High Heparanase Activity in Multiple Myeloma Is Associated with Elevated Microvessel Density

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ABSTRACT

Heparanase is an enzyme that cleaves heparan sulfate chains of proteoglycans, and its expression has been associated with increased growth, metastasis, and angiogenesis of some tumors. Because myeloma tumor cells express high levels of the syndecan-1 heparan sulfate proteoglycan and because these tumors grow as highly vascularized aggregates within the bone marrow, we analyzed the activity, expression, and function of heparanase in myeloma patients. Analysis of heparanase activity in the plasma isolated from bone marrow biopsies of 100 patients reveals 86 positive for heparanase activity and 14 negative. The bone marrow samples can be further divided into three categories of heparanase activity, high activity (42 patients), low activity (44 patients), and negative (14 patients). In contrast to the bone marrow plasma, levels of heparanase activity in peripheral blood plasma of 29 myeloma patients were either negative or low, suggesting that in multiple myeloma, heparanase functions in the local microenvironment of the bone marrow and its activity is not significantly elevated systemically. Immunohistochemistry reveals that patients with high levels of heparanase activity often have tumor cells with intense staining for the enzyme. Interestingly, a marked heterogeneity among tumor cells was noted, with clusters of highly stained cells surrounded by cells with weak or negative staining for heparanase. Analysis of microvessel density reveals a strikingly higher concentration of vessels in patients with high heparanase activity (78.96 vessels/mm²) as compared with patients negative for heparanase activity (25.03 vessels/mm²). When human myeloma cells transfected with the cDNA for heparanase are implanted in severe combined immunodeficient (SCID) mice, the resulting tumors exhibited a significantly higher microvessel density than tumors established with control transfectants, thus directly linking heparanase activity to the aggressive tumor phenotype (5–7). But syndecan-1 is also shed from the surface of myeloma cells, and this soluble form of syndecan-1 can be found in the serum of myeloma patients, in which it is an indicator of poor prognosis (8, 9). Shed syndecan-1 also binds to and accumulates within the bone marrow stroma of myeloma patients (10). Importantly, we have discovered that soluble syndecan-1 can promote the growth and metastasis of myeloma cells in vivo (11) which is consistent with the observation that soluble syndecan-1 is an indicator of poor prognosis. Although the mechanism of soluble syndecan-1 action is unknown, once bound to the marrow stroma, it may act to create reservoirs for factors that promote growth and angiogenesis (e.g., FGF-2).

It is becoming increasingly clear that heparan sulfate acts as a key regulator of cell behavior by fine-tuning the function of numerous proteins. Thus, mechanisms that regulate heparan sulfate structure and activity are of particular importance. One such mechanism is through the activity of heparanases, enzymes that cleave heparan sulfate chains. Recently a major heparanase was cloned from mammalian cells, and biochemical studies confirmed that it can degrade heparan sulfate proteoglycan (12–15). Cells synthesize a M₆ 65,000 pro-enzyme that undergoes proteolytic cleavage to produce a M₄ 50,000 active enzyme (14). Active heparanase cleaves the glycosidic bonds of heparan sulfate chains at only a few sites, producing fragments that are 10–20 sugar residues long (16). These soluble fragments of heparan sulfate are large enough to bind growth factors but are free from anchorage to cell surfaces or the extracellular matrix. In tumors, heparanase has been directly implicated in promoting invasiveness (14, 17, 18), angiogenesis (19, 20), and metastasis (12, 14, 21–23), and a recent study demonstrated that degradation of heparan sulfate can unveil cryptic fragments of heparan sulfate with unique biological activities that control tumor cell growth and metastasis (24).

We have discovered that a subpopulation of myeloma cells within the human bone marrow expresses heparanase as detected by immunohistochemistry. Heparanase enzymatic activity was detected in the bone marrow plasma of the majority of myeloma patients, but activity levels varied dramatically among patients. In addition, we find that high heparanase activity in myeloma correlates with altered gene expression that may promote an aggressive tumor phenotype, and that high heparanase activity correlates with high microvessel density. Moreover, when myeloma cells that overexpress active heparanase are implanted in severe combined immunodeficient (SCID) mice, they form tumors with higher microvessel density than their control transfected counterparts, thus directly linking heparanase activity to the increased microvessel density. Together, these studies indicate that heparanase likely plays an important role in regulating the growth and progression of myeloma.

INTRODUCTION

Multiple myeloma is a B-lymphoid malignancy characterized by tumor cell infiltration of the bone marrow, osteolytic lesions, and angiogenesis in the vicinity of the tumor cells (1, 2). Most myeloma cells express syndecan-1, a cell surface heparan sulfate proteoglycan that binds growth factors such as fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor to the cell surface through its heparan sulfate glycosaminoglycan chains (3, 4). Syndecan-1 on the cell surface promotes myeloma cell adhesion to type I collagen and to other cells and inhibits cell invasion, thus acting to oppose an aggressive tumor phenotype (5–7). But syndecan-1 is also shed from the surface of myeloma cells, and this soluble form of syndecan-1 can be found in the serum of myeloma patients, in which it is an indicator of poor prognosis (8, 9). Shed syndecan-1 also binds to and accumulates within the bone marrow stroma of myeloma patients (10). Importantly, we have discovered that soluble syndecan-1 can promote the growth and metastasis of myeloma cells in vivo (11) which is consistent with the observation that soluble syndecan-1 is an indicator of poor prognosis. Although the mechanism of soluble syndecan-1 action is unknown, once bound to the marrow stroma, it may act to create reservoirs for factors that promote growth and angiogenesis (e.g., FGF-2).

It is becoming increasingly clear that heparan sulfate acts as a key regulator of cell behavior by fine-tuning the function of numerous proteins. Thus, mechanisms that regulate heparan sulfate structure and activity are of particular importance. One such mechanism is through the activity of heparanases, enzymes that cleave heparan sulfate chains. Recently a major heparanase was cloned from mammalian cells, and biochemical studies confirmed that it can degrade heparan sulfate proteoglycan (12–15). Cells synthesize a M₆ 65,000 pro-enzyme that undergoes proteolytic cleavage to produce a M₄ 50,000 active enzyme (14). Active heparanase cleaves the glycosidic bonds of heparan sulfate chains at only a few sites, producing fragments that are 10–20 sugar residues long (16). These soluble fragments of heparan sulfate are large enough to bind growth factors but are free from anchorage to cell surfaces or the extracellular matrix. In tumors, heparanase has been directly implicated in promoting invasiveness (14, 17, 18), angiogenesis (19, 20), and metastasis (12, 14, 21–23), and a recent study demonstrated that degradation of heparan sulfate can unveil cryptic fragments of heparan sulfate with unique biological activities that control tumor cell growth and metastasis (24).

We have discovered that a subpopulation of myeloma cells within the human bone marrow expresses heparanase as detected by immunohistochemistry. Heparanase enzymatic activity was detected in the bone marrow plasma of the majority of myeloma patients, but activity levels varied dramatically among patients. In addition, we find that high heparanase activity in myeloma correlates with altered gene expression that may promote an aggressive tumor phenotype, and that high heparanase activity correlates with high microvessel density. Moreover, when myeloma cells that overexpress active heparanase are implanted in severe combined immunodeficient (SCID) mice, they form tumors with higher microvessel density than their control transfected counterparts, thus directly linking heparanase activity to the increased microvessel density. Together, these studies indicate that heparanase likely plays an important role in regulating the growth and progression of myeloma.

MATERIALS AND METHODS

Plasma from Bone Marrow Aspirates. Samples included the plasma from the bone marrow aspirates of 100 newly diagnosed multiple myeloma patients treated at the Myeloma Institute for Research and Therapy at our institution. Written and informed consent was obtained in keeping with institutional

Received 3/24/03; revised 10/3/03; accepted 10/16/03.

Grant support: NIH Grants CA55819 and CA68494.

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HEPARANASE IN MULTIPLE MYELOMA

policies. Bone marrow aspirates (10 ml) were treated with EDTA to prevent clotting and then were centrifuged at 900 rpm for 10 min at room temperature. Theuffy coat is visible after this treatment, and 2 ml of the topmost plasma, which is separate from the cells in thebuffy coat, was harvested. Bone marrow aspirate plasma was then snap-frozen in liquid nitrogen and stored at −80°C until analysis. Clinical parameters of the patients who provided bone marrow aspirate plasmas including β2 microglobulin, IgA, and IgG levels were determined by standard procedures in the clinical laboratory of the Department of Pathology (University of Arkansas for Medical Sciences, Little Rock, AR). Analysis of clinical variables was performed by Erik Rasmussen and Joth Jacobson of Cancer Research and Biostatistics (Seattle, WA) using the Pearson statistic.

Gene Expression Profile Analysis. Purification of myeloma plasma cells, purification of mRNA from these cells, and gene expression profile analysis from the patients evaluated in this study was performed as described previously using Affymetrix GeneChip HuGeneFL representing ~6800 genes (25). This analysis included the determination of syndecan-1 mRNA levels. Using the gene expression database established for these samples (25), we compared the gene expression profiles of purified myeloma plasma cells from the 18 patients with highest heparanase activities in the bone marrow aspirate plasma with the gene expression profiles of myeloma plasma cells from the 18 patients with the lowest heparanase activities. Comparisons between gene expression in these two groups were performed and evaluated by the significant analysis of microarrays (SAMS; Ref. 26).

Determination of Soluble Syndecan-1 Level in Bone Marrow Plasma. Levels of soluble syndecan-1 in patients’ bone aspirations were determined by ELISA using an EIl-pair kit from Diaclone (Cell Sciences, Inc., Norwood, MA). ELISA was performed on duplicate samples following the manufacturer’s instructions, and the absorbance at 450 nm was determined with an Auto-Reader II ELISA reader (Ortho Diagnostic Systems, Raritan, NJ). The standard curve was linear between 8 and 256 ng/ml, and all of the samples were diluted to concentrations within this range.

Peripheral Blood Plasma. Plasma was collected from 29 patients with newly diagnosed multiple myeloma. These patients were distinct from the 100 patients whose bone marrow aspirates were tested for heparanase activity. Blood was collected in sodium citrate buffer to prevent clotting, and the cells were separated from the plasma by standard procedures.

Immunohistochemistry. Sections were prepared from archival material of bone marrow biopsies taken from myeloma patients at the same time that the bone marrow aspirates were obtained. Bones were decalcified before embedding, and all fixed tissues were embedded in paraffin and sectioned. For immunohistochemical staining, the sections were warmed to 37°C for 12 h and 58°C for 20 min, deparaffinized with xylene, and then rehydrated through graded concentrations of ethanol and distilled water. Epitope retrieval was performed by heating the slides in alkaline phosphatase buffer solution (pH 6.0; Zymed). Slides were washed and incubated with 3% H2O2 to quench endogenous peroxidase activities and then were blocked with 5% nonfat dry milk in PBS. The slides were then stained with mouse monoclonal antibody directed against heparanase. This antibody to heparanase was generated against recombinant human heparanase and selected using an ELISA with recombinant heparanase (13, 27). Specificity of this monoclonal was confirmed by demonstrating that CAG human myeloma cells stain extensively with antibody after their transfection with the cDNA for human heparanase as compared with control-transfected cells that stain weakly with the antibody (not shown). A mouse monoclonal antibody directed against CD34 (Cell Marque, Austin, TX) was used to identify blood vessels. After staining with the primary antibody, slides were washed with PBS, stained with biotinylated goat antimouse IgG (Vector), and then with the Vector laboratories Vectastain Elite ABC kit (Burlington, CA). Reaction was accomplished using a 3,3′ diaminobenzidine substrate kit (Vector). Control stainings were performed with nonspecific hybridoma supernatant as the primary antibody.

Scoring for heparanase staining was determined in a blinded fashion by two different readers (Y. H., J. C., and Allison Theus, University of Arkansas, Little Rock, AR) for each immunohistochemistry sample. The readers assigned the samples scores of 0 for negative samples to 1+ for least intensely positive to 4+ for most intensely positive. Each section stained with antibody to heparanase was also compared with an adjacent section stained with an isotype-matched irrelevant antibody as a negative control. This level of staining was presumed to be negative and, therefore, 0. Samples previously identified as 4+ were used as positive controls to assure that the staining procedures were completed properly.

Heparanase Activity Assay. The heparanase activity assay used an immobilized [3H]heparan sulfate substrate and was performed as described previously (27). Purified recombinant heparanase (100 ng) was used as the positive control, and buffer (0.1 M sodium acetate, pH 5, 0.01% Triton X-100) was used as the negative control. Each sample was normalized to equal volume and tested in triplicate on at least two separate occasions.

Western Blot Analysis. Heparanase was enriched and separated from serum and plasma proteins by a conA “pull-down” procedure (13). Samples were prepared from bone marrow aspirate plasma or peripheral blood plasma by incubating 100 μl of either sample with 50 μl of Con A Sepharose beads (Pharmacia Biotech) overnight at 4°C. The beads were collected by centrifugation, and the supernatant was removed. The heparanase-bead complexes were washed twice in 0.5 M NaCl with 1 mM CaCl2 and MnCl2 in 20 mM Tris-HCl (pH 8) and then once in the same buffer adjusted to 50 mM Tris-HCl (pH 8). Heparanase was eluted by boiling the beads in 50 μl of SDS-PAGE sample buffer. Western blotting was performed as described previously (28) except that 4–20% gels (Invitrogen) were used. Heparanase was detected with an affinity-purified rabbit polyclonal antibody directed against recombinant human heparanase (13, 27) and a horseradish peroxidase-conjugated, donkey antirabbit IgG that does not cross-react IgG (Amersham). Specificity of the polyclonal antibody was confirmed by its strong immunoreactivity with M1, 50,000 and M6, 65,000 protein bands in CAG myeloma cells engineered to express heparanase and lower reactivity against the endogenous heparanase in control transfectants (Fig. 6). Immunoreactive bands were detected using a chemiluminescent system (ECL; Amersham Biosciences).

Analysis of Microvessel Density. Microvessel density was determined as described previously (29). Microvessels, visualized by CD34 (human) or CD31 (mouse model) immunohistochemical staining and distinguished from hematopoietic stem cells by the presence of a lumen, were counted in five nonoverlapping areas of tumor infiltration (1 mm2/area). The investigators, who were blind to the heparanase activity levels of the samples and the vessels were counted by at least two different individuals (Y. H. and Allison Theus), and the results of their findings were averaged. To determine the statistical significance of differences in observed microvessel densities, we performed the t test using two tailed, with Excel software to obtain Ps and SDs. Statistical significance was indicated by Ps ≤0.017 as determined by the Bonferroni correction for multiple comparisons.

Preparation of Heparanase-Expressing Multiple Myeloma Cells. Heparanase cDNA (HPSE1) was subcloned in the sense direction into pIRE2-EGFP (Clontech) vector, which allowed both the heparanase gene and the enhanced green fluorescent protein (EGFP) to be translated from a single bicistronic mRNA. The pIRE2-EGFP/HPSE1 construct was transfected into myeloma cells using Lipofectin reagent (Invitrogen) and Opti-MEM I (Life Technologies, Inc.) with 10 μg of DNA (pIRE2-EGFP vector only for Neo transfections or pIRE2-EGFP/HPSE1 construct for heparanase transfections) following the manufacturer’s instructions.

The CAG cells (2.0 × 105/transfection) were collected by centrifugation and were washed with 10 ml of RPMI medium. After washing, the cells were resuspended in 3 ml of the combined DNA/Lipofectin mixture, placed in a T-25 flask, and incubated for 5 h in a cell culture incubator at 37°C, 5% CO2 (2). At the end of this incubation 3 ml of RPMI with 10% fetal bovine serum were added, and the cells were placed back in the incubator overnight. Cells were collected by centrifugation and resuspended in fresh RPMI and 10% fetal bovine serum and were plated in a T-75 flask. Once cells reached 2.0 × 107 cells, they were sorted by green fluorescence using a flow cytometer. These cells were allowed to grow and were checked for green fluorescence after about a week. At least three sorts were performed to achieve greater than 60% cells exhibiting green fluorescence in the transfectants.

Expression of the HPSE1 protein was confirmed by lysing PBS-washed cells in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.2 mM PMSF, and 10 μg/ml leupeptin for 30 min on ice, spinning at 16,000 rpm at 4°C × 10 min, and retaining supernatants. Protein concentrations were determined using a BCA Protein kit (Fermentas). Western analysis was performed using 60 μg of protein/lane as described above.

Implantation of Myeloma Cells into SCID Mice. Seven-week-old female CB.17 SCID mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were housed and monitored in our animal facility. All of the
experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee. CAG cells (1 × 10⁶), transfected with either the Neo or heparanase-containing vector were injected s.c. into the left flank of the mice. Mice were sacrificed after 9 weeks, and microvessel density was assessed as described as above.

RESULTS

Heparanase Activity Is Detectable in the Bone Marrow. To determine whether active heparanase is present in the bone marrow, activity assays were performed on the plasma of bone marrow aspirates from 100 multiple myeloma patients. Cells were cleared from bone marrow aspirates by centrifugation, and the supernatants were tested for heparanase activity using an immobilized [³H]heparan sulfate substrate (27). Results from 18 samples representative of those with negative, low, and high heparanase activity are shown in Fig. 1, and a summary of results from the 100 samples is shown in Table 1.

Of the 100 samples, 86% were judged positive for heparanase because they released [³H]heparan sulfate at or above 10% of the level of the positive control, and fourteen samples were judged negative for heparanase because they had less than 10% of the activity of the positive control. Of the 86 positive samples, 44 were designated as having low heparanase activity because they had between 10 and 25% of the activity of 100 ng of recombinant heparanase, whereas 42 were designated as having high heparanase activity because they had greater than 25% of the activity of the positive control.

To assess the levels of heparanase protein and confirm its presence within the marrow, the marrow plasma samples were evaluated by Western blot analysis. A representative sample is shown in Fig. 1, inset. Heparanase was detected in all of the samples, although samples from some patients contained both the M₆ 65,000 and the M₇ 50,000 forms of the enzyme, whereas others contained only one detectable protein at either M₆ 65,000 or M₇ 50,000 (not shown).

In contrast to what was discovered in the marrow, peripheral blood plasma from a separate group of 29 patients were negative or were very low in heparanase activity (Fig. 2). However, the enzyme was detectable by Western blotting even in samples having no detectable heparanase activity (Fig. 2, inset). These enzyme activity results suggest that in myeloma, heparanase exerts its action primarily in the microenvironment of the bone marrow and not systematically, and that once in the circulation, the heparanase may be inactivated by serum factors.

Heparanase Is Expressed by a Subpopulation of Myeloma Cells. The heparanase activity detected in the marrow plasma could have been derived from several sources including tumor cells, bone marrow stroma, or other cells within the bone marrow. Staining with monoclonal antibody directed against heparanase revealed that heparanase was expressed by tumor cells within the bone marrow. In tumors that expressed high levels of heparanase, a striking heterogeneity was found. Groups of intensely labeled myeloma cells were found clustered together (Fig. 3A) and were often surrounded by tumor cells that were negative or that expressed low levels of heparanase. In contrast, tumors from some patients expressed low levels of heparanase, again with heterogeneity among the tumor cells (Fig. 3B). Overall, the level of immunostaining for heparanase correlated with the level of heparanase enzyme activity (as determined by the solid-phase assay, Table 1). For example, subpopulations of myeloma cells with strong staining for heparanase were generally detected in patients that had high heparanase activity, but strong immunostaining for heparanase was never observed in patients with low or negative heparanase activity. In all of the samples assessed, immunostaining for heparanase was not seen in the bone marrow stroma or on inflammatory cells within the marrow. However, as expected, staining for heparanase was apparent in megakaryocytes. Together, these studies indicate that the heparanase activity within the bone marrow plasma likely arises predominantly from the expression of heparanase by the myeloma tumor cells.

![Fig. 1](image1.png)

**Fig. 1.** Heparanase activity is present in the plasma from bone marrow aspirates of most myeloma patients. Heparanase activity in 18 bone marrow (BM) aspirates representative of the range of heparanase activities found in 100 samples. Heparanase activity is measured by release of [³H]heparan sulfate. Columns represent the mean of triplicate determinations ± SDs (bars). Values for recombinant heparanase (positive control) and buffer only (negative control) were 113 ± 123 and 168 ± 11, respectively. Inset, Western blot of bone marrow aspirate plasma from sample BM274 identifying the M₆ 65,000 and M₇ 50,000 forms of heparanase. kDa, molecular weight in thousands.

![Fig. 2](image2.png)

**Fig. 2.** Heparanase activity is absent or low in the circulation of myeloma patients. Heparanase activity in peripheral blood plasma from 16 myeloma patients. Columns with bars, the mean of triplicate determinations ± SDs for recombinant heparanase (positive control) and buffer only (negative control), 149 ± 46 and 20 ± 2, respectively. Inset, Western blot of peripheral blood plasma from sample SR602 identifies the M₆ 65,000 and M₇ 50,000 forms of heparanase. kDa, molecular weight in thousands; [³H]-HS, [³H]heparin sulfate.

**Table 1** Heparanase activity levels in the plasma from bone marrow aspirates of myeloma patients

<table>
<thead>
<tr>
<th>Activity Level</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative &lt;10%</td>
<td>14</td>
</tr>
<tr>
<td>Low 10–24%</td>
<td>44</td>
</tr>
<tr>
<td>High &gt;25%</td>
<td>42</td>
</tr>
</tbody>
</table>

- Negative activity is judged when heparanase activity is not detected.
- Low activity is judged as present when heparanase activity is detected but less than 10% of the activity of 100 ng of recombinant heparanase.
- High activity is judged as present when heparanase activity is greater than 25% of the activity of the positive control.
syndecan-1 mRNA, and soluble syndecan-1 in bone marrow aspirate plasmas were also evaluated. A Pearson correlation statistic measured the linear association between heparanase activity levels and these variables (Table 2). Only the level of syndecan-1 mRNA showed a statistically significant and positive correlation with heparanase activity levels (Table 2). In addition, heparanase activity did not correlate with the clinical stage of the myeloma as judged using the Durie-Salmon staging I, II, IIIA, and IIIB criteria (not shown). Thus, the variability among patients in heparanase activity within the bone marrow was not simply a measure of tumor burden.

**High Heparanase Activity Promotes Enhanced Microvessel Density in Myeloma Tumors.** Because heparanase is known to participate in promoting angiogenesis, myeloma tumors were assessed for microvessel density using antibodies to CD34 (Table 3). Analyses revealed that patients with high heparanase activity in their bone marrow plasma had tumors with a high mean microvessel density (78.96 ± 24.46 vessels/mm²). This microvessel density in the marrow of the high heparanase patients was significantly higher than that found in patients negative for heparanase activity (25.03 ± 10.48 vessels/mm²) as well as in those having low levels of activity (34.31 ± 19.97 vessels/mm²) in their marrow (Fig. 4; Table 3). As a confirmation of CD34 staining to assess angiogenesis, some tumors were also stained for factor VIII-related antigen. Results of these analyses were similar to those using anti-CD34 (not shown). The correlation of high heparanase activity with high microvessel density is consistent with heparanase acting to promote angiogenesis, thereby sustaining tumor growth and promoting tumor cell dissemination.

On the basis of these findings, we predicted that if heparanase levels in myeloma cells were increased, then there would also be an increase in the microvessel density in tumors of these cells relative to that of tumors of cells with lower heparanase. Thus, to determine whether heparanase expression is linked directly to the extent of microvessel density in myeloma tumors, CAG human myeloma cells were stably transfected with heparanase (CAGhpse) in the pIRES2-EGFP bicistronic plasmid or the empty vector control (CAGneo). CAGneo and CAGhpse transfectants were sorted based on the fluorescence of EGFP. Sorted cultures achieved 86% positive cells for CAGhpse and 87% positive cells for CAGhpse (Fig. 5, A and B). The activity assay revealed ~4-fold greater heparanase activity in the CAGhpse cells as compared with the CAGneo cells, which had an activity level similar to that of the negative control (Fig. 5C). Western

**Heparanase Activity Correlates with Syndecan-1 mRNA Levels.** Several continuous clinical parameters of the patient population used in this study can serve as indicators of tumor burden including levels of β2 microglobulin, IgA, and IgG; these were examined for possible correlations between very high or very low heparanase activities in the bone marrow aspirate plasmas. In addition, levels of syndecan-1 mRNA, and soluble syndecan-1 in bone marrow aspirate plasmas were also evaluated. A Pearson correlation statistic measured the linear association between heparanase activity levels and these variables (Table 2). Only the level of syndecan-1 mRNA showed a statistically significant and positive correlation with heparanase activity levels (Table 2). In addition, heparanase activity did not correlate with the clinical stage of the myeloma as judged using the Durie-Salmon staging I, II, IIIA, and IIIB criteria (not shown). Thus, the variability among patients in heparanase activity within the bone marrow was not simply a measure of tumor burden.

**Table 2 Heparanase activity correlates with syndecan-1 mRNA levels**

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>n</th>
<th>Pearson a</th>
<th>P b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta2 microglobulin</td>
<td>92</td>
<td>−0.005</td>
<td>0.96</td>
</tr>
<tr>
<td>IgA level</td>
<td>91</td>
<td>−0.189</td>
<td>0.07</td>
</tr>
<tr>
<td>IgG level</td>
<td>97</td>
<td>−0.063</td>
<td>0.54</td>
</tr>
<tr>
<td>Syn-1 mRNA</td>
<td>97</td>
<td>0.228</td>
<td>0.02</td>
</tr>
<tr>
<td>sSyn-1</td>
<td>98</td>
<td>0.176</td>
<td>0.08</td>
</tr>
</tbody>
</table>

a Pearson correlation statistic measures strength of linear association. It runs from −1 to 1 with 1 being perfect positive correlation and −1 perfect negative correlation.

b P, the general cutoff to determine statistical significance is a P < 0.05.

c sSyn-1, soluble syndecan-1 in marrow plasma.

**Table 3 Mean microvessel densities in bone marrow of multiple myeloma patients relative to heparanase activity level**

Microvessel densities were determined by counting CD34 positive cells that were associated with a lumen as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Heparanase activity</th>
<th>Negative (n = 10)</th>
<th>Low (n = 10)</th>
<th>High (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.03 (±10.48) Vessels/mm²</td>
<td>34.31 (±19.97) Vessels/mm²</td>
<td>78.96 (±24.46) Vessels/mm²</td>
<td></td>
</tr>
<tr>
<td>P = 0.206 (Neg-Low)</td>
<td>P = 0.0044 (Low-High)</td>
<td>P = 0.000042 (Neg-High)</td>
<td></td>
</tr>
</tbody>
</table>

Neg, negative.
... blot analyses of samples loaded to equal protein revealed high levels of heparanase in the CAG hpse transfectants as compared with the CAG neo cells (Fig. 5D). Both the M₆ 65,000 form and a high level of the M₆ 50,000 form of heparanase were readily detected in the CAG hpse extract, whereas a lower level of a single M₆ 50,000 band of endogenous heparanase was detected in the CAG neo extract (Fig. 5D). Thus, the increased heparanase activity in the CAG hpse transfectants was due to increased production of active heparanase protein by these cells.

To establish tumors, CAG hpse or CAG neo cells were implanted s.c. into the flanks of SCID mice. Three tumors of each cell type were harvested 9 weeks after implantation and were evaluated for microvessel density. A total of 30 microscopic fields of CAG hpse and CAG neo tumors were evaluated for microvessel density by two blinded investigators (Y. H. and Allison Theus) revealing a 35% increase in microvessel density when heparanase levels are elevated (mean of 112 vessels/mm² in the CAG hpse tumors and 83 vessels/mm² in CAG neo tumors; P = 0.0005). Representative photomicrographs are shown in Fig. 6. These results parallel those found in patients in whom elevated levels of heparanase correlated with elevated microvessel density (Table 3; Fig. 4). Thus, the studies with heparanase-transfected CAG myeloma cells support the notion that higher microvessel density in myeloma patients may be the direct result of elevated heparanase expression.

**Gene Expression Profiles Suggest High Heparanase Activity Is Associated with an Aggressive Myeloma Phenotype.** The finding that heparanase promotes enhanced microvessel density in myeloma tumors suggests that heparanase expression may be associated with an aggressive myeloma phenotype. To further examine this possibility, we turned to the gene microarray results generated from the mRNA of the purified myeloma cells (25). Cells harvested for these analyses were from the same bone marrow aspirates used to generate the...
The best SAM score was for regulated and down-regulated genes with the highest SAM scores are patients with high levels of heparanase activity as compared with cells and 12 genes significantly down-regulated in tumor cells from patients with high heparanase activity. A total of 6800 genes probed, we found 11 genes significantly up-regulated and 12 genes significantly down-regulated in tumor cells from patients with high levels of heparanase activity as compared with cells from patients with low heparanase activity. Those significantly up-regulated and down-regulated genes with the highest SAM scores are listed in Table 4. The best SAM score was for GADD45A, a growth arrest gene that is down-regulated in patients having high heparanase activity. Deletion of GADD45A leads to centrosome amplification and consequent abnormal mitosis and aneuploidy. The second best SAM score was another down-regulated gene that encodes a transcriptional regulator named transforming growth factor-β-stimulated clone 22 (TSC22). This protein has a leucine zipper structure, is translocated to the nucleus during apoptosis, and may act as a tumor suppressor gene by regulating apoptosis.

These results suggest that elevated levels of heparanase activity in tumors are associated with changes in gene expression that may, along with enhanced microvessel density, contribute to an aggressive myeloma phenotype.

**DISCUSSION**

This work provides the first evidence that high levels of heparanase activity can promote enhanced microvessel density in myeloma bone marrow. This is of particular importance because high microvessel density is associated with poor prognosis in myeloma. Our work demonstrates that heparanase is expressed by a subpopulation of multiple myeloma cells in the bone marrow and that high enzymatic

![Fig. 5. Heparanase levels in CAGhpse and CAGneo human myeloma cells. A, B, fluorescence-activated cell sorting of CAGhpse (A) and CAGneo (B) reveals high enhanced green fluorescent protein (EGFP) fluorescence in the transfectants (A and B, bold traces). C, heparanase activity in controls, buffer, and recombinant heparanase (rHPSE), and transfectants, CAGhpse cell extract (Neo), and CAGneo cell extract (HPSE). D, Western blot analysis of extracts of CAGhpse (HPSE) and CAGneo (Neo) using 60 μg of protein in each lane and a polyclonal antibody to heparanase as described in the “Materials and Methods.” kDa, molecular weights in thousands.

Fig. 6. Increased heparanase is associated with increased microvessel density. A, identification of vessels (stained brown) with antibody to CD31 in a tissue section of CAGhpse tumor. These cells have high heparanase activity (400 cpm [3H]heparan sulfate released or 80% of the activity of the positive control, see Fig. 5C). Blue, nuclei stained by hematoxylin. B, identification of vessels with antibody to CD31 in a sample of CAGneo tumor. These cells have low heparanase activity (100 cpm [3H]heparan sulfate released or 20% of the activity of the positive control, Fig. 5C).

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The gene expression profiles of myeloma plasma cells from the 18 patients with the highest heparanase activities were compared with that of the 18 patients with the lowest heparanase activities.
activity of heparanase in myeloma patient bone marrow aspirates correlates with high tumor microvessel density. Moreover, our in vivo studies reveal that elevation of heparanase activity in myeloma tumor cells promotes the generation of tumors having a significantly higher microvessel density than tumors established from control cells having low heparanase activity. These findings are consistent with studies indicating that heparanase stimulates angiogenesis (16, 19, 20, 35, 36) and supports the notion that heparanase contributes directly to an aggressive myeloma tumor phenotype. Importantly, the high levels of heparanase expression by myeloma cells as judged by immunohistochemistry correlate with high levels of heparanase activity in the marrow. Thus, it is likely that the myeloma cells themselves are producing the heparanase that contributes to increased angiogenesis. Also, the finding that high heparanase activity was present in 42% (42 of 100) of the samples tested indicates that this enzyme plays a role in a significant fraction of myeloma cases. This is in contrast to chronic lymphocytic leukemia and non-Hodgkin lymphomas that do not express detectable levels of heparanase (37). Comparison of gene expression profiles between patients with high and low heparanase activity also support the conclusion that high heparanase activity marks an aggressive tumor phenotype.

The finding that high heparanase activity in myeloma bone marrow tumors correlates with high microvessel density is consistent with the known role of this enzyme in promoting angiogenesis (19, 20). Moreover the studies using the mouse model, confirm that high heparanase activity promotes enhanced microvascular density. Increased microvessel density occurs even though, in the mouse model, the tumors are growing s.c. without the benefit of the bone marrow microenvironment. The difference in microenvironments may explain why heparanase levels correlate more directly with extent of angiogenesis in the bone marrow of human patients as compared with tumors grown s.c. in mice. In bone marrow of myeloma patients a 3-fold increase in heparanase activity in primary tumors correlates with a 3-fold increase in microvascular density. However, in the mouse model when transfected cell lines are implanted s.c., a 4-fold increase in heparanase activity correlates with a 35% increase in microvascular density in tumors. It will be interesting to more extensively examine the role of heparanase in metastasis and angiogenesis within the human bone by using the severe combined immunodeficient mouse with a living human bone implanted (SCID-hu) model for myeloma (11).

Our findings indicate that heparanase may have a central role in promoting angiogenesis needed for the sustained growth of multiple myeloma. First, cleavage of heparan sulfate within the extracellular matrix and particularly within the basement membrane may be required for the migration of metastasizing tumor cells and for remodeling of the vasculature during angiogenesis. Second, heparanase can directly promote angiogenesis by releasing heparin-binding angiogenic growth factors such as FGF-2 and vascular endothelial growth factor that are trapped within the extracellular matrix or on the cell surface (38). This idea is supported by the finding that heparanase increases the angiogenic response to tumors and that enhanced heparanase mRNA expression correlates with tumor vascularity (36, 39). Moreover, hepatocyte growth factor, FGF-2, and vascular endothelial growth factor are overexpressed in multiple myeloma cell lines and in the serum of multiple myeloma patients (40–42). Most myeloma cells express the heparan sulfate proteoglycan syndecan-1 (CD138; Ref. 10), that can act to concentrate these growth factors on the surface of myeloma cells or within the bone marrow stroma. Subsequent cleavage of heparan sulfate by heparanase would result in the liberation of the immobilized growth factors (5).

The finding that heparanase stimulates enhanced microvessel density in myeloma raises the question as to the source of these vessels. Because the bone marrow is highly vascularized, it is possible that the observed microvessels arise from buds formed from existing vessels (neoangiogenesis). However, de novo vessel growth arising from mesenchymal stem cells (vasculogenesis) is also a possibility given the accessibility of stem cells to the marrow compartment. Given the fact that the marrow represents a rich milieu of growth factors, vascularity and stem cells, it would not be surprising if the enhanced microvessel density seen with high levels of heparanase expression is due to both angiogenic and vasculogenic mechanisms. However, to date, there are no studies that indicate heparanase preferentially promotes either form of blood vessel growth.

All of the studies in the present work were performed on newly diagnosed myeloma patients at our center. Thus, we do not yet know whether heparanase activity correlates with clinical progression or long-term survival in myeloma. However, our finding that heparanase promotes angiogenesis in myeloma, coupled with reports that high microvessel density in the bone marrow is an indicator of poor prognosis in myeloma (33, 34, 43), suggests that inhibition of heparanase is a rational strategy for myeloma therapy. Candidate inhibitors include PI-88, a yeast-derived phosphomannopentose currently in clinical trials as a potential inhibitor of tumor progression (19). PI-88 was identified based on its ability to inhibit heparanase activity and metastasis of rat mammary adenocarcinoma cells to draining popliteal lymph nodes (19). Inhibition of heparanase with PI-88 was also effective in reducing blood-borne metastases (19). Two other heparanase inhibitors, laminarin sulfate and a phosphorothioate homopolymer of cytidine (SdC28), inhibit lung colonization after i.v. injection of melanoma and breast carcinoma cells (23).

The mechanisms by which heparanase facilitates cancer progression likely involves more than just remodeling extracellular matrix or releasing growth factors. There is evidence that the fragments of heparan sulfate released on heparan sulfate degradation maintain bioactivity and may in fact be more active than the native heparan sulfate chain from which they are derived. In a landmark study, it was discovered that the intact heparan sulfate on the ectodomain of shed syndecan-1 potently inhibits heparin-mediated FGF-2 mitogenicity because of the poorly sulfated domains in its heparan sulfate chains (44). However, subsequent degradation of these poorly sulfated regions by heparanase generates heparan sulfate fragments that markedly activate FGF-2 mitogenicity (44). Importantly, these “activating fragments” of heparan sulfate are present in wound fluids. Similarly, it was recently demonstrated that the action of bacterial heparinase I (which cleaves heparan sulfate in regions similar to those cleaved by human heparanase) yields biologically active fragments that promote growth of melanoma cells and their subsequent metastasis to the lungs (24). In contrast, the action of a different enzyme, bacterial heparinase III, which produces heparan sulfate fragments similar to those cleaved by human heparanase) yields biologically active fragments that promote growth of melanoma cells (23). Thus, encoded within intact heparan sulfate chains are cryptic structural elements that have the power to either positively or negatively impact the behavior of cancer cells, and the action of heparanase can liberate these cryptic fragments with resulting biological consequences. Thus, it will be important to determine the specific function of heparan sulfate fragments generated by the action of heparanase on human myeloma cells, and how these fragments modulate tumor cell behavior.

Lastly, in light of the fact that heparanase can generate biologically active fragments of heparan sulfate, it is reasonable to speculate that heparanase may also modulate the osteolysis that accompanies myeloma progression. Many of the factors that regulate bone turnover have the capacity to bind to heparan sulfate (e.g., heparanase growth factor, interleukin-8, interleukin-6, osteoprotegrin). Myeloma tumor cells express high levels of syndecan-1, and the shed syndecan-1 accumulates in the bone marrow (10). Thus, the myeloma marrow is replete with heparan sulfate that is strategically located to modulate growth factor activities in the bone compartment. Evidence from our
laboratory supports this hypothesis. For example, in a murine bone marrow culture system, medium from syndecan-1-expressing cells inhibits osteoclast formation within culvaria; and syndecan-1, or intact heparan sulfate chains from syndecan-1, inhibit osteoclastogenesis and promote osteoblastogenesis (46). Degradation of heparan sulfate chains by heparanase would likely alter these effects, similar to what occurs with FGF-2, which is inhibited by the intact heparan sulfate of syndecan-1 but is activated when the heparan sulfate is degraded by heparanase (44). Thus inhibitors of heparanase activity may have a dual beneficial effect in myeloma. In addition to reducing myeloma tumor growth, they may also suppress osteolysis, a major complication of this disease.

ACKNOWLEDGMENTS

We thank Erik Rasmussen and Joth Jacobson (Cancer Research and Biostatistics, Seattle, WA) for their statistical assistance correlating clinical parameters with heparanase activity, and Rudy Parrish (University of Arkansas for Medical Sciences, Division of Biometry) for help with statistical analysis of microvessel densities. We thank Josh Epstein and Richard C. Kurten (University of Arkansas for Medical Sciences, Little Rock, AR) for help with photomicroscopy.

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High Heparanase Activity in Multiple Myeloma Is Associated with Elevated Microvessel Density

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