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Stabilins are expressed in bone marrow sinusoidal endothelial cells and mediate scavenging and cell adhesive functions

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ABSTRACT

Bone marrow sinusoidal endothelial cells have a specific function as a site of transmigration of hematopoietic stem and progenitor cells and mature blood cells between bone marrow and blood stream. However, the specific characteristics of bone marrow sinusoidal endothelial cells are still largely unclear. We here report that these cells express stabilin-1 and stabilin-2, which in liver sinusoidal endothelial cells have been identified as endocytic scavenger receptors for several ligands, including SPARC and hyaluronan. We show here that intravenously injected formaldehyde-treated serum albumin, advanced glycation end-products, and collagen I α -chains were taken up by bone marrow sinusoidal endothelial cells, showing that these cells have a scavenging function and thereby may modulate bone marrow vascular stem cell niches. Importantly, we show that hyaluronan mediated adhesion of hematopoietic stem and progenitor cells to stabilin-2-transfected cells, suggesting that stabilin-2 has a role in adhering circulating stem and progenitor cells to bone marrow.

INTRODUCTION

Endothelial cells are specialized according to the tissue-specific functions. A distinct type of endothelial cell makes up the sinusoids of liver, spleen, and bone marrow. Distinguishing features of these sinusoidal endothelial cells (SECs) are sieve plates of fenestrae without diaphragms and a non-continuous or missing basement membrane(1).

In the liver, SECs have a key role in the endocytic removal of waste products, like connective tissue molecules, atherogenic lipids, and advanced glycation end products (AGEs) from the circulation(2). In BM, SECs function as a transit site for newly produced mature blood cells and for transmigration of hematopoietic stem and progenitor cells (HSPCs) between blood stream and bone marrow (BM) intersinusoidal spaces(3). In contrast to most endothelia, BM SECs constitutively express VCAM-1 and SDF-1(4) which promote migration of HSPCs to BM. However, the molecules mediating specific BM SEC functions, including extra- and intravasation, are still incompletely characterized.

Stabilin-1 and -2 are endocytic receptors expressed in non-continuous sinusoidal endothelium. We therefore studied whether BM SECs may express these receptors and have scavenging functions similar to SECs in other tissues. We also studied the possibility that

stabilin-2 through its hyaluronan-binding ability might have a role in cell adhesive interactions with HSPCs.

DESIGN AND METHODS

Tail vein injections and immunofluorescence microscopy.

The animal experiments were approved by the animal ethics committees in Uppsala (C-192/3) and Lund (M215-05). TRITC-labelled formaldehyde-treated BSA (FSA) and DTAF-labelled collagen I α -chains were produced according to standard protocols (5, 6). FITC-labelled AGE-modified BSA (AGE-BSA) was a kind gift from Dr. Berit Hansen, Heidelberg, Germany. C57Bl6 female mice (8-10 week old) were given single intravenous injections of the ligands in PBS (140 μ g/200 μ l for DTAF-labelled collagen I and 100 μ g/200 μ l for other ligands).

Frozen sections (7-8 μ m) of BM were prepared as described (7). The sections were fixed 5 minutes in -20°C methanol, blocked in PBS, 10% FCS (Gibco), and incubated 1 hour with antibodies against stabilin-1 and -2 (8, 9) and 30 min with Alexa-Fluor 488-goat anti-rabbit antibodies (Molecular Probes, Invitrogen). The sections were photographed using a Zeiss Axioplan microscope equipped with Zeiss Plan-APOCHROMAT 20x/0.75 and 40x/0.95 lenses, ORCA-1394ER digital camera and Openlab-3 software.

RT-PCR.

RNA was purified from mouse liver and BM using the Qiagen RNeasy Mini-Kit and treated with DNase I. cDNA was produced using Superscript II Reverse Transcriptase kit (Invitrogen). PCR primers were: stabilin-1 forward (exon 67) 5'-GTGCTGGGATCTGAGCCTCC, stabilin-1 reverse (exon 69) 5'-GTGTCGGGGAAGTCCTCCTC, stabilin-2 forward (exon 68) 5'-CTTTGGCAAGCAGCAGCCTG, and stabilin-2 reverse (exon 69) 5'-GTTCTCCAGCTCCCGTTCTC. The protocol was: denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 minute / 68°C for 2 minutes / 72°C for 5 minutes, and a final elongation for 7 minutes at 72°C.

Cell adhesion assay.

BM cells from 8-12 week old β -actin GFP transgenic mice(10) were enriched for c-kit by using anti-CD117 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Treatment with streptomyces hyaluronidase (H1136, Sigma-Aldrich) (3U/ml in PBS, 0,5% BSA) was performed at 37°C, 5% CO₂ for 30-45 min.

HEK293 cells stably expressing human stabilin-1 or mouse stabilin-2 (8) and nontransfected cells were grown in DMEM with 4,5g/L glucose, 10% FCS, L-glutamine, β -ME (10^{-2} M) at 37°C with 5% CO₂. For cell adhesion assay, the cells were plated in 24-well tissue culture plates (1×10^5 cells/well) and grown overnight. The medium was changed to IMDM. After 4-6 hours, c-kit-enriched cells (2×10^5 cells/well) were added and incubated for 2 hours. The non-adherent cells were harvested by collecting the medium and washing the wells twice with PBS, 5% FCS. The lineage negative, kit^{hi} Sca-1^{hi} (LSK) HSPCs (11) in c-kit enriched input and nonadherent cells were analyzed by BD FACSLSR II (BD Biosciences, San Jose, CA) using FowJo software (TreeStar Inc. San Carlos, CA). Antibodies used (from BD, Biosciences, unless otherwise stated) were rat anti-mouse CD16/32 (clone 2.4G2) , pacific blue- or FITC-Sca-1 (D7) and APC-CY7- or APC-c-Kit (2B8) (E-Bioscience, San Diego, CA), PE-Cy5-rat anti-CD4 (H129.9), -CD3 (17A2), -220/CD45 (RA3-6B2, Biolegend, San Diego, CA), -Mac-1 (M1/70, Biolegend), -TER119 (TER-119, Biolegend), -GR1 (RB6-8C5, Biolegend) and -CD8a (Ly2/53-67). 7-amino-actinomycin (7-AAD; Sigma-Aldrich Co, St Louis, MO) was used to exclude dead cells. Expression of hyaluronan was analyzed using biotinylated hyaluronan-binding protein (Sigma, H9910) and streptavidin-PE.

RESULTS AND DISCUSSION

RNA expression of mouse BM stabilins was studied by RT-PCR. PCR products obtained from BM and liver, used as a positive control (Figure 1A), matched the sizes expected for the selected primers (289 and 118 base pairs for stabilin-1 and -2, respectively). By Western blotting of BM lysates, stabilin-1 was detected as a band of 280 kDa and stabilin-2 as two bands of 300 and 175 kDa (not shown), similar to their appearance in liver (9). Immunofluorescent staining showed ubiquitous expression of both stabilin-1 and stabilin-2 in BM SECs (Figure 1B, D). Control staining with the anti-rabbit antibody gave no specific staining (Figure 1H).

Stabilin-1 was originally identified in non-continuous SECs in spleen(12) and was subsequently found in liver and lymph node SECs. In addition, its expression is induced in angiogenic endothelium associated with wound healing, tumour vascularisation and chronic

inflammation, and in macrophages after “alternative activation”(13). In contrast, expression of stabilin-2 in endothelium has been found restricted to SECs(14), in agreement with the expression in SECs in BM.

To investigate the scavenging function of the bone marrow SECs, the uptake of selected scavenger receptor ligands was analyzed after intravenous administration. Accumulation of TRITC-FSA in the BM SECs was observed within one hour (Figure 1C, E, F). There was little or no fluorescence in the intersinusoidal spaces. Injection of unlabelled FSA together with TRITC-FSA reduced the fluorescence in the SECs (Figure 1G), indicating that the uptake was due to specific recognition of FSA. Likewise, labeled AGE-BSA accumulated in BM SECs (Figure 1I). These two ligands are primarily cleared by the stabilins. Uptake of DTAF- collagen I α -chains, which are eliminated by the receptor for non-native collagens,(6) was also seen in BM SECs (Figure 1I).

Several extracellular matrix components have been identified as ligands for stabilins. One example is SPARC, which is expressed by hematopoietic progenitors, megakaryocytes, osteoblasts, adipocytes, and endothelial cells(15). SPARC mediates diverse functions, including inhibition of cell proliferation and modulation of cell-matrix interactions(15), and its clearance by stabilin-1 may thus influence proliferation and migration of BM cells(16). Stabilin-2 has been identified as a receptor for hyaluronan, collagen propeptides(9), chondroitin sulphate(17) and heparin. Hyaluronan is synthesized and deposited on the cell surface by HSPCs and has been shown to mediate their mobilization and homing into BM(18). Our FACs analysis showed expression of hyaluronan in one third of LSK cells (Fig 2A), in agreement with published results(19). Importantly, we found significantly increased adhesion of c-kit enriched cells and LSK cells to cells transfected with stabilin-2 compared to cells expressing stabilin-1 or non-transfected cells. Pretreatment with hyaluronidase resulted in significantly reduced adhesion of both c-kit enriched cells and LSK cells to stabilin-2 expressing cells. These results suggest that stabilin-2 may be an important adhesion molecule for initial recruitment of circulating HSPCs to SECs, promoting their homing into BM. Similarly, binding of tumour cell-associated hyaluronan by stabilin-2 might cause tumour cells in blood to halt in bone marrow, thereby increasing the likelihood for metastasis at this site. In support of this, high level of hyaluronan synthesis in prostate cancer cells has been correlated with increased metastasis to the bone marrow(20).

In conclusion, this study shows a novel function for BM SECs as a scavenging endothelium with implications for the molecular environment in BM stroma. Further, the specific cell adhesive interactions mediated by hyaluronan on HSPCs and stabilin-2 expressing cells in vitro suggest a new role for this receptor in HSPC homing to BM.

AUTHORSHIP AND DISCLOSURES

Author's contributions: HQ. was the principal investigator and takes primary responsibility for the paper. HQ, SoJ, and ME performed laboratory work for this study. HQ, SoJ, BS, PM, StJ and ME designed research, analyzed data and wrote the paper.

Declaration of commercial interest: All authors declare that they have no conflicting interests.

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FIGURE LEGENDS

Figure 1. Expression of stabilin-1 and stabilin-2 in bone marrow (BM) sinusoidal endothelial cells (SECs). (A). cDNA fragments generated from liver and bone marrow (BM) using PCR primers for stabilin-1 (lanes 1 and 4) and stabilin-2 (lanes 2 and 3) were separated on 1.5% agarose gel. Bp: 100-300 base pair molecular weight markers. (B-E) Immunostaining of stabilin-1 (B) and stabilin-2 (D) of in mouse bone marrow SECs and uptake of TRITC-FSA by SECs in the corresponding tissue sections (C, E). The bone marrow was isolated 60 minutes after the injection of TRITC-conjugated FSA in the tail vein and the frozen sections were stained by antibodies against stabilin-1 and stabilin-2. (H) Control staining with the anti-rabbit antibody. (F, G). Competition of uptake of TRITC-FSA by unlabelled FSA. TRITC-FSA (0.5 mg/ml) was injected in the tail vein alone (F) or together with 6.4 mg/mL of unlabelled FSA (G), and the accumulation of fluorescence in BM after 60 minutes was analyzed. (I). Localization in mouse BM of intravenously injected AGE-BSA. The bone marrow was collected 60 minutes after injection of the FITC-conjugated protein. (J, K), Localization in BM (J) and liver (K) of intravenously injected DTAF-collagen α -chains. The tissues were collected 30 minutes after injection of the protein. The sections were photographed using a Zeiss Axioplan microscope equipped with Zeiss Plan-APOCHROMAT 20x/0.75 and 40x/0.95 lenses, ORCA-1394ER digital camera and Openlab-3 software. Original magnification was (B-H, J, K) x40, (I) x20.

Figure 2. Expression of hyaluronan (HA) on bone marrow (BM) stem and progenitor cells and adhesion of BM c-kit-enriched and lineage negative, c-kit^{hi} Sca-1^{hi} (LSK) cells to HEK293 cells and HEK293 cells transfected with stabilin-1 or stabilin-2. (A). HA expression in LSK cells. Left panel: FACS profile showing gating of SCA-1^{high}c-Kit^{high} of lineage negative (not shown) cells. Right panel: HA expression (dashed line) and control staining with the secondary streptavidin-PE antibody (solid line) in the gated LSK cells. (B). The number of nonadherent c-kit enriched BM cells (KIT) (left) and LSK cells (right) after incubation in wells plated with HEK293 cells (293) or cells transfected with stabilin-1 (ST1) or stabilin-2 (ST2). ST2+HASE; adhesion of cells treated with hyaluronidase to HEK cells expressing stabilin-2. ** p<0.01; *** p<0.001. Data are from 2 experiments performed in 3-6 replicates; horizontal bars show mean values. Each dot represents nonadherent cells from one well. Except for two samples pooled in

(293) group, the frequency of LSK cells was analyzed separately from each well. The mean number of LSK cells within 2×10^5 c-kit enriched cells was $13.8-15.5 \times 10^3$. (C). FACS profiles showing the frequency of LSK cells in the corresponding nonadherent cell populations.

Figure 1

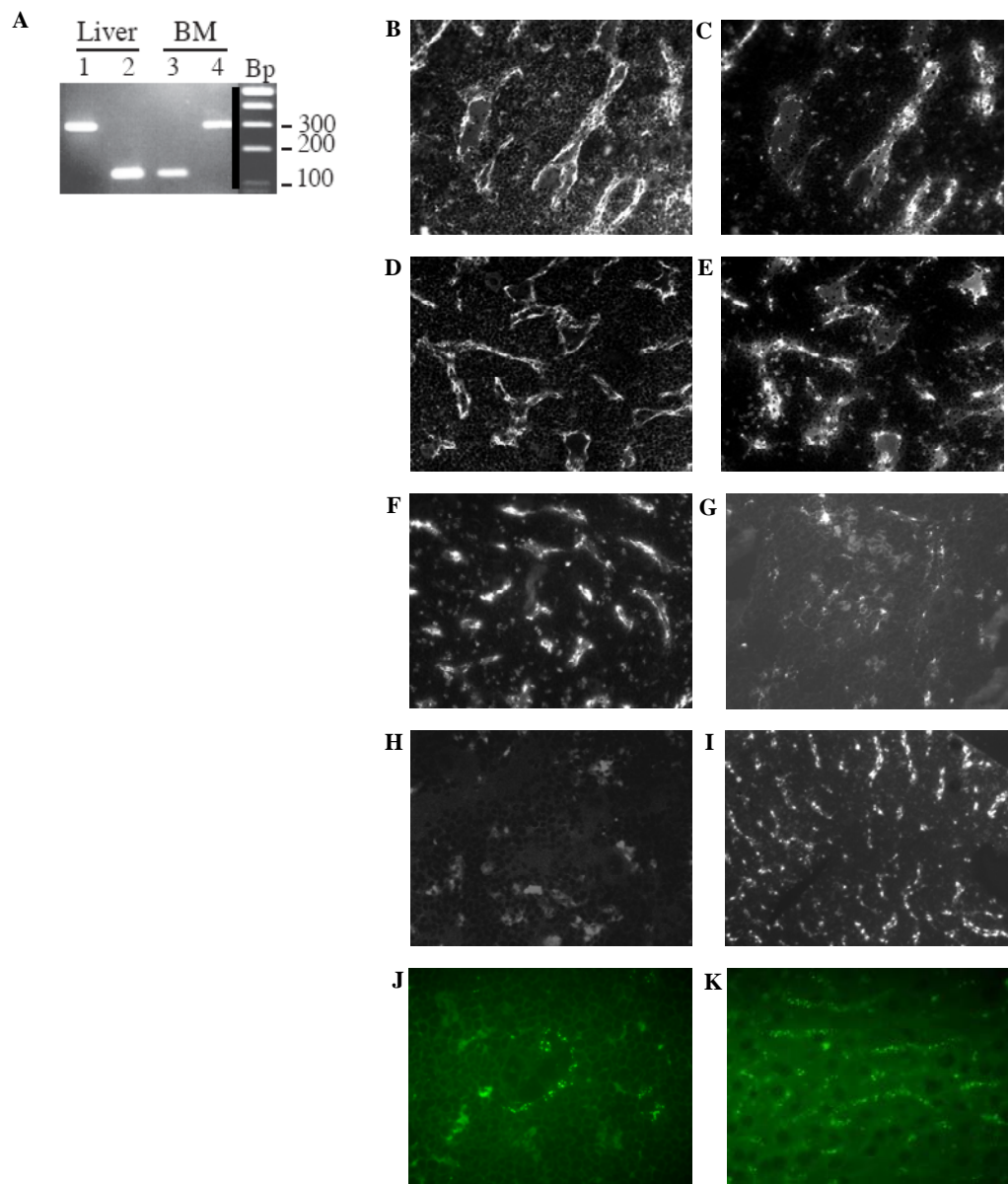


Figure 2.

