

Heparanase expression in human leukemias is restricted to acute myeloid leukemias

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Objective. Matrix metalloproteinases and an endo- β -D-glucuronidase (heparanase) are enzymes that degrade the protein and carbohydrate constituents of basement membranes, thereby facilitating transendothelial migration of blood-borne cells. Heparanase activity was found to correlate with the metastatic potential of solid tumors. We evaluated heparanase expression, at the levels of gene and protein expression and activity in a variety of leukemias, and compared it with normal hematopoietic cells.

Materials and Methods. Heparanase expression was evaluated in leukocytes isolated from peripheral blood of 71 patients with myeloid and lymphoid leukemias, or non-Hodgkin's lymphoma. Analysis was performed at two levels: heparanase RNA was determined by reverse transcriptase polymerase chain reaction, and heparanase protein was evaluated by immunocytochemistry and flow cytometry.

Results. In eight peripheral blood samples from normal donors, heparanase RNA was detected, and protein was found within the cytoplasm of granulocytes. In mononuclear cells derived from various leukemias, heparanase RNA was expressed in 14 of 15 acute myeloid leukemia (AML) samples. In contrast, cells derived from all 33 chronic lymphoblastic leukemia, all 7 non-Hodgkin's lymphoma, 7 of 8 chronic myeloid leukemia, and 6 of 8 acute lymphoblastic leukemia patients showed no detectable expression of the heparanase RNA. Heparanase protein was detected primarily within the cytoplasm of AML cells, indicating that the enzyme is produced and stored within the cytoplasm of myeloid cells, with limited expression on the cell surface.

Conclusion. We propose that heparanase expression is associated with the myeloid lineage and may serve as an independent marker to support the identification of AMLs. © 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Heparan sulfate proteoglycans (HSPGs) are macromolecules composed of a core protein covalently O-linked to repeating hexuronic and D-glucosamine disaccharide units [1–3]. HSPGs are located at the subendothelial basement membrane, where they cross-link a variety of extracellular matrix (ECM) molecules (e.g., collagen, laminin, fibronectin), thereby contributing to the formation and preservation of blood vessel walls [4]. The extravasation of circulating metastatic tumor cells is accompanied by degradation of

various components of the subendothelial basement membrane [5]. Thus, highly metastatic cells produce and secrete a variety of ECM-degrading enzymes, including members of the matrix metalloproteinase (MMP) family (e.g., MMP-2 and MMP-9) [6]. MMP-2 and MMP-9 are produced by lymphoid and myeloid neoplasias, as well as by normal hematopoietic cells, including granulocytes, monocytes, and lymphocytes [7].

HSPG degradation disintegrates the subendothelial basement membrane, consequently facilitating transendothelial migration of blood-borne cells [8,9]. Heparanase is an endo- β -D-glucuronidase that degrades the heparan sulfate (HS) side chains of HSPG. Heparanase activity has been detected in several types of normal hematopoietic cells, including neutrophils, megakaryocytes, and activated lymphocytes and may mediate their extravasation during inflammatory

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and immune responses [9]. Heparanase expression correlates with the metastatic potential of lymphoma, fibrosarcoma, breast carcinoma, and melanoma cells [8,10–14]. However, heparanase expression pattern in hematologic proliferative disorders has not been investigated.

Recently, the gene encoding the human heparanase was cloned. Its deduced amino acid sequence includes a potential signal peptide, indicating that heparanase can be secreted outside the plasma membrane [13–16]. Heparanase genomic locus spans ~40 kb and is localized on human chromosome 4q21.3 [13,14]. Transfection of heparanase cDNA into a nonmetastatic lymphoma cell line resulted in an increase in the metastatic potential, demonstrating a direct link between heparanase expression and malignancy of hematopoietic cells [13].

In the present study, we investigated whether heparanase expression is associated with specific types of hematologic malignancies. We found that heparanase expression in leukemias was restricted predominantly to cells of the myeloid lineage. Heparanase RNA, protein, and activity were not detected in chronic lymphoblastic leukemia (CLL) and most other leukemias, or in hemato-oncologic disorders. Therefore, heparanase may be potentially utilized as an additional marker for acute myeloid leukemias (AMLs), independent of more common membrane markers.

Materials and methods

Antibodies

Mouse anti-heparanase monoclonal antibody (mAb) 130 (IgG1) was previously described [13]. Fluorescein isothiocyanate (FITC)-labeled mAb 130 was provided by InSight Ltd. (Rabin Science Park, Rehovot, Israel). FITC-conjugated anti-CD3, CD14, CD15, CD19, and CD34 mAbs and isotype control IgG1 were purchased from Dako (Denmark) and utilized according to the manufacturer's instructions. FITC and phycoerythrin (PE)-conjugated goat anti mouse antibodies were obtained from Dako and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively.

Blood samples

Peripheral blood and bone marrow samples were obtained from discarded material utilized for routine laboratory tests at the Hematology Laboratory, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel, and the Hematology Department, Hadassah-Hebrew University Hospital, Jerusalem, Israel. Use of discarded material is approved by our Institutional Review Board.

RNA isolation and reverse transcriptase polymerase chain reaction

Total RNA was isolated utilizing TRIzol (Life Technologies, Gaithersburg, MD, USA) and subjected to reverse transcriptase polymerase chain reaction (RT-PCR) as previously described [13]. Briefly, reverse transcription of 500 ng total RNA from normal and leukemia mononuclear cells samples was performed by oligo(dT) priming, followed by PCR utilizing the following oligonucleotide primers: HPU-355 (5'-TTCGATCCCAAGAAGGAATCAAC-3')

and HPL-229 (5'-GTAGTGATGCCATGTAAGTGAATC-3'). Aliquots (10 μ L) of the amplification products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Only RNA samples that gave completely negative results in PCR without RT were further analyzed. The following conditions were applied to heparanase PCR amplifications: 94°C for 4 minutes, 29 cycles of 94°C for 45 seconds, 60°C for 1 minute, and 72°C for 45 seconds. PCR products then were incubated at 4°C for 1 hour. The total numbers of samples evaluated for heparanase RNA expression were CLL 33, acute lymphoblastic leukemia (ALL) 8, AML 15, chronic myeloid leukemia (CML) 8, non-Hodgkin's lymphoma (NHL) 7, and normal donors 8.

Isolation of peripheral blood monocytes

Mononuclear cells were isolated from normal donor peripheral blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient and washed two times in phosphate-buffered saline (PBS). Adherent cells were collected from the mononuclear cell fraction by incubation at 37°C for 2 hours in RPMI on plastic tissue culture flasks. FACS analysis of the monocyte cell preparation was performed using CD14/CD45 antibodies. Fewer than 5% of cells were CD14⁻CD45⁺.

Flow cytometry of peripheral blood and bone marrow samples

For staining with the FITC-conjugated anti-heparanase mAb 130, 50- μ L samples were incubated with 10 μ L of mAb 130 (or 10 μ L of isotype control) for 30 minutes at 4°C. The samples then were incubated with 450 μ L of erythrocyte lysis solution (Becton-Dickinson, San Jose, CA, USA) for 12 minutes at 4°C, followed by wash with 2 mL of PBS. The samples were subjected by flow cytometric analysis utilizing FACS Calibur (Becton-Dickinson). For analysis of normal and leukemia peripheral blood and bone marrow, samples were purified by Ficoll-Paque density gradient centrifugation and washed twice with PBS. Indirect immunofluorescence staining was performed by incubating 0.5 to 1 \times 10⁶ cells with 10 μ g/mL mAb 130 for 30 minutes at 4°C. The samples were washed twice with PBS, followed by incubation with 50 μ L of 1:100 diluted PE- or FITC-conjugated goat anti-mouse antibody for 30 minutes at 4°C. The cells were washed with PBS and analyzed by flow cytometry utilizing FACS Calibur (Becton Dickinson). The total numbers of samples evaluated for heparanase surface expression were CLL 10, ALL 2, AML 10, CML 2, myelodysplastic syndrome (MDS) 4, multiple myeloma 2, and lymphoma 2.

Immunofluorescence staining of heparanase in normal circulating human granulocytes

Granulocyte-enriched fraction was purified from normal blood samples by Ficoll-Paque density gradient centrifugation and washed twice with PBS. Immunofluorescence staining with anti-heparanase mAb 130 was performed as previously described [17].

Immunocytochemistry

Cytospins prepared from peripheral blood cells were fixed in acetone:methanol (1:1) for 10 minutes at 24°C. Slides were washed in PBS and endogenous peroxidases were blocked with 0.3% H₂O₂ in methanol for 15 minutes. The slides were incubated for 15 minutes at 24°C with PBS containing 10 mM glycine and 10 μ g/mL bovine serum albumin, followed by blocking for 30 minutes with normal goat serum in PBS containing 1% bovine serum albumin.

Affinity-purified monoclonal anti-heparanase antibodies were added (1:50–1:200 dilution) for 4 hours at 4°C, followed by incubation for 1 hour at room temperature with biotinylated secondary goat anti-mouse IgG antibodies and 1 hour with streptavidin-peroxidase conjugate (1:200 dilution) (Histostain-SP; Zymed Laboratories Ltd.) [13,18].

Heparanase activity

Dishes coated with metabolically labeled ECM were prepared as previously described [8–10,13]. Platelet-depleted preparations of leukocytes were suspended ($2.5 \times 10^6/\text{mL}$) in serum-free RPMI medium and incubated (24 hours, 37°C, pH 6.6) with ^{35}S -labeled ECM. The incubation medium was centrifuged and the supernatant analyzed by gel filtration on a Sepharose CL-6B column (0.9×30 cm) [8–10,13]. Fractions (0.2 mL) were eluted with PBS and counted for radioactivity [8–10]. Degradation fragments of HS side chains were eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II). A nearly intact HSPG was eluted just after the V_0 ($K_{av} < 0.2$, peak I) [8–10,13]. Each experiment was performed at least three times, and the variation of elution positions (K_{av} values) did not exceed $\pm 15\%$.

Results

Heparanase expression in normal circulating leukocytes

We analyzed heparanase RNA expression in normal peripheral blood leukocytes. RNA was extracted from eight samples of peripheral blood leukocytes and semiquantitative RT-PCR was performed using previously described oligonucleotide primers [13]. As expected, a single band of 585-bp PCR product was detected (Fig. 1). Relatively high levels of heparanase RNA were found in all samples derived from normal donors of peripheral blood leukocytes (Fig. 1, lanes 1–6; two additional positive samples are not shown).

RNA analysis, however, is not informative with respect to the specific cell type expressing heparanase due to the heterogeneity of peripheral leukocytes. Therefore, we independently determined heparanase cellular localization in hematopoietic cells by immunofluorescence staining. For this purpose, polymorphonuclear enriched Ficoll fraction was applied to glass slides and permeabilized with 0.5% Triton X-100 followed by immunofluorescence staining with mAb

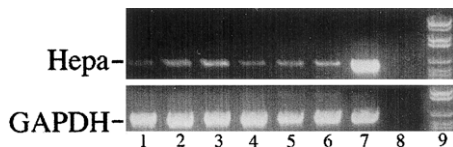


Figure 1. Heparanase RNA expression in peripheral white blood cells of normal donors. Peripheral white blood cells were isolated by Ficoll-Paque from six healthy donors. Total RNA was assessed by RT-PCR, as described in the Materials and methods section, using primers specific for heparanase (585 bp) (lanes 1–6, top) and GAPDH (598 bp) (lanes 1–6, bottom). Lane 7, top: control pcDNA3 plasmid containing the full-length heparanase cDNA; lane 7, bottom: control human melanoma cDNA as template; lane 8: reaction-containing reagents only without cDNA; Lane 9, DNA molecular weight markers.

130. As shown in Figure 2A, mAb 130 specifically stained the cytoplasm of peripheral blood polymorphonuclear cells (granulocytes). In contrast, heparanase was not detected in mononuclear cells or erythrocytes (Fig. 2A). To examine whether monocytes express heparanase, we isolated circulating adherent monocytes. As shown in Figure 2B, profound heparanase activity was detected in the monocytes, which was inhibited by heparin. In addition, heparanase RNA expression was detected in isolated monocytes (not shown). These results indicate that most of the heparanase in circulating mature leukocytes apparently is stored within the cytoplasm of granulocytes and monocytes.

Heparanase RNA and protein expression in leukemic cells

Aberrant expression of ECM-degrading metalloproteinases may characterize specific types of malignant hematopoietic

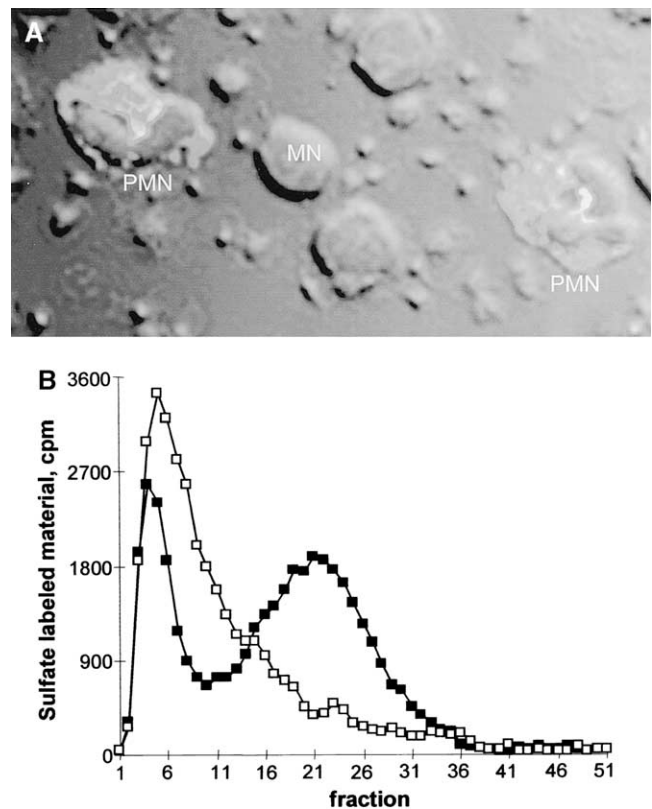


Figure 2. Heparanase expression in peripheral blood leukocytes. (A) Normal blood samples enriched for polymorphonuclear cells by Ficoll-Paque density gradient centrifugation were spread on glass slides, permeabilized, and stained for heparanase as described in the Materials and methods section. Note the strong cytoplasmic staining of the polymorphonuclear (PMN) cells and the lack of heparanase expression in mononuclear (MN) cells and erythrocytes. (B) Peripheral blood monocytes were isolated as described in the Materials and methods section. Monocytes (2.5×10^6 cells/mL) were incubated (24 hours, 37°C, pH 6.6) with sulfate-labeled ECM in the absence (filled square) or presence (open square) of $2.5 \mu\text{g/mL}$ heparin. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration over Sepharose 6B, as described in the Materials and methods section.

cells [7]. We evaluated heparanase expression at both the RNA and protein levels in samples obtained from patients with a variety of hematologic disorders, predominantly leukemias. Heparanase RNA expression was evaluated in mononuclear cells by RT-PCR. As shown in Figure 3A, we found heparanase RNA expression primarily in AML samples. We detected heparanase RNA in 14 AML samples classified as M1, M2, M3, M4, M5, and M7. Heparanase expression was not detected in a single case of AML classified as M6 (erythroleukemia). Similarly, we did not detect heparanase expression and activity in the K562 erythroleukemia cell line (not shown). Heparanase RNA was not detected in any of the CLL samples, regardless of their clinical stage. As summarized in Table 1, no heparanase RNA was detected in all CLL (0/33) and NHL (0/7) samples examined. Only 2 (25%) of 8 ALL samples were positive for heparanase expression. The six heparanase-negative samples were newly diagnosed patients, and the two heparanase-positive ALL samples were from patients in relapse. Eight CML samples were examined for heparanase expression, which were from patients in the chronic phase of the disease; all were positive for BCR/Abl. Under the PCR conditions applied in this study, only 1 of 8 CML samples (patient in the blast crisis of the disease) was positive for heparanase RNA expression. These data were confirmed by immunostaining of leukocytes applied on slides (Fig. 3B). Heparanase staining also indicated that the enzyme was located mostly within the cytoplasm of AML blasts (Fig. 3B,

Table 1. Expression of heparanase RNA (RT-PCR) in human leukemias and lymphomas

Type	No. of patients	No. of hpa positive	No. of hpa negative
CLL	33	0	33
AML	15	14	1
ALL	8	2	6
CML	8	1	7
NHL	7	0	7

Heparanase RNA (hpa) expression was evaluated in peripheral blood mononuclear cells of patients with leukemias and lymphomas as described in the Materials and methods section. ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphoblastic leukemia; CML = chronic myeloid leukemia; NHL = non-Hodgkin's lymphoma.

no. 5), similar to normal granulocytes and monocytes (Figs. 2 and 3B, no. 1). In contrast, no heparanase staining was detected in normal circulating lymphocytes (Fig. 3B, no. 2), or in CLL (Fig. 3B, no. 3) or CML (Fig. 3B, no. 4) cells.

To evaluate whether expression of heparanase RNA and protein in AML results in production of a functional enzyme, we examined representative AML and CLL samples for heparanase enzymatic activity. Peripheral blood leukocytes were isolated by Ficoll-Paque from patients with AML M4 and CLL, and heparanase activity was examined as previously described [8–10,13]. As shown in Figure 4 (inset), heparanase RNA was detected in the AML (lane 1), but not CLL sample (lane 2). As expected, extensive degra-

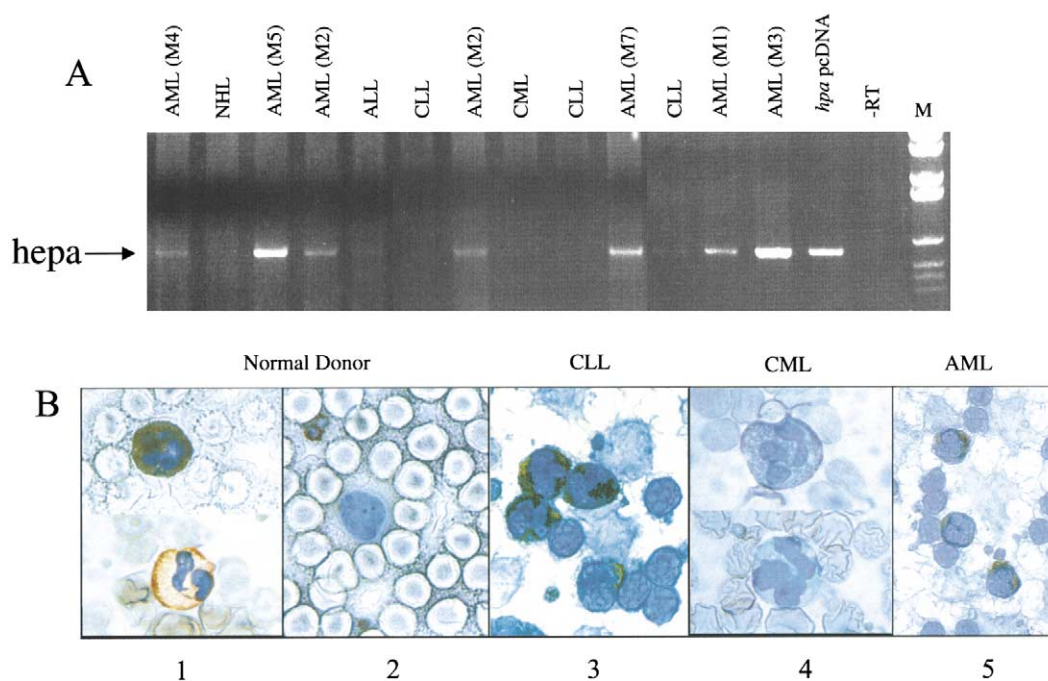


Figure 3. Expression of heparanase by normal and leukemic leukocytes. White blood cells isolated from patients with various leukemias as well as healthy donors were analyzed by (A) RT-PCR for the presence of heparanase transcripts and (B) immunohistochemistry (mono-clonal anti-heparanase antibodies) for expression of the heparanase protein. A selection of representative cases is shown. The heparanase RNA is expressed in mononuclear cells from patients with AML, but not CLL, ALL, NHL, or CML. Specific staining of the heparanase protein is seen in neutrophils (no. 1) and platelets (no. 2), but not lymphocytes (no. 2) in samples from normal donors. Staining is seen in the cytoplasm of AML blasts (no. 5) but not of CML (no. 4) and CLL (no. 3) leukemic cells.

dation in intact ECM of HS into low molecular weight S³⁵ labeled fragments was exerted by AML but not by CLL cells (Fig. 4). Degradation of HS by AML cells was inhibited in the presence of heparin, indicating specific heparanase activity (Fig. 4) [8–10,13].

These results suggest that heparanase transcription, translation, and activity are characteristic features of AML. Interestingly, some of the CML samples, which also are associated with the myeloid lineage, were negative both for heparanase RNA and protein.

Cell surface heparanase expression is restricted to rare cases of AML

The coding sequence of heparanase indicates that the enzyme contains several stretches of hydrophobic amino acids (i.e., Pro⁵¹⁵-Ile⁵³⁴) that potentially could serve as membrane anchoring domains [13,14]. However, our immunocytochemical stainings revealed that heparanase is predominantly a cytoplasmic protein (Figs. 2 and 3). Cell surface heparanase localization was evaluated directly by flow cytometric analysis performed on normal peripheral blood samples, utilizing FITC-conjugated mAb 130 [13]. We utilized FITC-conjugated IgG1 as an isotype control. Figure 5 shows a representative flow cytometric analysis of cell surface heparanase on the entire population of circulating leukocytes. Figure 5A

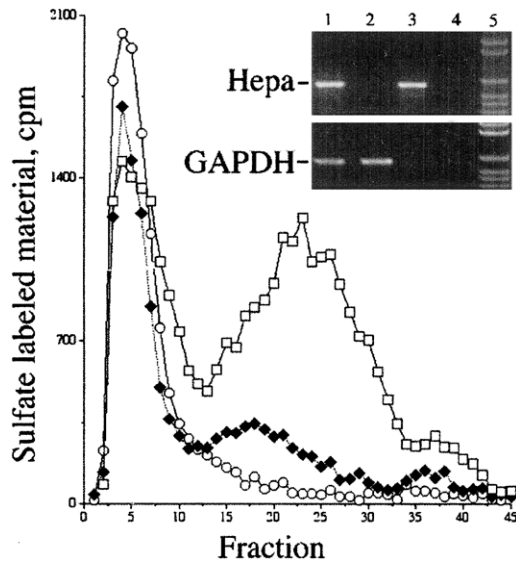


Figure 4. Heparanase activity and RNA expression in cells from AML vs CLL patients. Leukemic cells were isolated by Ficoll-Paque from patients with AML-M4 (open squares) and CLL (open circles). Cells (2.5×10^6 cells/mL) were incubated (24 hours, 37°C, pH 6.6) with-sulfate labeled ECM in the absence (open squares) or presence (filled diamond, AML cells) of 2.5 μg/mL heparin. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration over Sepharose 6B, as described in the Materials and methods section. Inset: Total RNA assessed by RT-PCR using primers specific for heparanase (top) and GAPDH (bottom). AML M4 (lane 1), CLL (lane 2), control pcDNA3 containing the heparanase cDNA (lane 3, top), reaction containing reagents only without cDNA (lane 4), and DNA molecular weight markers (lane 5) are shown

indicates low surface heparanase expression on peripheral blood leukocytes. Next, we evaluated cell surface heparanase expression on the three major types of circulating leukocytes, defined by their size (forward scatter) and granularity (side scatter): granulocytes (region Gr, Fig. 5A), monocytes (region Mo, Fig. 5A), and lymphocytes (region Ly, Fig. 5A). As shown in Figure 5B, very low levels of surface heparanase were detected on granulocytes (Fig. 5B, left) and to an even lesser extent on monocytes (Fig. 5B, middle). No significant amount of heparanase was detected on the cell surface of lymphocytes (Fig. 5B, right).

In a similar manner, we evaluated cell surface heparanase expression on samples obtained from patients with a variety of hematologic disorders. Cell surface heparanase was detected in 2 of 10 AML samples but not in any of the other 22 samples from patients with hematologic disorders, which included 2 ALL, 2 CML, 10 CLL, 4 MDS, 2 NHL, and 2 multiple myeloma samples (not shown).

As shown in Figure 6, peripheral blood blasts of an AML M4 patient were double stained with CD14 and heparanase (Fig. 6, bottom left panel), indicating cell surface heparanase expression on the leukemic cells. Cell surface heparanase

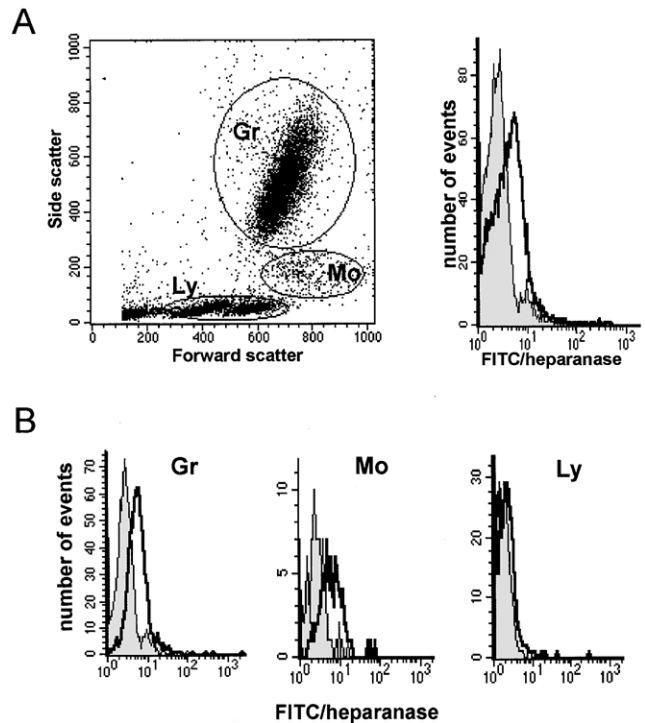


Figure 5. Flow cytometric analysis of cell surface heparanase expression on normal circulating leukocytes. Normal blood sample was stained with FITC-conjugated anti-heparanase mAb 130 or isotype control. The cells were analyzed by flow cytometry. (A) Left: Forward scatter and side scatter of normal circulating leukocytes. Regions Gr, Mo, and Ly define granulocytes, monocytes, and lymphocytes, respectively; right: heparanase cell surface expression on normal circulating leukocytes. (B) Heparanase expression on specific populations of granulocytes (Gr), monocytes (Mo), and lymphocytes (Ly). Filled areas = isotype controls; solid lines = heparanase staining.

nase-expressing cells also were positive for CD15 (not shown). In contrast, normal CD3⁺ lymphocytes of the same patient were negative for cell surface heparanase expression (not shown). Cell surface heparanase was detected by flow cytometric analysis of a bone marrow sample from the same patient (Fig. 6, bottom middle panel). The same sample also contained bone marrow resident CD14⁺ cells that express a very low level of cell surface heparanase (Fig. 6, bottom middle panel). Interestingly, the double-positive heparanase⁺/CD14⁺ cell population disappeared while the same patient was analyzed during clinical remission, indicating that the cells expressing a high level of surface heparanase within the bone marrow were indeed leukemia cells (Fig. 6, bottom right panel). Therefore, it appears that the factors that control aberrant cell surface heparanase expression in leukemic cells are not confined to a specific microenvironment within the circulation or the bone marrow.

These data indicate that heparanase transcription, translation, and activity are associated with normal myeloid cells and are retained following malignant transformation of AML. Cell surface heparanase expression is limited to rare cases of AML.

Discussion

Metastatic spread of blood-borne tumor cells involves degradation of blood vessel wall constituents, including basement membrane molecules such as collagen type IV, laminin, and HSPG [8–11,19,20]. Hence, malignant cells constitutively express a variety of ECM-degrading enzymes, including MMPs and heparanase [7–12,19,21–23].

Previous studies evaluated the expression of MMPs in malignant hematopoietic cells [7]. MMP-9 expression was detected in bone marrow samples obtained from normal individuals and patients with several hematologic disorders, including AML, CML, and MDS [7]. In contrast, MMP-2 expression was detected predominantly in AML and MDS, but not in normal or CML bone marrow samples [7]. Due to the lack of anti-heparanase mAbs and molecular probes, heparanase expression in aberrant hematopoietic cells has not been investigated. As we now have these tools, we evaluated heparanase expression at the RNA, protein, (intracellular and cell surface) and activity levels in leukemias and normal circulating leukocytes.

We detected heparanase RNA expression in all samples of AML, except for erythroleukemia (M6). We did not detect any heparanase in the erythroblastic cell line (K562) or in mature erythrocytes, indicating a lack of heparanase expression in cells of the erythroid lineage (Fig. 7). Similarly, heparanase expression was not detected in leukemias from the lymphoid lineage, as well as in normal circulating lymphocytes. However, mature lymphocytes may acquire heparanase expression upon activation [9]. Heparanase expression was detected in AML cells at both early and late stages of myeloid differentiation (M1, M2, M3, M4, M5, and M7), as well as in mature circulating granulocytes. These data indicate an early heparanase gene expression checkpoint within the myeloid differentiation pathway (Fig. 7). Heparanase expression is sustained throughout the differentiation of the myeloid lineage (Fig. 7). Surprisingly, we did not detect heparanase expression in most of the CML

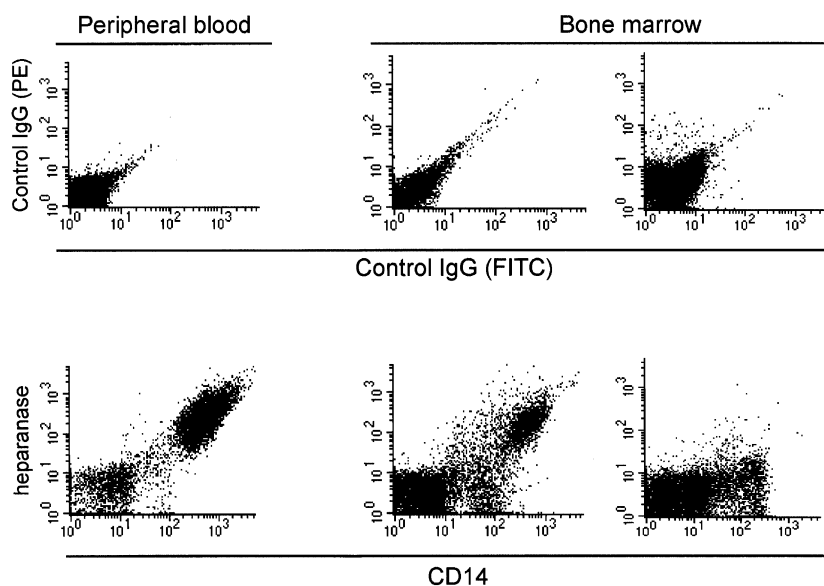


Figure 6. Cell surface heparanase expression in circulating and bone marrow AML M4 cells. Samples obtained from AML M4 peripheral blood (left column), bone marrow (middle column), and bone marrow following clinical remission (right column) were purified by Ficoll-Paque density gradient centrifugation. The purified cells were double labeled with anti-heparanase mAb 130, followed by PE-conjugated goat-anti mouse antibody and then FITC-conjugated anti CD14 mAb (bottom row). The matching isotype control stainings are shown in the top row. Note that although the peripheral blood sample contained one population of double-positive heparanase⁺/CD14⁺ cells, the bone marrow contained a population of heparanase⁻/CD14⁺ cells and double-positive heparanase⁺/CD14⁺ cells. The latter cell population disappeared while the patient underwent clinical remission.

samples, except from a patient in the blast crisis of the disease. It is well known that CML cells lack certain enzymatic activities such as the leukocyte alkaline phosphatase, which are associated with late stages of myeloid differentiation [24,25]. Because heparanase RNA and protein were detected in relatively less differentiated AML cells, we suggest that some characteristics associated with early differentiation of myeloid cells (in our study, heparanase expression) also may be affected in CML. This may indicate that CML originates from a very early myeloid stem cell, prior to the differentiation-related activation of heparanase gene expression (Fig. 7).

No heparanase expression was detected in normal and aberrant circulating lymphoid cells, or in CD34⁺ progenitor cells (our unpublished data). Our data indicate that early hematopoietic progenitors, lymphoid cells, and most leukemic cell types do not depend on heparanase for transmigration from the bone marrow into the circulation. Therefore, trafficking of cells from the bone marrow into the circulation during normal and aberrant hematopoiesis probably is controlled by other cellular functions, such as integrins and the cytoskeleton, rather than being regulated by ECM-degrading enzymes. For example, transmigration of CML cells from the bone marrow into the circulation has been attributed to a loss of their normal cytoskeletal/integrin functions due to the activity of the BCR/*Abl* oncogene [26,27]. In line with this putative mechanism, we did not detect heparanase expression in most of the CML samples.

Cell surface heparanase was detected in only a few of the AML samples, although the same cells express both heparanase

nase RNA and activity. These results are in agreement with the immunofluorescent staining pattern of heparanase, which shows a predominantly cytoplasmic granular localization pattern in both normal and aberrant myeloid cells. Thus, heparanase is produced and stored within the cytoplasm of myeloid cells, with only very low amounts of the enzyme present on the cell surface. Apparently, the presence of a signal peptide at the N-terminus of the heparanase protein does not necessarily direct the molecule to the cell surface, but rather to its storage within the cytoplasm in a mechanism not yet defined.

Previous studies indicated that neutrophils are the major cell type expressing heparanase in the circulation [28]. Neutrophil heparanase was colocalized with gelatinase in tertiary granules [29,30]. The amino acid sequence of human heparanase contains a stretch of hydrophobic amino acids at the C-terminus, and complete solubilization of the enzyme required detergent extraction, which points to its possible association with the plasma membrane [13,14]. It was suggested that in highly malignant breast and bladder carcinoma cells, cytoplasmic enzymes such as cathepsin B are translocated to the cell surface [31,32]. However, only a small amount of heparanase was detected on the cell surface of normal and most aberrant hematopoietic cells. We rarely observed high levels of cell surface heparanase in AML leukemias. Therefore, it appears that in some malignancies, including breast and bladder carcinomas [31,32] and certain types of leukemias (as demonstrated in the present study), enzymes that normally are stored within cytoplasmic granules can be translocated to the cell surface.

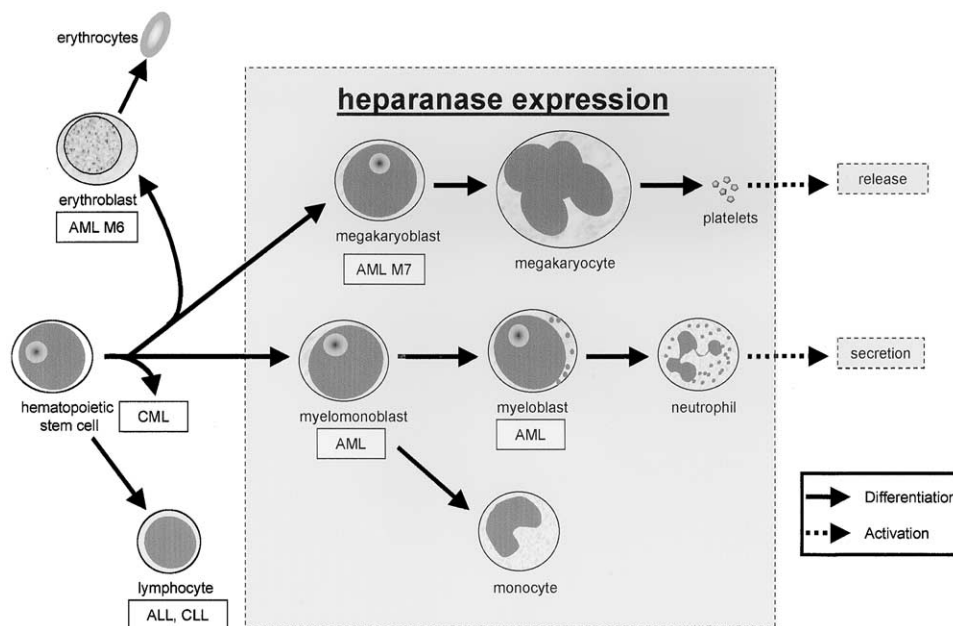


Figure 7. Heparanase expression in hematopoietic cells. Heparanase gene and protein are not expressed in early hematopoietic CD34⁺ progenitors. Apparently, there is a checkpoint for heparanase expression early in the monocyte/myeloid/megakaryocyte lineage. CML onset may take place in cells prior to this checkpoint, resulting in heparanase-negative CML progenies. Cellular activation may turn-on heparanase expression in nonexpressing cells (e.g., lymphocytes [9]) or induce its release from heparanase-expressing cells (e.g., neutrophils [28]).

We did not detect heparanase expression and activity in all CLL and most other types of leukemias. We propose that heparanase expression, both at the RNA and protein levels, may serve as an independent marker applied to support the identification of AML and to distinguish this class of leukemias from CML or leukemias of the lymphoid lineage. In addition, the lack of heparanase expression may help to identify circulating CML cells (chronic phase) independently of other common but rather problematic techniques (e.g., alkaline phosphatase staining [33]). Other enzymes (e.g., metalloproteinases) comprise multigene families with variable degrees of sequence homologies and structural similarities, making their usage in diagnosis difficult. In contrast, only one heparanase gene has been detected to date, enabling the development of efficient and reliable molecular diagnostic methodology.

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