Function of Heparanase in Prostate Tumorigenesis: Potential for Therapy

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Abstract Purpose: Heparanase is the predominant enzyme that cleaves heparan sulfate, the main polysaccharide in the extracellular matrix. Whereas the role of heparanase in sustaining the pathology of human cancer is well documented, its association with prostate carcinoma remains uncertain. Our research was undertaken to elucidate the significance of heparanase in prostate tumorigenesis and bone metastasis.

Experimental Design: We applied immunohistochemical analysis of tissue microarray, in vitro adhesion and invasion assays, as well as mouse models of intraosseous growth and spontaneous metastasis of prostate cancer, monitored by whole-body bioluminescent imaging. Electroporation-assisted administration of anti-heparanase small interfering RNA in vivo was applied as a therapeutic approach.

Results: We report a highly statistically significant (P < 0.0001) prevalence of heparanase overexpression in prostate carcinomas versus noncancerous tissue, as well as strong correlation between tumor grade and the extent of heparanase expression. We observed >5-fold increase in the metastatic potential of PC-3 prostate carcinoma cells engineered to overexpress heparanase. Notably, overexpression of a secreted form of the enzyme also led to a dramatic increase in intraosseous prostate tumor growth. Local in vivo silencing of heparanase resulted in a 4-fold inhibition of prostate tumor growth, representing the first successful application of anticancer therapy based on heparanase small interfering RNA and validating the potential of heparanase as a target for prostate cancer treatment.

Conclusions: Heparanase directly contributes to prostate tumor growth in bone and its ability to metastasize to distant organs. Thus, anti-heparanase strategy may become an important modality in the treatment of prostate cancer patients, particularly those with bone metastases.

Prostate cancer is responsible for more gender-specific cancer-related deaths in men than any other cancer. The outcome of the disease is mainly determined by metastases, with the most frequent involvement of bone (90%), lungs (46%), and, to a less extent, other soft viscera (1, 2). Although curable if detected when confined to the prostate gland, the attempts of treatment have met with a limited success once the disease has spread outside the prostate. Improvement in prostate cancer patient survival requires the identification of new therapeutic targets based on a detailed understanding of the biological mechanisms involved in metastatic dissemination and tumor growth in the bone and other target organs.

Heparanase is the predominant mammalian enzyme degrading heparan sulfate, the main polysaccharide component of the extracellular matrix (ECM; refs. 3, 4). Heparan sulfate is a ubiquitous macromolecule associated with the cell surface and ECM of a wide range of tissues and organs. In the ECM, particularly the subendothelial and epithelial basement membranes, heparan sulfate binds to and assembles structural ECM proteins, thus contributing to ECM integrity and barrier function. In bone tissue, heparan sulfate is localized in the intercellular space between osteoblasts and osteoclasts, as well as on the surface of cells undergoing osteoblastic differentiation (5, 6). Enzymatic cleavage of heparan sulfate by heparanase leads to disassembly of the ECM and is therefore involved in biological phenomena associated with tissue remodeling and cell movement, including cancer progression, metastasis, and angiogenesis (3, 4, 7–10). Preferential expression of heparanase was shown in tissue specimens derived from several carcinoma types (11–14). A possible link between heparanase activity and prostate carcinoma progression was previously discussed (15–18), either supporting (17) or opposing (18) the enzyme contribution. Our research was undertaken to elucidate...
the biological significance of heparanase in prostate tumorigenesis. Applying prostate carcinoma tissue microarray analysis, as well as a number of in vitro and in vivo models, we show that heparanase is preferentially expressed in prostate cancer, its expression levels are in strong positive correlation with tumor grade, and its activities directly contribute to prostate tumor take and growth in bone, as well as subsequent metastasis to distant organs. Local administration of anti-heparanase small interfering RNA (siRNA) resulted in a marked regression of transplantable human prostate tumor growing in mouse host. To our knowledge, this study represents the first successful application of anticancer therapy based on in vivo delivery of heparanase siRNA. Taken together, our data suggest that, apart from its potential application as a diagnostic tool, heparanase is a promising therapeutic target in prostate carcinoma patients, particularly those bearing bone metastases.

Materials and Methods

Tissue arrays, immunostaining, and statistics. Low-density LandMark prostate tissue microarrays were purchased from Ambion and contained 122 formalin-fixed, paraffin-embedded nonselected specimens of prostate carcinoma tissues (33 tumors scored with Gleason grade 3, 46 tumors with grade 4, and 43 with grade 5), as well as 49 specimens of benign prostate, six of which harbored a presumptive precursor lesions, called prostatic intraepithelial neoplasia. Bone biopsy specimens from patients with metastatic prostate cancer were provided by the Department of Pathology, Hadassah Medical Center. The use of these specimens was approved by the Ethics Committee of the Hadassah Medical Center. Immunodetection of heparanase was done as described (3) with minor modifications. Briefly, sections of the tissue array blocks were deparaffinized and rehydrated. Tissue was then incubated in 3% H2O2, denatured by boiling (3 min) in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0), and blocked with 10% goat serum in PBS. Sections were incubated by using the Zymed AEC substrate kit (Zymed Laboratories) for 10 min, followed by counter staining with Mayer's hematoxylin. Slides were photographed using fluorescent microscopy.

Visualized with a Zeiss axioscope microscope and manually scored by three groups (7). The antibody was diluted 1:100 in 10% goat serum in PBS. Control slides were incubated with 10% goat serum alone. Color was developed by ethidium bromide staining. The experiments were repeated thrice, and counted in quadruplicates with a Coulter counter (Coulter Electronics Ltd.).

Proliferation assay. Cells were seeded in complete growth medium (5 × 10^4 cells per well of 24-well plate). Starting on day 1 of the experiment and everyday for 7 days thereafter, the cells were harvested and counted in quadruplicates with a Coulter counter (Coulter Electronics Ltd.).

Heparanase activity. Tumor cell lysates prepared from 1 × 10^6 cells by three cycles of freeze and thaw in reaction buffer (20 mmol/L phosphate-citrate buffer (pH 6.6) containing 1 mmol/L DTT, 1 mmol/L CaCl2, and 50 mmol/L NaCl) were incubated (5 h, 37°C) with Na3SO4-labeled ECM, prepared as described (21). The incubation medium was centrifuged at 14,000 rpm 4°C for 15 min, and the supernatant containing 35S-labeled heparan sulfate degradation fragments was analyzed by gel filtration on a Sepharose CL-6B column (0.9 × 30 cm). Fractions (0.2 mL) were eluted with PBS, and the amount of radioactivity in each fraction was counted in a β-scintillation counter (21). Each experiment was done at least thrice, and the variation in elution positions (Kav values) did not exceed 15%. Reaction buffer with or without recbinomiant human heparanase (1 ng/mL) was routinely used as a positive and negative control, respectively.

Invasion through Matrigel. Tumor cells (3 × 10^5 cells in 1 mL DMEM containing 0.1% bovine serum albumin) were assayed in triplicates for Matrigel invasion at 37°C in a humidified incubator (95% air, 5% CO2) for 6 h by using Boyden blind-well chemotaxis chambers (NeuroProbe) and polycarbonate filters (13 mm in diameter, 8 μm pore size; Costar Scientific) coated with Matrigel, as described (22, 23). Medium conditioned by NIH 3T3 fibroblasts was used as a chemoattractant and placed in the lower compartment of the Boyden chamber. For negative control, serum-free DMEM containing 0.1% bovine serum albumin was placed in the lower compartment of the Boyden chamber instead of chemoattractant. After 6 h, the filters were removed, and the cells on the lower surface of the filter were stained with Diff-Quik kit (American Scientific Products). The total cells from six microscopic fields at magnification of 100×, selected at random, was counted for each filter.

Cell adhesion. PC-3-Hpa, PC-3-Sp, and PC-3-Vo cells (7 × 10^4) were resuspended in DMEM containing 10% FCS, fluorescently labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbozycyanine perchlorate (Invitrogen) for 30 min in room temperature (0.75 mg/mL) and incubated (30 min, 37°C, pH 7.2) with confluent bovine aortic endothelial cell monolayers. After incubation, cells were washed (3×) with serum-free medium, and the remaining firmly attached cells were fixed in methanol. Ten arbitrarily chosen fields were viewed and photographed using fluorescent microscopy.

RNA isolation and reverse transcription–PCR. RNA was isolated with TRI-Reagent (Medical Research Council) according to the manufacturer’s instructions. After reverse transcription of 1 μg total RNA by oligo(dT) priming, cDNA was amplified using Taq DNA polymerase (Promega). The primers used were H1PPU-355, 5'TTCGATCCAGAAAG-GAACTCAAC-3' and H1PP-229, 5'GTAGTGTGATCGATTAGCAGT-3' for heparanase; GAPDHU: 5'CCACCATGCAAAATTCATTGCAGCA-3' and GAPDHL: 5'TCTAGACCGCGAGTCTGACCC-3' for GAPDH; L19UL5: ATGGCCACTCTTGTCCAAAC-3' and L19UL: 5'GCGTCTTCTG-GCTTCTTCT-3' for L19. The PCR conditions were an initial denaturation at 94°C for 2 min, denaturation at 94°C for 15 s, annealing for 45 s at 58°C, and extension for 1 min at 72°C. Aliquots (15 μL) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The experiments were repeated thrice, yielding similar results.

Intratibial injection of PC-3 prostate carcinoma cells. Six-week-old male severe combined immunodeficient (SCID) mice were divided into three groups (n = 7 per group), anesthetized and injected into the left tibia with 0.5 × 10^6 of either PC-3-Vo, PC-3-Hpa or PC-3-Sp cells resuspended in 0.02 mL PBS. Tumor size was monitored for 46 days. As intrasosseous tumor growth could not be measured directly, at least
using the Acid Phosphatase Leukocyte kit (Sigma). Osteoclasts were identified by staining for tartrate-resistant acid buffer formalin for 24 h, decalcified in 14% EDTA solution for 2 weeks at room temperature and proceeded for histologic analysis. tibias and lungs were removed. The tibias were fixed in 10% and left representative images of heparanase immunostaining in prostatic tissue microarray staining intensity (white columns, no staining = 0; gray columns, medium staining = 1; black columns, high staining = 2). $\chi^2$ analysis confirmed highly statistically significant ($P < 0.0001$) 3-fold prevalence of heparanase overexpression in prostate carcinomas versus noncancerous prostatic tissue. In addition, specimens were graded according to Gleason score (27), revealing a strong positive correlation between tumor grade and the extent of heparanase expression (Spearman rank correlation coefficient = 0.5215; Table 1). Inset, heparanase immunostaining of bone biopsy specimens from patients with metastatic prostate cancer.

during early stages of the experiment, leg circumference, which reflects tumor burden, was measured by a caliper twice a week, 0.5 cm below the knee in two dimensions. Tumor volume ($V$) was calculated by the equation $V = 4/3 \pi r^2$. On day 46 of the experiment, mice were sacrificed, and left tibias and lungs were removed. The tibias were fixed in 10% buffered formalin for 24 h, decalcified in 14% EDTA solution for 2 weeks at room temperature and proceeded for histologic analysis. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase Leukocyte kit (Sigma) as described (24). The number of osteoclasts per millimeter of bone surface was determined by visual inspection of digital images of stained tissue sections at a magnification $\times 200$: (a) in bone portion at least 3 mm away from the tumor mass and (b) at the tumor-bone interface. Lungs were fixed in Bouin's solution and scored for the number of metastatic nodules on the lung surface under a dissecting microscope (23, 25). All experiments were done in accordance with the guidelines of the Animal Care and Use Committee of the Hebrew University. The experiments were repeated twice, and identical results were obtained.

In vivo imaging. The cooled-charged-coupled device camera model LN/CCD-1300EB, equipped with ST-133 controller and a 50-mm Nikon lens (Roper Scientific, Princeton Instrument) and supported with comparable software, was used for light detection as described (26). In all experiments, animals were anesthetized before light detection. The exposure conditions (including exposure time, distance of lens from the object, and time after injection of luciferin) were kept identical. Ten minutes before monitoring light emission, the animals were injected i.p. with Beetle luciferin (Promega Corp.) in PBS at 126 mg/kg body weight. Animals were placed in a dark box, supplemented with a controlled light to take pictures of the background gray-scale image, and then exposed to the cooled-charged-coupled device to generate a pseudocolor image that represents light intensity. For colocalization of the bioluminescent emission on the animal body, gray-scale and pseudocolor images were merged by using the appropriate imaging software.

In vivo administration of heparanase siRNA to DU145 prostate carcinoma. Six-week-old male SCID mice were injected intradermally with DU145 cells (1.6 $\times$ 10$^6$ cells per mouse). Two weeks later, when palpable tumors of 2 to 3 mm in diameter were detected, mice were divided into three groups at eight mice per group. DU145 tumors were electrooporated with either heparanase siRNA vectors or empty vector pSUPER or remained untreated. For in vivo electroporation, mice were anesthetized and plasmid DNA was intradermally injected with a 0.3-ml syringe and 30-gauge needle into the tumor (15 $\mu$g per tumor in 20 $\mu$L PBS). To keep variability to a minimum, the same skilled operator did all injections. A 30-s time interval lapsed between injection and initiation of electroporation. The in vivo electroporation system (Genetronics Inc.) consisted of a square wave pulse generator (ECM 830) and a caliper electrode applied topically. The caliper electrode (model 384; BTX/Harvard Apparatus) consisted of two 1-cm$^2$ brass plate electrodes. Electroporation was done by squeezing the tumor between two plates and applying six pulses of 75 V with a pulse length of 20 ms and interval of 1 s and polarity reversal after three pulses.

### Results

Heparanase overexpression in clinical samples of prostate carcinoma. Applying immunohistochemical staining of commercially available tissue microarrays with anti-heparanase antibody, we examined heparanase expression in 171 specimens of neoplastic and nonneoplastic prostate tissues. Scoring of heparanase protein levels in the specimens was done by an expert pathologist (scores: 0, no staining; 1, low heparanase

### Table 1. Frequency and extent of heparanase expression in nonmalignant and malignant prostate tissue samples

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal</th>
<th>Prostatic intraepithelial neoplasia</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Grade 5</th>
</tr>
</thead>
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<tr>
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<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
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<tr>
<td>N</td>
<td>31</td>
<td>11</td>
<td>1</td>
<td>6</td>
<td>0</td>
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<tr>
<td>% per condition</td>
<td>72.1</td>
<td>25.6</td>
<td>2.3</td>
<td>100</td>
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staining; 2, high heparanase staining). Both the frequency and intensity of heparanase staining were significantly enhanced in prostate carcinomas compared with benign lesions or normal prostate tissue (Fig. 1A). Heparanase protein was easily detected in 86% of prostate carcinoma specimens, whereas among nonmalignant prostate tissue samples, only 24% expressed detectable levels of heparanase (χ² test, \( P < 0.0001 \)). We also found a strong positive correlation between Gleason grade (27) and the level of heparanase expression in the tumor tissue (Spearman correlation coefficient = 0.5215; Table 1).

In a separate set of experiments, strong heparanase immunostaining was observed in bone biopsy specimens from patients with metastatic prostate cancer (Fig. 1B).

**Effect of heparanase overexpression on PC3 prostate carcinoma cell adhesion and invasion.** The observed preferential expression of heparanase in prostate carcinoma tissue led us to examine the possible mechanistic involvement of heparanase in prostatic tumor progression. For this purpose, we used PC3 prostate carcinoma cell line, which expresses relatively low levels of endogenous heparanase (Supplementary Fig. S1) and, upon intraosseous injection in vivo, produces osteolytic bone tumors that do not metastasize to distant organs (28). This model is, therefore, suitable for studying the effects of heparanase on prostate carcinoma–induced bone turnover, destruction, and metastasis. PC3 cells were stably transfected with empty pCDNA3 expressing vector (Vo), vector encoding for human heparanase (Hpa), or vector encoding for a secreted form of heparanase (Sp; ref. 20). Upon selection, >100 stable transfected clones were pooled (to avoid possible effects of insertional mutagenesis) and the cells were examined for heparanase enzymatic activity in vitro measured by incubation (16 h, 37°C) of cell lysates with a metabolically sulfate-labeled ECM. Sulfate-labeled degradation products released into the incubation medium were subjected to gel filtration on Sepharose 6B columns (3, 21). We have previously shown that labeled fragments eluted in fractions 15 to 30 (peak II) are degradation products of heparan sulfate, as they were (a) 5-fold to 6-fold smaller than intact heparan sulfate side chains, (b)

![Fig. 2](attachment:image.png)

**Fig. 2.** Effect of transfection with pCDNA3-Hpa and pCDNA3-Sp vectors on heparanase activity and PC-3 cell invasion. A and B, heparanase enzymatic activity. Lysates (A) or conditioned medium (B) of PC-3 cells stably transfected with pCDNA3-Hpa, pCDNA3-Sp, or pCDNA3-Vo were incubated with \(^{35}\)S-labeled ECM, as described in Materials and Methods. Sulfate-labeled degradation fragments released into the incubation medium were analyzed by gel filtration. Each experiment was done thrice, and the variation in elution positions (K\(_{av}\) values) did not exceed 15%. C, adhesion of PC3 cells, stably transfected with pCDNA3-Hpa, pCDNA3-Sp, or pCDNA3-Vo vectors to bovine aortic endothelial cells, was measured as described in Materials and Methods and quantitated in 10 random microscopic fields (100×). Columns, mean of three independent experiments; bars, SD. D, Matrigel invasion. PC-3 cells stably transfected with pCDNA3-Hpa, pCDNA3-Sp, or pCDNA3-Vo vectors were tested for in vitro invasiveness through Matrigel. Briefly, the cells were incubated (3 × 10⁵ cells/mL) on top of Matrigel-coated filters for 6 h, and the membranes were then fixed and stained, as described in Materials and Methods. Cell migration was quantified in six random microscopic fields (100×) of triplicate filters. Columns, mean of three independent experiments; bars, SD.
resistant to further digestion with papain and chondroitinase disulfide-avidin complex method, and (c) susceptible to deamination by nitrous acid (21). In contrast, sulfate-labeled material released from ECM eluted in fractions 3 to 15 (peak 1) is produced by proteolytic enzymes degrading the proteoglycan core protein and residing in the ECM and cell lysates (21). As expected, increased heparanase enzymatic activity was detected in lysates prepared from both PC3-Hpa and PC3-Sp cells compared with PC3-Vo cells (Fig. 2A). In accordance with the observed difference in enzymatic activity, elevated levels of heparanase mRNA were observed in PC3-Hpa and PC3-Sp cells compared with a very low level in the PC3-Vo cells (Fig. 2A, inset). Heparanase activity was detected in medium conditioned by PC3-Sp, but not PC3-Hpa or PC3-Vo, transfectants (Fig. 2B), validating secretion of heparanase by PC3-Sp cells. Secretion and cell surface localization of heparanase in PC3-Sp cells was also confirmed by their increased adhesion to endothelial cells (29) compared with the adhesive ability of PC3-Hpa cells, which was similar to that of PC3-Vo cells (Fig. 2C). Interestingly, expression of either secreted or nonsecreted forms of heparanase conferred a similar increase in the ability of PC3-Sp and PC3-Hpa cells (respectively) to invade through Matrigel (a reconstituted basement membrane preparation; Fig. 2D).

Effect of heparanase on intraosseous PC3 prostate carcinoma growth and osteoclastogenesis. PC3-Vo, PC3-Hpa, and PC3-Sp cells were injected into the marrow cavity of the tibia of male SCID mice to evaluate the effect of heparanase on intraosseous prostate tumor growth. On day 22 after injection, PC3-Sp cells formed palpable tumors whereas no grossly observable tumors were detected in mice injected with control PC3-Vo or PC3-Hpa cells (Fig. 3A). In fact, both PC3-Vo and PC3-Hpa cells formed tumors of detectable size only by day 32 of the experiment. The statistically significant difference between PC3-Sp and either PC3-Vo or PC3-Hpa tumor size persisted throughout the duration of the experiment (at day 42, the calculated P values were 0.00461 and 0.02592, respectively; Fig. 3A). On day 23 postinjection, six randomly selected mice from each group were sacrificed and their tibia were removed and processed for histology. Histologic examination of PC3-Sp bone tumors revealed that the bone tissue was almost entirely replaced by a tumor that consisted of prostate carcinoma cells, occasionally admixed with bone tissue remnants (Fig. 3A, inset, left and B, left). In contrast, in bones injected with either PC3-Vo or PC3-Hpa cells, tumor masses of limited size were found within the bone, the shape of the bone remained unchanged, and extensive bone destruction was noted, and proper organization of the bone tissue was maintained (Fig. 3A, inset, right and B, middle and right). To ensure that the observed effects were not a result of a difference in the proliferative capacity of PC3-Vo, PC3-Hpa, and PC3-Sp cells, we did proliferation assay and found no statistically significant difference in proliferation rates of the three cell types (not shown). To test whether heparanase acts in this system by activating osteoclastogenesis, sections of the tumor-bearing bones were processed to identify TRAP-positive osteoclasts. In tibia injected with PC3-Sp cells (and to a lesser extent, with PC3-Hpa cells), but not PC3-Vo cells, numerous TRAP-positive osteoclasts were identified in a region at least 3 mm away from the tumor mass, as well as in the neighboring femur (Fig. 3C). When the number of osteoclasts was determined in areas of direct contact between the bone and the tumor tissue, no difference was found between PC3-Vo-
cells, activates osteoclastogenesis in the bone portion neighboring the initial metastatic lesion.

**Effect of heparanase on metastatic ability of PC-3 prostate carcinoma cells.** We examined the effect of heparanase on spontaneous metastatic dissemination of PC3 prostate carcinoma growing in bone. We injected PC3-Vo, PC3-Hpa, or PC3-Sp cells, genetically engineered to express the luciferase gene (LUC), into the tibia of SCID mice and used the whole-body bioluminescent LUC imaging to monitor prostate tumor progression in vivo. Once LUC bioluminescence was detected in the lungs of PC3-Hpa–injected (Fig. 4A), but not of PC-Vo–injected (not shown), animals (day 46 of the experiment), the mice of all three groups were euthanized and their lungs were stained with Bouin’s solution and evaluated for the number of surface metastatic colonies. As shown in Fig. 4B and C, the lungs of mice injected with either PC3-Hpa or PC3-Sp cells were massively colonized by prostate carcinoma cells versus no or very few metastatic nodules detected in the lungs of PC3-Vo–injected mice.

Altogether, these results show the ability of heparanase to accelerate prostate carcinoma progression and metastasis, emphasizing the particular importance of the secreted enzyme in enhancing bone-residing tumor growth.

**Effect of heparanase gene silencing on DU154 prostate carcinoma growth in vivo.** We next evaluated the therapeutic potential of heparanase targeting in prostate cancer treatment. For this purpose, we used the siRNA approach to silence the heparanase gene in DU145 human prostate carcinoma cell line, which expresses high levels of the endogenous enzyme (Supplementary Fig. S1). We have used the previously described pSi2 and pSH1 vectors, encoding for siRNA species Si2 and SH1 and capable of effective and specific silencing of the mouse (23, 25) and human (25) heparanase gene, respectively. To deliver the pSi2 and pSH1 vectors into DU145 tumors growing in a mouse host, we applied an electroporation technique, previously used for administration of expression vectors in vivo (25, 26). To show that this technique ensures actual delivery of the electroporated DNA and its lasting expression in vivo, we first electroporated the tumors produced by DU145 cells in SCID mice with SV40-LUC construct, encoding for the luciferase gene under the constitutive SV40 promoter, and visualized the expression of luciferase in the tumors on day 3 postelectroporation (Fig. 5C; ref. 25). In the subsequent experiments, 24 SCID mice were injected with DU145 cells, as described in Materials and Methods. Upon development of palpable tumors (~2-3 mm in diameter), mice were divided into three groups at eight mice per group. The tumors in the experimental group were electroporated with a mixture of the siRNA-expressing vectors pSH1 and pSi2 to target both the tumor-derived (human) and host-derived (mouse) heparanase gene, respectively, tumors in the control group were electroporated with empty vector pSUPER, and animals of the third group remained untreated (the tumors in this group served as a control for possible inhibitory effect of the electroporation procedure itself on tumor growth). Electroporations were done on days 14 and 21 of the experiment. Tumor growth was monitored for 28 days. Then, mice were sacrificed and the tumors were photographed (Fig. 5B), weighed (Fig. 5A), and processed for immunohistology (Fig. 5D). As shown in Fig. 5, treatment with anti-heparanase siRNA resulted in a significant inhibition of DU145 tumor growth; weight of tumors electroporated with the siRNA vectors decreased significantly compared to the control groups.

![Fig. 4. Overexpression of heparanase increases pulmonary metastasis in SCID mice. PC3-Vo, PC3-Hpa, and PC3-Sp cells, stably cotransfected with LUC expressing vector, were injected into the right tibia of SCID mice. A, at 46 d postinjection, when the presence of lung metastases was detected by real-time in vivo bioluminescence imaging in mice injected with either PC3-Hpa or PC3-Sp cells, but not PC3-Vo cells, all the mice were euthanized and their lungs were fixed and examined for the number of carcinoma colonies on the lung surface. B, gross appearance of lungs of mice injected with PC3-Vo (top), PC3-Hpa (middle), or PC3-Sp (bottom) cells. C, columns, represent the mean number of colonies per lung (n = 5 mice); bars, SE. A statistically significant difference in the number of colonies per lung was observed between PC3-Vo–injected and either PC3-Sp (P = 0.019)–injected, or PC3-Hpa (P = 0.0415)–injected mice.](www.aacjournals.org)
was >4-fold lower compared with tumors that were left untreated (Student’s test, \( P = 0.020 \)) or electroporated with control pSUPER vector (Student’s test, \( P = 0.016 \)).

To test the local expression of heparanase in siRNA-treated tumors and to ensure that electroporation of the siRNA-expressing plasmids resulted in heparanase silencing throughout the \textit{in vivo} experiment, we compared heparanase immunostaining in tissue sections of the untreated DU145 tumors versus tumors electroporated with empty pSUPER vector or with a mixture of the siRNA-expressing plasmids (pSi2/pSH1) on day 28 of the experiment. Intense heparanase staining was observed in both untreated or pSUPER-electroporated tumors (Fig. 5D, left and middle) versus a very weak staining in pSi2/pSH1 electroporated tumors (Fig. 5D, right). In the latter group, decreased staining intensity was evident in both mouse-derived (i.e., epidermis) and human-derived (i.e., tumor) cells (Fig. 5D, right), consistent with the administration of siRNA species targeting the mouse and human heparanase.

**Discussion**

The role of heparanase in sustaining the pathology of cancer is being extensively studied during the last decade and supported by numerous reports describing overexpression of...
the enzyme in malignant tumors, including those of the gastric tract (14), bladder (11), breast (12), colon (13), and pancreas (30). Moreover, elevated levels of the enzyme have been found in body fluids of cancer patients (31–34), reflecting secreted nature of heparanase protein.

The relationship between heparanase and prostate carcinoma progression remains less unequivocal and was disputed in recent publications, favoring (17) and opposing (18) the involvement of the enzyme in prostate cancer. Here, using tissue microarray technology, we revealed a highly statistically significant ($P < 0.0001$) 3-fold prevalence of heparanase overexpression in prostate carcinomas versus noncancerous prostatic tissue, as well as a strong positive correlation between tumor grade and the level of heparanase expression. Although further retrospective clinical studies involving higher numbers of clinical samples are needed to define the precise diagnostic and, possibly, prognostic value of heparanase analysis in prostate cancer, our findings imply that heparanase may become an important molecular marker in clinical decision-making process for prostate carcinoma patients. Moreover, our results indicate that heparanase may also be applied as a target for intervention. Applying heparanase gene overexpression and silencing in animal models of prostate carcinoma, we have shown the important role of heparanase in prostate tumor progression. Of particular interest is the function of heparanase in accelerating bone colonization and secondary bone-to-lung metastasis of prostate carcinoma.

Bone homing of prostate tumors involves complex interactions between osteoclasts, osteoblasts, ECM, and cancer cells (35). Although bone metastases of prostate cancer are often osteoblastic, biochemical and histologic studies clearly show that both bone resorption and formation occurs during bone colonization by prostate carcinoma cells (36–39). Moreover, it was suggested that osteoblastic metastases are formed in trabecular bone at sites of previous osteoclastic resorption and that such resorption is required for subsequent formation of osteoblastic metastases (reviewed in ref. 35). Bone resorption seems to be an integral part of the hypothesized vicious cycle through which prostate tumor cells stimulate bone turnover, and bone turnover stimulates further tumor progression. Tumor cells secrete factors that promote the activity of osteoclasts, a unique cell type derived from hematopoietic stem cells and expresses a large number of highly specific enzymes involved in bone breakdown. As the bone matrix is destroyed, it releases growth factors and other stimulatory substances capable of enhancing growth of prostate cancer cells that have colonized the bone (35). In the present study, we found that bone-residing prostate carcinoma cells express high levels of heparanase protein (Fig. 1C). Moreover, we showed that overexpression of secreted heparanase confers an increased osteolytic response and accelerated growth of prostate carcinoma cells growing in mouse tibia (Fig. 3). Increased number of osteoclasts per millimeter of bone tissue was detected in the tibia portion distant from the tumor in mice injected with PC3-Sp cells, but not with control PC3-Vo cells (Fig. 3C). Interestingly, in subcutaneous rather than intraosseous setting of breast carcinoma models, overexpression of heparanase, even in the absence of secretion, was sufficient to accelerate the growth of breast tumors (40, 41). These findings, together with the previously reported ability of heparanase to facilitate recruitment of osteoclasts (41, 42), suggest that heparanase secretion by prostate carcinoma cells augments progression of the bone tumor by activating osteoclastogenesis and bone resorption in the vicinity of the initial metastatic lesion, thus providing both space and growth-promoting bioactive molecules, which further accelerate bone colonization by the prostate carcinoma.

Promotion of cell adhesiveness by secreted heparanase may represent additional explanation for the observed difference between PC3-Sp and PC3-Hpa intraosseous tumor growth. Cells overexpressing a secreted form of the heparanase protein exhibit increased adhesion potential (Fig. 2C; ref. 43), which in turn is known to activate numerous biological responses (i.e., cell proliferation, survival, signaling) that may contribute to the enhanced intraosseous growth of PC3-Sp tumor.

The intratibial injection model used in this study resembles the formation of bone cortex lesions by bone marrow–residing carcinoma cells, enabling to show the role of heparanase (particularly the secreted enzyme) in this process. However, the limitation of this system is that it does not provide information on the earlier events in bone colonization process, including arrival of blood-born carcinoma cells and formation of bone marrow metastases. Bone marrow is closely implicated in the spread of tumor cells to the skeleton, as exemplified by clinical observations that bone metastatic lesions most frequently occur at sites where bone marrow and vascularity are prominent. The contribution of heparanase to dissemination of carcinoma cells via blood circulation is well established (3, 23). Thus, we speculate that heparanase contributes to the ability of carcinoma cells to arrive and establish metastases in the bone marrow, in addition to its role in bone cortex colonization showed by the intratibial injection model. In support of this notion is the observation that heparanase overexpression also conferred an increased ability of prostate tumor cells to metastasize to the lungs. Unlike bone tumor growth, which was preferentially augmented by the secreted form of the enzyme, metastatic potential was augmented in prostate carcinoma cells expressing either the secreted or nonsecreted species of heparanase. These in vivo observations are in agreement with data obtained in applying the in vitro Matrigel invasion assay (Fig. 2D), representing an important step of the metastatic process (i.e., basement membrane penetration) in which PC3-Hpa and PC3-Sp cells exhibited a similar ability to invade Matrigel.

Worth mentioning, none of the cell types exhibited noticeable bone metastases upon orthotopic injection into the mouse prostate (not shown), suggesting that in prostate carcinoma bone metastasis, heparanase activity is relevant mostly for the late stages of the metastatic cascade (i.e., tumor take and growth in the bone tissue), which are better represented by the intratibial injection model (Fig. 3).

Altogether, our results suggest that in prostate carcinoma patients, anti-heparanase therapies, currently under extensive development (44–46), may suppress metastatic dissemination and progression of bone homing tumors.

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