asked whether the heparanase is involved in macrophage extravasation by use of macrophage-like cell lines. Regulation of heparanase activity during macrophage differentiation and attachment to basement membranes was also investigated. A major regulatory process seems to be its unique spatial distribution.

Materials and Methods

Chemicals

RPMI 1640 medium was purchased from Nissui Pharmaceuticals (Tokyo, Japan), FCS from BioWhittaker (Walkersville, MD), PMF from Wako Chemicals (Osaka, Japan), PMA (PS139), heparin (H3400), EDC (0.2 mg), and fluorescein sodium salt (FL, Fluca, Tokyo, Japan) (5 μg) were dissolved in water, and stirred for 1 h at room temperature, followed by dialysis overnight with water. The solution was then concentrated with a Centrican 30 concentrator (Amicon, Bedford, MA). The ratio of attached fluorescein to unmodified carboxyl group was determined using the carbazole-sulfuric acid method.

An enzymatic reaction was conducted in a 100 μl mixture containing 25 mM sodium acetate buffer (pH 5.5), 5 μg of FL-HS, 20 mM t-saccharic acid 1,4-lactone (Sigma-Aldrich), and cell lysates at 37°C for 24 h. The reaction was stopped by the addition of 100 μg of heparin and subsequent boiling for 5 min, followed by addition of solution (5 M NaCl, 250 mM Tris-HCl, 0.5% Triton X-100, pH 7.4). The mixture was then centrifuged at 1000 rpm for 5 min to precipitate the insoluble material. The products of FL-HS yielded by this reaction were analyzed by high-speed gel permeation chromatography. Briefly, a 50–100 μl aliquot of the supernatant was injected into Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated with PBS and eluted at 0.5 ml/min. Eluates were monitored by fluorescence intensity at 520 nm (excitation at 492 nm). The activity was determined by HPLC chromatograms by measuring an area of the peak of the intact FL-HS and degraded FL-HS.

Immunocytochemistry

U937 cells (5 × 10^5 cells/well) cultured on a chamber slide (154534; Nalge Nunc, Naperville, IL) or those cultured in suspension and fixed on a chamber slide by centrifugation (500 rpm, 5 min) were washed with Dulbecco’s modified PBS (PBS containing 0.91 mM CaCl2 and 0.49 mM MgCl2), and fixed with 4% paraformaldehyde supplemented with 0.1% saponin. After washing with Dulbecco’s modified PBS, the cells were blocked with 10% normal mouse IgG (Zymed Laboratories) in Dulbecco’s modified PBS for 30 min, treated with the first Abs (10 μg/ml) in PBS containing 3% BSA and 0.1% normal goat serum overnight at 4°C, then treated with 5 μg/ml FITC-conjugated anti-mouse IgG(Fc) Ab F(ab')2, for 15 min at 22°C. In permeabilized conditions, 0.1% saponin was added throughout the whole procedure. Stained cells were examined on a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, U.K.) equipped with a krypton/argon laser.

For cell polarization assays, differentiated U937 cells (5 × 10^5 cells/well) were cultured in a 1 well in a well of a 4-well chamber slides in which solid agarose gel was prepared on one side of the well. (154526; Nalge Nunc). A chemotactic peptide fMLP was added to the gel (10^-7M), and the chamber slides were incubated for 6 h. After the removal of the gel, cells were fixed with 4% paraformaldehyde supplemented with 8% glucose and stained for heparanase by the same method previously described.

Flow cytometry

U937 cells cultured in suspension were fixed in 4% paraformaldehyde supplemented with 8% sucrose. Normal goat IgG (Zymed Laboratories) at 10 μg/ml in Dulbecco’s modified PBS was applied to block nonspecific bindings for 30 min. The cells were incubated with Abs (10 μg/ml) in PBS containing 3% BSA and 2% normal goat serum for 30 min on ice, then treated with 5 μg/ml FITC-conjugated anti-mouse IgG(Fc) Ab F(ab')2, for 15 min on ice. Five thousand cells were analyzed using an Epics Elite flow cytometer (Coulter, Miami, FL).

Transmission assay

Transwells (8-μm pore size, d = 6.4 mm; Asahi Techno Glass, Tokyo, Japan) were added with 50 μg of Matrigel (BD Labware, Bedford, MA) solubilized in 200 μl of PBS, and air-dried at room temperature. The transwells were then assembled in a 24-well plate (MS-80240; Sumitomo Bakelite). The lower chambers were filled with 600 μl of RPMI 1640 medium supplemented with 10 μg/ml fibronectin and the upper chamber of each transwell was filled with 200 μl of U937 or differentiated U937 (2 × 10^5 cells/ml). The plates were incubated under humidified conditions at 37°C with 5% CO2 for 24 h. Nonmigrating cells on the upper surface were scraped gently and removed. The filters were fixed in ethanol and stained with Giemsa solution. The number of cells per magnification at ×400 high-power field that migrated to the lower surface of the filter was counted under a microscope. A mean of cell counts in five independent high-power fields per filter was obtained and indicated.

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Preparation of peripheral blood monocytes

Peripheral blood monocytes were isolated from venous blood drawn from normal healthy volunteers at the Tokyo Metropolitan Red Cross Blood Center (Tokyo, Japan) by using an anti-CD14 mAb-coated microbeads and magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) as described elsewhere (22, 23). Purity of monocytes was 91.3 ± 1.7% (n = 3, mean ± SD), judged as the percentage of CD14+ cells by flow cytometric measurements.

Results

PMA-treated U937 and THP-1 cells degrade ECM HS

Promonocytic leukemia cell lines U937 and THP-1 before or after PMA treatment were used in this study. Size distribution of released 35S-sulfated materials from endothelial ECM was determined after incubation with these cells. The released radioactivity from ECM after incubation with untreated U937 or THP-1 cells eluted at the void volume fraction (Fig. 1, A and B). The size distribution of 35S-sulfated materials released by the presence of PMA-treated U937 or THP-1 cells indicated that the degraded ECM components had a molecular mass of ~5 kDa (Fig. 1, C and D). Degradation of the 35S-sulfated component was not observed when incubation was performed in the presence of heparin, an inhibitor for heparanase (Fig. 1, ○).

Expression level of heparanase gene did not alter after PMA treatment

The degradation of HS by these PMA-treated cells should be dependent on heparanase because only one heparanase gene was identified in humans (24). Using an internal sequence of the heparanase gene as a competitor, semiquantitative RT-PCR of heparanase mRNA expression was performed. The products were found to migrate to a similar position as human heparanase mRNA from human breast carcinoma cell lines (8) (Fig. 2A). The amount of PCR-amplified products of U937 and THP-1 cells before and after the PMA treatment did not differ (Fig. 2B), although degradation of ECM HS by these cells was observed only after treatment with PMA.

Heparanase activity levels in cell lysates were not altered by PMA treatment

A possibility that processing of heparanase proenzyme (7) modified heparanase activity after PMA treatment was assessed. Cell lysates from untreated and PMA-treated cells were compared for heparanase activity. FL-HS was used as a substrate (25).

Percentages of degraded HS were estimated and used as indicators of heparanase activity in the lysates. The degradation seemed to be due to heparanase because pretreatment of the U937 cell lysate with anti-heparanase mAb (10 μg/ml) inhibited as much as 80% of the degradation (data not shown). PMA treatment did not influence the relative quantity of the enzymatic activity in U937 and THP-1 cell lysates (Fig. 3). Therefore, we concluded that untreated and PMA-treated cells contained similar amounts of active heparanase.

PMA-treated cells express heparanase molecule on the cell surface

Because the difference in HS degradation between untreated and PMA-treated U937 cells was observed only when intact cells were used, cell surface distribution or secretion of heparanase was thought to be responsible for the difference. We therefore performed immunocytochemical detection of this enzyme using anti-heparanase mAb.

When the cells were permeabilized, heparanase associated with unidentified granule-like structures in the cytoplasm irrespective of PMA treatment (Fig. 4, A and D). When the cells were not permeabilized, heparanase was distributed discretely on the surfaces of PMA-treated cells (Fig. 4E). Flow cytometric analysis confirmed the surface expression of heparanase (Fig. 4F). Cell surface localization of heparanase was not observed in untreated cells (Fig. 4, B and C). The clear difference suggested that the difference in cell-mediated HSPG degradation by untreated and PMA-treated cells may be explained by the distribution of this enzyme.
Transport of heparanase to the cell surfaces was inhibited by pretreatment with nocodazole before the PMA treatment under conditions in which microtubule structures were completely disrupted (Fig. 5C). Expression of heparanase and HSPG degradation was completely blocked by the treatment (Fig. 5, D and E).

**Cell adhesion-dependent capping of heparanase**

When PMA-treated U937 cells were detached from the surface, placed on the surface of a glass plate by centrifugation, and cell surface localization of heparanase was examined, a discrete pericellular staining around the rim of the cells was observed as described earlier. However, when the cells were placed on a glass plate and incubated, cell surface heparanase (Fig. 6D) relocated and became concentrated on one of the pericellular rims of the cells. This so-called capping was observed as early as 5 min after incubation at 37°C. Approximately 50% of the cells showed this profile within 1 h (Fig. 6E). When the cells were placed on plates coated with fibronectin, laminin, Matrigel, or BSA, we observed similar capping distribution. When agarose gel containing a chemotactic peptide IMLP was placed on the glass plate, the localization of heparanase on the cellular rim was directed toward the concentration gradient of IMLP (Fig. 6F). This apparently indicated that heparanase accumulated on the leading edge of the migrating cells. Such unidirectional capping was not observed when a chemotactic stimulus was not provided (Fig. 6E).

**Heparanase is involved in transmigration through ECM**

Two chamber culture systems separated by membranes coated with Matrigel were used to examine transmigration of U937 cells. Human fibronectin was used as a chemoattractant in these experiments. The number of PMA-treated U937 cells that migrated through the membrane was approximately double that of untreated cells. The number of cells in the lower chamber was significantly smaller when heparin or anti-heparanase mAb was added to the upper chamber (p < 0.05) (Fig. 7). These results suggest that cell surface heparanase is involved in the transmigration of PMA-treated U937 cells through basement membranes.

**Capping of heparanase on the cell surface of peripheral blood monocytes**

A remaining question to be clarified is whether the cell surface expression and capping of heparanase occurs in nontransformed macrophages and related cells. CD14+ peripheral blood monocytes were stained with anti-heparanase mAb under nonpermeabilized condition. A large portion of monocytes (68.7 ± 7.1%, n = 4, mean ± SD) was stained with anti-heparanase mAb. Of the heparanase-positive monocytes, 34.3 ± 10.3% (n = 4, mean ± SD) of the cells showed capping formation (Fig. 6, G and H).

**FIGURE 4. Intracellular and cell surface distribution of heparanase.** Intracellular (A and D) and cell surface (B and E) heparanases were immunostained and observed using confocal laser scanning microscopy. Simultaneously, the fixed U937 cells in suspension were also stained with anti-heparanase mAb (bold lines) or mouse IgG1 (broken lines) and applied to flow cytometer (C and F). U937 (A-C) and U937 treated with PMA (D-F) for 24 h are shown. Horizontal bar indicates 10 μm. The experiments are repeated three times and similar results were obtained.
results were obtained. Horizontal bar indicates 10 μm. G and H, Monocytes were allowed to adhere on a chamber slide for 1 h. Cell surface expression of heparanase in peripheral blood monocytes was visualized by immunostaining followed by alkaline phosphatase colorization (red). Arrowheads indicate capping sites of the heparanase molecule. The experiments are repeated three times and similar results were obtained. Horizontal bar indicates 10 μm.

**Discussion**

Heparanase is an endo-β-glucuronidase specific for HS. Heparanase in metastatic melanoma cells is thought to be constitutively active outside cell surfaces and capable of destroying surrounding tissue, whereas the enzyme should be strictly regulated in cells of the immune system. We used U937 and THP-1 cells as models to study the spatial regulation of heparanase in macrophages. PMA-treated cells exhibited degradation of HSPGs that were contained in endothelium-derived ECM (Fig. 1). This was not found with untreated cells. Heparanase mRNA expression and the enzyme activity in cell lysates before and after the PMA treatment was very similar (Figs. 2 and 3). The supernatant of PMA-treated cells did not show any detectable heparanase activity (data not shown), therefore degradation of HSPGs was not due to released enzymes. A most remarkable difference between the two populations was cellular distribution of heparanase, i.e., cell surface expression in PMA-treated cells (Fig. 4). The requirement of microtubule-dependent molecular migration for the cell surface expression of the enzyme is achieved by the use of an inhibitor nocodazole (Fig. 5). After adhesion of the PMA-treated cells, capping of heparanase was induced (Fig. 6, A–F). Heparanase seems to be required for macrophage transmigration through Matrigel that contains HS because invasion was significantly inhibited by the addition of anti-heparanase mAb (Fig. 7). Taken together, we postulate that cell surface transport and capping of the heparanase are a series of important regulatory mechanisms for heparanase activity in macrophages. As the heparanase is expressed on the cell surface in a large portion of peripheral blood monocytes (Fig. 6, G and H), the cellular distribution of heparanase we demonstrate in this study seem to represent a unique feature of a certain subpopulation of monocytes.

Heparanase was previously reported to be expressed in cells in the immune system, and these cells are capable of transmigrating through vascular basement membranes in response to chemotactants and inflammatory stimuli. For example, expressions in neutrophils, monocytes, macrophages, Langerhans cells, and activated (but not resting) T lymphocytes have been previously reported (12, 14–18). This enzyme is potentially involved in the degradation of HSPGs in ECM by all of these cells. Alternatively, heparanase might function as an adhesion molecule to ECM that facilitates leukocyte adhesion as predicted previously (26). We demonstrated that migration of PMA-treated U937 cells through the reconstituted basement membrane (Matrigel) was significantly inhibited by addition of the anti-heparanase mAb (Fig. 7). Therefore, heparanase is proven to be involved in the cell transmigration through the reconstituted basement membrane. This was not due to the cytotoxic effect of the Ab because previous studies indicated that the transmigration was dependent at least in part on matrix metalloproteinases (MMP) and other matrix degrading enzymes (27). Our immunocytochemical study showed that one of the membrane-associated MMP, MT1-MMP, colocalized with heparanase in PMA-treated U937 cells (data not shown). Cellular distribution of MT1-MMP in macrophages has not been extensively studied. In tumor cells, MT1-MMP has been shown to be an enzyme that localizes preferentially in caveolin-rich membrane (28), accumulates at the invadopodia (29), degrades and activates MMP-2, and thus facilitates ECM degradation (30). Other proteolytic enzymes such as seprase and DPP IV distribute at the invasive edge of tumor cells and fibroblasts (31, 32). Colocalization of a number of matrix degradation enzymes at the leading edge of macrophages would be preferable to achieve effective transmigration through basement membrane. Untreated U937 cells also showed some invasive capacity that was not inhibited by either anti-heparanase Ab
or heparin. This would be due to nonproteolytic migration reported recently with U937 cells (33).

Heparanase was previously shown to be localized primarily in perinuclear acidic endosomal and lysosomal granules, and in the tertiary granules of human neutrophils together with MMP-9 (34, 35). We examined heparanase localization in U937 cells differentiated into macrophage-like cells by immunostaining with anti-heparanase mAb. Intracellular heparanase distributed in the granule-like structure in both PMA-treated and untreated cells (Fig. 4). In PMA-treated cells, heparanase was distributed on the cell surface and subsequently capped in an adhesion-dependent manner (Fig. 4). After the appearance on the cell surface, heparanase seems to behave as a membrane-associated protein, as the supernatant of PMA-treated U937 cells scarcely showed heparanase activity (data not shown). Heparanase is a transmembrane protein with a hydrophobic stretch of 20 aa near its C terminus (7–9). Alternatively, heparanase may bind putative cell surface receptors. A 300-kDa mannose phosphate receptor on activated T lymphocytes and an unidentified receptor that is expressed on human fibroblast cell surfaces were reported to contribute to an uptake of heparanase (36, 37). Our conclusion that cell surface expression of heparanase regulates transmigration of macrophages would be consistent with previous findings showing the importance of cellular distribution of the heparanase molecule. Vlodavsky’s group (38, 39) reported that transfection of chimeric constructs composed of human heparanase and chicken heparanase signal peptide facilitated cell surface expression of the heparanase and enhanced invasive capacity of the transfected cells. Further studies will be needed to clarify the mechanism of the cell surface transport and capping.

In conclusion, we show that macrophage-like cells express heparanase on the cell surface, and this was essential for the invasive properties after differentiation.

References