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Accumulation of Ym1 and formation of intracellular crystalline bodies in alveolar macrophages lacking heparanase

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ABSTRACT

Heparanase is a heparan sulfate (HS) degrading endoglucuronidase that has been implicated in cell migration and inflammatory conditions. Here we used mice deficient of heparanase (*Hpse*$^{-/-}$) to study the impact of heparanase on airway leukocytes. Normal numbers of macrophages and lymphocytes were present in bronchoalveolar lavage fluid of *Hpse*$^{-/-}$ mice, indicating that heparanase is not essential for proper homing of leukocytes to airways. While lymphocytes from *Hpse*$^{-/-}$ mice displayed normal morphology, *Hpse*$^{-/-}$ alveolar macrophages showed a striking, age-dependent appearance of granule-like structures in the cytoplasm. Transmission electron microscopy revealed that these structures corresponded to membrane-enclosed crystalline bodies that closely resembled the intracellular crystals known to be formed by the HS-binding protein Ym1, suggesting that heparanase deficiency is associated with intracellular Ym1 deposition. Indeed, applying immunocytochemistry, we found markedly higher levels of intracellular Ym1 protein in *Hpse*$^{-/-}$ versus *WT* alveolar macrophages, and there was a significant correlation between levels of Ym1 protein detected by immunoblotting and amounts of crystalline material in BAL cells. Biosynthetic radio-labeling of the macrophages revealed accumulation of non-degraded HS chains in *Hpse*$^{-/-}$ macrophages. Together, these findings implicate heparanase in normal processing of HS in macrophages, and indicate that heparanase regulates intracellular Ym1 accumulation and crystal formation in airways.

1. Introduction

Heparan sulfate proteoglycans (HSPGs) consist of protein cores to which sulfated and thereby negatively charged glycosaminoglycans (GAGs) of heparan sulfate (HS) type are attached. HSPGs are ubiquitously expressed, being present on the cell surface of most cell types as well as being one of the main components of the extracellular matrix (ECM). Cell surface HSPGs, e.g. of the syndecan or glypican families (Alexopoulou et al., 2007; Filmus et al., 2008), mediate cell–cell and cell–matrix contacts and function as co-receptors for various growth factors (Mythreye and Blobe, 2009). In addition, cell surface HSPGs lining the vascular endothelium act as sites for leukocyte attachment, thereby facilitating migration of blood-borne cells into tissues (Wang et al., 2005).

Heparanase is an endoglucuronidase that cleaves HS chains (Pikas et al., 1998). Heparanase may thus modify the structure of preformed HSPGs and hence regulate the plethora of processes in which HSPGs are implicated. Indeed, previous studies have suggested a role for heparanase in various pathophysiological conditions, including tumor metastasis (Escobar Galvis et al., 2007; Vlodavsky and Friedmann, 2001), experimental autoimmune disease (Lider et al., 1989), amyloidosis (Li et al., 2005) and delayed type hypersensitivity (DTH) reactions (Edovitsky et al., 2006). A role for heparanase in tumor metastasis is supported by the strong correlation between levels of heparanase expression and metastatic potential of B16 melanoma (Vlodavsky et al., 1999) and T lymphoma (Goldshmidt et al., 2002b) cells. Further, it has been shown by RNA interference that suppression of heparanase expression ameliorates DTH reactions (Edovitsky et al., 2006) as well as reduces lung colonization of B16 melanoma cells (Edovitsky et al., 2004). The role of heparanase in normal cell and tissue physiology is however not well characterized, although a role in tissue remodeling, vascularization and hair growth has been suggested applying transgenic mice overexpressing heparanase (Zcharia et al., 2004).

Abbreviations: BAL, bronchoalveolar lavage; BMMØ, bone-marrow-derived macrophage; DTH, delayed type hypersensitivity; ECM, extracellular matrix; GAG, glycosaminoglycan; *Hpse*, heparanase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; TEM, transmission electron microscopy.

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It is important to point out that most of the functional implications for heparanase have been derived from correlative or *in vitro* observations, and that detailed investigations of the *in vivo* function of heparanase have been hampered by the lack of relevant tools. The recent generation of a mouse strain lacking heparanase expression has opened new possibilities for determining its *in vivo* function (Zcharia et al., 2009). Heparanase-deficient ($Hpse^{-/-}$) mice do not demonstrate any major abnormalities under normal conditions, possibly due to an increased expression of matrix metalloproteases, compensating for the loss of heparanase-mediated ECM degradation (Zcharia et al., 2009). Moreover, heparanase deficiency results in accumulation of non-degraded HS chains in different tissues.

Heparanase is highly expressed by numerous types of tumor cells, and by various cells of the immune system (Vlodavsky et al., 1992), including mast cells (Gong et al., 2003), eosinophils (Temkin et al., 2004), dendritic cells (Benhamron et al., 2006) and monocytes/macrophages (Sasaki et al., 2004). However, the role of heparanase in the context of immune cell function is only partially understood. Here we addressed this issue by studying the impact of heparanase deficiency on various leukocyte populations present in airways. We show that the absence of heparanase does not affect homing of lymphocytes or macrophages into the airways, nor does it affect lymphocyte morphology. However, lack of heparanase caused an accumulation of Ym1, a chitinase-like protein, accompanied by a striking appearance of intracellular crystalline bodies in alveolar macrophages. Moreover, the HS chains in heparanase mutant macrophages were longer than in *WT* macrophages. Together, these findings implicate heparanase in the regulation of macrophage homeostasis.

2. Materials and methods

2.1. Mice and collection of bronchoalveolar lavage

Heparanase-deficient ($Hpse^{-/-}$) mice were generated as described (Zcharia et al., 2009) and were bred and maintained in the animal facility at Uppsala Biomedical Centre. Experimental groups were age and sex matched and $Hpse^{+/+}$ littermates (*WT* mice) were used as controls. All animal experiments were approved by the local ethical committee. Bronchoalveolar lavage (BAL) fluid was collected by rinsing the lungs twice with 1 ml HBSS. Total numbers of cells were counted and cytospin slides were prepared. In separate experiments the remaining cells were centrifuged and prepared for TEM or immunoblot analysis, and the cell-free BAL-supernatants were stored in −20 °C until used.

2.2. Transmission electron microscopy (TEM)

BAL cells from 7 or 17 months old $Hpse^{-/-}$ and *WT* mice were pooled from two mice per group and prepared for TEM as described (Braga et al., 2007). Electron micrographs were generated using a Hitachi electron microscope (Hitachi Ltd., Tokyo, Japan) (Lukinius et al., 1995).

2.3. Differential cell count and morphology

Cytospin slides were stained with May-Grünwald/Giemsa (Merck, Darmstadt, Germany) and the numbers of different airway cells were determined by differential counting of at least 200 cells per slide. The altered morphology of alveolar macrophages, i.e. the presence of granules in the cytoplasm, was quantified by blinded scoring of at least 200 cells per slide. Cells with visual granules were given scores (1–3) corresponding to the degree of granulation and no score (0) was given for cells lacking granules. An average score per counted cell was determined. To evaluate the presence of highly sulfated GAGs, cells were stained with Toluidine blue or Alcian blue as described (Braga et al., 2007; Reimer et al., 2006).

2.4. Immunocytochemistry

For immunocytochemistry, cytospin slides were fixed in ice-cold acetone for 5 min. Cells were permeabilized and blocked by incubation for 30 min with PBS containing 0.1% saponin, 0.5% BSA, and 10% goat serum. Endogenous peroxidase was blocked with PBS containing 0.3% H_2O_2 and 0.3% goat serum for 5 min. Cells were stained for 1 h with anti-Ym1 antibodies (StemCell Technologies, Vancouver, Canada) or anti-mouse IgG (H + L) antibodies (Chemicon International, Temecula, CA, USA) diluted 1:200 in a blocking buffer (PBS with 0.1% saponin, 0.5% BSA, 2% goat serum, and 0.42 mM NaCl). Rabbit IgG (Vector Laboratories, Burlingame, CA, USA) was used as a negative control. Cells were then incubated for 1 h with biotinylated goat anti-rabbit IgG diluted 1:200 in blocking buffer. The Vectastain® Elite ABC reagent and DAB substrate kit for peroxidase were used for development (Vector Laboratories). Slides were counterstained for 2 min with Mayer's hematoxylin solution (Sigma–Aldrich, St. Louis, MO, USA) and mounted with VectaMount (Vector Laboratories).

2.5. Generation of bone-marrow-derived macrophages

To generate conditioned medium for macrophage differentiation, we cultured the colony stimulating factor (CSF)-1 secreting cell line L929 (provided by Dr Ulrich Blank, Inserm U699, Paris, France) in DMEM supplemented with 5% FCS, 50 μg/ml penicillin, 60 μg/ml streptomycin and 2 mM L-glutamine (SVA, Uppsala, Sweden). Culture densities were initially 3×10^5 cells/ml, and after 8 days of culture, supernatants were collected, filtered and stored at −20 °C until used. Bone marrow cells from 8 months old *WT* or $Hpse^{-/-}$ mice were collected from femura and tibia by flushing the bones with 2.5 ml PBS. Red blood cells were removed by incubating cells for 2 min in 200 μl lysis buffer (0.15 M NH_4Cl, 0.1 mM EDTA, pH 7.4). Cells were cultured overnight in complete medium (DMEM supplemented with 15% FCS, 10% L929-conditioned medium, 50 μg/ml penicillin, 60 μg/ml streptomycin and 2 mM L-glutamine). Non-adherent cells, including monocyte precursors, were collected and 3×10^5 cells/ml were added to Petri dishes and cultured for 10 days in complete medium before used in further analyses.

2.6. FACS analysis

To confirm the purity of bone-marrow-derived macrophages, flow cytometry was used to identify presence of cells expressing the macrophage marker CD11b. Cells were washed twice in PBS and diluted in flow cytometry buffer (0.5%FCS/PBS). Samples of 0.2×10^6 cells were stained for 30 min on ice with 0.5 μg PE-labeled anti-CD11b antibodies or with 0.5 μg PE-labeled isotype controls (ImmunoTools, Friesoythe, Germany). After washing two times, cells were analyzed using a FACScan® flow cytometer and the CELLQuest™ 3.3 software (Becton Dickinson, San Jose, CA, USA).

2.7. Biosynthetic labeling and analysis of HS

Bone-marrow-derived macrophages from *WT* or $Hpse^{-/-}$ mice were cultured at 0.56×10^6 cells/ml in sulfate free medium supplemented with 2 mM L-glutamine, 1% pest, 10% L929-conditioned medium, 10% FCS (dialyzed against PBS), and 100 μCi/ml $Na^{35}SO_4$ (specific activity 1500 Ci/mmol; PerkinElmer, Waltham, MA, USA). After 24 h, HS was isolated from medium or cell lysates as described