

Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma

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The heparan sulfate (HS) proteoglycan, syndecan-1, plays a major role in multiple myeloma (MM) by concentrating heparin-binding growth factors on the surface of MM cells (MMCs). Using Affymetrix microarrays and real-time reverse transcriptase–polymerase chain reaction (RT-PCR), we show that the gene encoding heparanase (HPSE), an enzyme that cleaves HS chains, is expressed by 11 of 19 myeloma cell lines (HMCLs). In *HPSE*^{pos} HMCLs, syndecan-1 gene expression and production of soluble syndecan-1, unlike expression of membrane syndecan-1, were significantly increased.

Knockdown of *HPSE* by siRNA resulted in a decrease of syndecan-1 gene expression and soluble syndecan-1 production without affecting membrane syndecan-1 expression. Thus, *HPSE* influences expression and shedding of syndecan-1. Contrary to HMCLs, *HPSE* is expressed in only 4 of 39 primary MMC samples, whereas it is expressed in 36 of 39 bone marrow (BM) microenvironment samples. In the latter, *HPSE* is expressed at a median level in polymorphonuclear cells and T cells; it is highly expressed in monocytes and osteoclasts. Affymetrix data were validated at the protein level,

both on HMCLs and patient samples. We report for the first time that a gene's expression mainly in the BM environment (ie, *HPSE*) is associated with a shorter event-free survival of patients with newly diagnosed myeloma treated with high-dose chemotherapy and stem cell transplantation. Our study suggests that clinical inhibitors of *HPSE* could be beneficial for patients with MM. (Blood. 2007;109:4914-4923)

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Introduction

Multiple myeloma (MM) is a B-cell neoplasia characterized by the growth of a clone of malignant plasma cells (PCs) in the bone marrow. Syndecan-1, a transmembrane heparan sulfate proteoglycan (HSPG), is expressed by both normal and malignant PCs and is now widely used to identify and purify these cells.^{1,2} We have previously shown that among the 10 known HSPGs, syndecan-1 is the main one present on the surface of PCs.³ Syndecan-1 binds to extracellular matrix proteins and to growth factors that have a HS-binding domain, thus promoting their activity.⁴ In particular, it has been shown that syndecan-1 mediates hepatocyte growth factor (HGF) binding and promotes c-met signaling in myeloma cells (MMCs).⁵ We and others have shown that syndecan-1 is necessary for the MMC growth activity of HGF and epidermal growth factor (EGF) family ligands that have a HS-binding domain.^{3,5-7} Syndecan-1 concentrates high amounts of growth factors at the cell surface and then likely facilitates the activation of their receptors. The ectodomain of syndecan-1 can be cleaved from the MMC surface into a soluble form by metalloproteinases that remain to be identified in MMCs.^{8,9} Soluble syndecan-1 is present at high levels in the serum of patients with MM and is an indicator of poor prognosis.^{8,10,11} It also accumulates in the bone marrow microenvironment where it might sequester heparin-binding growth factors in the proximity of the tumor.¹² Accordingly, it has been shown that

soluble syndecan-1, which retains biologically active HS chains, can promote tumor growth in a myeloma severe combined immunodeficiency–hu mouse model.¹³

The function of HSPGs is regulated by extracellular enzymes that modulate the structure of HS chains. One such enzyme is heparanase (HPSE), encoded by only one gene in mammalian cells.^{14,15} HPSE is an endoglucuronidase that cleaves HS chains at only a few sites, resulting in HS fragments of 10 to 20 sugar units long.^{14,16} These fragments are long enough to bind growth factors and can be more biologically active than the native HS chains.¹⁷ *HPSE* is overexpressed in numerous tumors where it increases the angiogenic and metastatic potential of tumor cells.¹⁸ In patients with MM, *HPSE* activity could be found in bone marrow aspirates.¹⁹ *HPSE* increases microvessel density¹⁹ and promotes growth and metastasis of MMCs to bone in vivo.²⁰

Here, we show for the first time that *HPSE* is involved in the regulation of syndecan-1 gene expression and controls the production of soluble syndecan-1 in myeloma cell lines. In patients with newly diagnosed MM, *HPSE* is expressed mainly by cell fractions of the bone marrow microenvironment, especially monocytes and osteoclasts, but weakly by MMCs. High *HPSE* expression in bone marrow of patients with MM correlates with a shorter event-free survival (EFS) and overall survival (OAS).

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Materials and methods

Cell samples

Whole bone marrow (WBM) samples and purified MMCs were obtained from 39 patients with MM at diagnosis (presenting at the University Hospitals of Heidelberg, Germany, and Montpellier, France) after written informed consent was given in accordance with the Declaration of Helsinki. These patients were treated with high-dose chemotherapy (HDC) and autologous stem cell transplantation (ASCT). The study was approved by the ethics boards of both universities. According to the Durie-Salmon classification, 8 patients were in stage IA, 6 in stage IIA, 22 in stage IIIA, and 3 in stage IIIB. Four patients had IgA κ MM, 3 IgA λ MM, 19 IgG κ MM, 7 IgG λ MM, 4 Bence-Jones κ MM, 1 Bence-Jones λ MM, and 1 nonsecreting MM. MMCs were purified with anti-CD138 magnetic cell sorting microbeads (Miltenyi-Biotec, Paris, France). Normal WBM samples were obtained from healthy donors after informed consent was given. For 7 additional patients with newly diagnosed MM, BM environment (ENV) cells were obtained by removing myeloma cells with CD138 Miltenyi microbeads (< 1% plasma cells). For 5 other patients with newly diagnosed MM, BM T cells, monocytes, and polymorphonuclear cells were purified as described previously.²¹ Osteoclasts and BM stromal cells (BMSCs) were generated *in vitro* as described.²¹ Immature dendritic cells (DCs) were generated from leukapheresis products of patients with MM, as previously described.²² Briefly, 8×10^6 G-CSF-mobilized leukapheresis cells were plated in 2 mL X-VIVO15 medium (BioWittaker, Walkersville, MD) per well in 6-well flat-bottomed plates (Nunc, Roskilde, Denmark). Nonadherent cells were discarded by gentle rinsing after a 2-hour incubation at 37°C in 5% CO₂. Adherent cells were cultured in X-VIVO15 medium with 2% human albumin, 100 ng/mL GM-CSF (LEUKINE; Berlex, Montville, NJ) and 25 ng/mL IL-4 (Cellgenix, Freiburg, Germany) for 5 days. Mature DCs were obtained by a further 24-hour maturation with GM-CSF, IL-4, and TNF α . Human IL-6-dependant XG myeloma cell lines (HMCLs) were obtained in our laboratory.^{23,24} They were routinely maintained in RPMI1640, 10% fetal calf serum, and 2 ng/mL IL-6 (Abcys, Paris, France). U266, SKMM, OPM2, LP1, and RPMI8226 HMCLs were purchased from ATTC (Rockville, MD).

Microarray hybridization

RNA was extracted with the RNeasy Kit (Qiagen, Hilden, Germany), the SV-total RNA extraction kit (Promega, Mannheim, Germany), and Trizol (Invitrogen, Karlsruhe, Germany) in accordance with the manufacturer's instructions. Biotinylated complementary RNA (cRNA) was amplified with double *in vitro* transcription, according to the Affymetrix small sample labeling protocol (Affymetrix, Santa Clara, CA). The biotinylated cRNA was fragmented and hybridized to the human genome U133 set (for HMCLs) or U133 Plus 2.0 (for patient samples) microarrays according to the manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GCOS software (Affymetrix).

Real-time reverse transcriptase-polymerase chain reaction

RNA was extracted using the RNeasy Kit (Qiagen). We generated cDNA from 100 ng total RNA using Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France). For real-time reverse transcriptase-polymerase chain reaction (RT-PCR), we used Assay-on-Demand primers and probes and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions. Gene expression was measured using the ABI Prism 7000 Sequence Detection System. For comparative analysis of gene expression, data were obtained by using the $\Delta\Delta C_T$ method derived from a mathematical approach previously described. For each sample, the C_T value for the gene of interest was determined and normalized to its respective C_T value for β_2 -microglobulin (B2M) ($\Delta C_T = C_T - C_{T\text{B2M}}$) or GAPDH ($\Delta C_T = C_T - C_{T\text{GAPDH}}$) and compared with a cell type used as a positive control. The formula used was $1/2^{\Delta\Delta C_T \text{ sample} - \Delta C_T \text{ control cell line}}$. C_t values were collected during the log phase of the cycle. The results were expressed as the relative mRNA levels to control cell sample mRNA.

Western blot

Cells were lysed in 10 mM Tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM NaPPi, 1% Triton X-100, 5 μ M ZnCl₂, 100 μ M Na₃VO₄, 1 mM DTT, 20 mM β -glycerophosphate, 20 mM PNPP, 20 μ g/mL aprotinin, 2.5 μ g/mL leupeptin, 0.5 mM PMSF, 0.5 mM benzamide, 5 μ g/mL pepstatin, and 50 nM okadaic acid. Lysates were resolved on 12% sodium dodecyl sulfate-polyacrylamide by gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Schleicher and Schuell, Kassel, Germany). Membranes were blocked for 2 hours at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1% TBS-T, 5% nonfat milk, and then immunoblotted with a rabbit polyclonal antiheparanase antibody (InSight Biopharmaceuticals, Rehovot, Israel) at a 1:1000 dilution. As a control for protein loading, we used a mouse monoclonal anti- β -actin antibody (Sigma, St Louis, MO). The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies by an enhanced chemiluminescence detection system.

HPSE activity assay

HPSE activity in HMCLs was measured with a commercial assay based on the measurement of HPSE-induced degradation of biotinylated-HS (b-HS) fragments (Takara Bio, Kyoto, Japan). Briefly, 10×10^6 cells were collected and suspended in 1 mL extraction buffer. After cell debris removal by centrifugation, the supernatant was incubated with biotinylated-HS (b-HS) for 45 minutes at 37°C. The mixture was then incubated on a FGF-coated plate and undegraded b-HS was detected by HRP-streptavidin. This b-HS fragment is specifically designed so that it cannot bind to FGF when being degraded by HPSE, thus resulting in a lower HRP signal. Correspondence between absorbance at 450 nm and HPSE activity was determined by comparison to a standard curve established using unlabeled heparan sulfate as a standard substitute.

siRNA experiments

MMCs were transfected with 1 nM SMARTpool HPSE siRNA or 1 nM nontargeting siRNA (siGLO) (Dharmacon, Lafayette, CO). For transfections, Amaxa nucleofection technology was used (Amaxa, Cologne, Germany). U266 myeloma cells were resuspended in the nucleofector T solution, available as part of the Amaxa cell optimization kit, and were electroporated using the T-001 protocol. Briefly, 4×10^6 cells were divided into aliquots of 100 μ L with 15 μ g siRNA and were transferred to a cuvette and nucleofected with the Amaxa Nucleofector device. Cells were immediately transferred into wells containing 37°C prewarmed culture medium in 24-well plates. All experiments were performed in duplicate. At days 1 to 2 after electroporation, (1) the gene expression level of HPSE, syndecan-1, B2M, and GAPDH were analyzed by real-time PCR; (2) membrane syndecan-1 expression levels at the cell surface were analyzed with a FACS-Calibur fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA), using PE-conjugated anti-CD138 (Beckman Coulter, Marseilles, France); (3) cells were counted and viability was assessed using annexin V-FITC (Boehringer, Mannheim, Germany); (4) soluble syndecan-1 was measured by enzyme-linked immunosorbent assay (ELISA) from cell line culture supernatants.

Production of soluble syndecan-1 by myeloma cell lines

HMCLs in the exponential growth phase were cultured for 3 days. The cell count, the density of membrane syndecan-1, and the concentration of soluble syndecan-1 were measured every day for 3 days. Soluble syndecan-1 was measured using an ELISA kit with a detection level of 8 ng/mL (Dialone, Besançon, France).

Immunohistochemistry

HPSE expression was assessed by immunohistochemistry on bone marrow biopsies from 20 patients with newly diagnosed myeloma, after written informed consent was given. Biopsies were fixed in Bouin fixative, decalcified, and routinely processed for paraffin embedding. Sections of

Bouin-fixed, paraffin-embedded HMCLs (XG-2 and XG-7) were used as positive and negative controls, respectively. Tissue sections were deparaffined in xylene and rehydrated through graded concentrations of ethanol and distilled water. Epitope retrieval was performed by incubating the slides for 40 minutes in 10 mM EDTA buffer solution (Euromedex, Souffelweyheim, France), pH 7.2, at 99.8°C, in a double boiler. The slides were then incubated for 30 minutes in a blocking solution containing 10% goat serum (Dako Cytomation S.A.S., Trappes, France). Slides were washed, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol followed by 15-minute incubation in CAS Block (Zymed Laboratories, San Francisco, CA). The slides were stained with a rabbit polyclonal anti-HPSE antibody (InSight Biopharmaceutical) at a concentration of 4.5 µg/mL. All stainings were performed with the NEXES Ventana Medical Systems automaton using the iVIEW DAB Detection Kit (Ventana Medical Systems, Illkirch, France) with goat antirabbit secondary Abs and diaminobenzidine as chromogen, according to the manufacturer's instructions. The immunohistochemical reaction was counterstained with hematoxylin. Heparanase staining was determined by 2 pathologists (P.R. and Thérèse Rousset, CHU, Montpellier, France). Each section stained with anti-HPSE was compared with an adjacent section stained with irrelevant rabbit serum as a negative control.

Images were visualized using an Olympus BX-51 microscope (Olympus, Tokyo, Japan) equipped with one of the following: a UplanApo 40×/0.85 NA air objective (Figure 6, except panel Bvi) or a UplanApo 10×/0.40 NA air objective (Figure 6Bvi). All images were taken at room temperature using an Olympus CMA-D3 camera (Tokyo, Japan) and were processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

Statistical analysis

Gene Expression Profiles were analyzed with our bioinformatics platform (RAGE, remote analysis of microarray gene expression; <http://rage.montp.inserm.fr>) designed by T. Reme (INSERM U475, Montpellier, France) or SAM (Significance Analysis of Microarrays) software. SAM analysis was applied to 22 530 probesets with at least 3 presences in the 19 HMCLs samples and a variation coefficient of 20 or greater. We performed a 2-class comparison between *HPSE^{pos}* and *HPSE^{neg}* HMCLs, with 1000 permutations and a false discovery rate of 7.7%. Statistical comparisons were made with the nonparametric

Mann-Whitney test or the Student *t* test for pairs. The statistical significance of differences in event-free survival between groups of patients was estimated by the log-rank test. An event was defined as relapse or death (for EFS) or as death (for OAS). The survival curves were plotted using the Kaplan-Meier method.

Results

HPSE expression in myeloma cell lines

Expression of *HPSE* was evaluated with Affymetrix U133 set microarrays on 19 HMCLs. Eleven of 19 HMCLs displayed a "present" call for the *HPSE* probeset (*HPSE^{pos}*) with a median expression level of 90 (range, 48-448; Figure 1A). The other 8 cell lines displayed an "absent" call (*HPSE^{neg}*) with a low expression level (median, 6; range, 2-28) (Figure 1A). The call (present or absent) is determined by Affymetrix GCOS software and indicates whether a gene is reliably expressed.²⁵ The data were validated by real-time RT-PCR ($r = 0.87$, $P < .01$; Figure 1B). HPSE expression at the protein level was evaluated by Western blot analysis. HPSE was detected in 3 of 3 HMCLs with a present Affymetrix detection call (U266, XG-2, and XG-20) but not in those with an absent Affymetrix call (XG-1 and XG-7; Figure 1C). The 50-kDa MW active form of the enzyme was predominant, but the 65-kDa MW proenzyme could also be detected (Figure 1C). Furthermore, HMCLs were tested for HPSE activity using an assay based on the measurement of HPSE-induced degradation of biotinylated-HS fragments (b-HS). In this assay, undegraded b-HS is bound to an FGF-coated plate, and bound b-HS is detected by HRP-streptavidin. The b-HS fragment is specifically designed so that it does not bind to FGF when degraded by HPSE, thus resulting in a lower HRP signal. HPSE activity was high in the *HPSE^{pos}* XG-2 and XG-20 HMCLs (0.6 U/mL and 0.82 U/mL, respectively). It was intermediate in U266 (0.14 U/mL) and below the

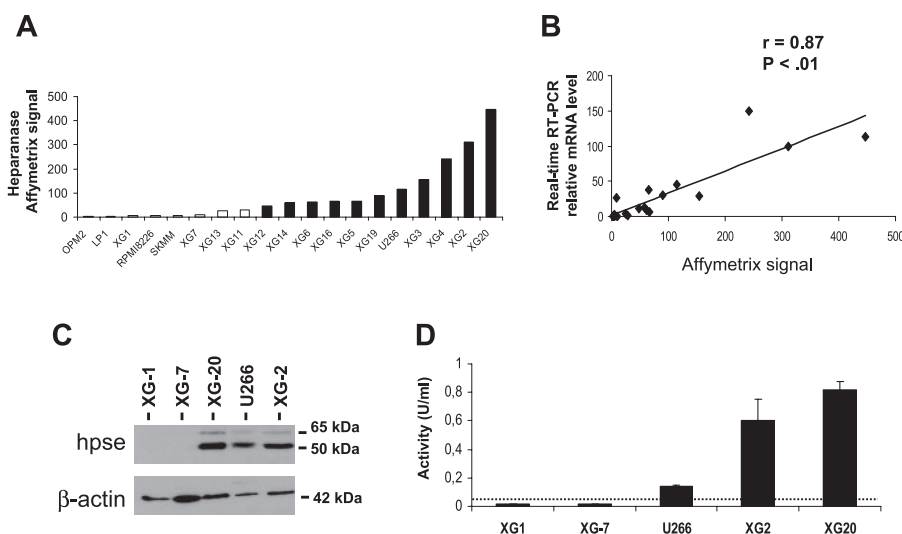


Figure 1. HPSE expression and activity are heterogeneous in HMCLs. (A) Expression of *HPSE* in 19 HMCLs was determined using Affymetrix U133 set microarrays. □ or ■ indicates that the Affymetrix call is absent or present, respectively. (B) Correlation between Affymetrix and real-time RT-PCR *HPSE* expression data. For real-time RT-PCR, *HPSE* expression in each sample was normalized to that of *GAPDH*, and the XG-2 cell line was used as a reference with the arbitrary value of 100. The correlation line is indicated in the graph. (C) HMCLs were lysed, and the lysates were separated on a 12% SDS-PAGE and analyzed by Western blot with a polyclonal anti-HPSE antibody. Both the 65-kDa MW proenzyme and the 50-kDa MW active form were identified. β-Actin was used as a loading control. Results are of 1 experiment representative of 3. kDa indicates molecular weight in thousands. (D) HPSE activity was determined using an ELISA-type detection assay. HMCL lysates were incubated with b-HS and then only undegraded b-HS could bind an FGF-coated ELISA plate. Bound b-HS was detected with HRP-streptavidin followed by a colorimetric assay. HPSE activity corresponding to absorbance at 450 nm was determined by comparison with a standard curve as described in "Materials and methods." One unit is defined as the activity that can degrade 0.063 ng b-HS when reacted at pH 5.8 at 37°C for 1 minute. The detection limit (dotted line) was 0.05 U/mL. Results are of 1 experiment representative of 3. Data are means ± SD of HPSE activity determined on triplicates.

detection limit (0.05 U/mL) in the *HPSE^{neg}* XG-1 and XG-7 HMCLs (Figure 1D). Thus, HPSE activity correlated with HPSE expression in HMCLs.

Gene expression profile associated with *HPSE* expression in myeloma cell lines

Because the *HPSE* signal expression in HMCLs is either present or absent, these HMCLs are of interest to study the biologic function of *HPSE* in MM. We compared the gene expression profile of the 11 *HPSE^{pos}* HMCLs with those 8 *HPSE^{neg}* HMCLs in a supervised analysis performed with the SAM software using 1000 permutations. Only probesets that were present in more than 3 samples and had at least a 2-fold change in expression were retained in the analysis. This resulted in a list of 41 probesets that were all overexpressed in *HPSE^{pos}* HMCLs, with a false discovery rate (FDR) of 7.7%. These 41 probesets interrogated 33 genes and 3 expressed sequence tags (ESTs), which are listed in Table 1. Interestingly, syndecan-1 was among those 41 genes.

Correlation between soluble syndecan-1 production and *HPSE* gene expression in HMCL

As shown in Figure 2A syndecan-1 gene was significantly overexpressed 4.3-fold in *HPSE^{pos}* HMCLs (median = 1320) compared with *HPSE^{neg}* HMCLs (median = 304). Because syndecan-1 is expressed as a membrane protein that is cleaved by metalloproteinases, we quantified membrane expression and soluble syndecan-1 production in HMCLs (Figure 2B-2C). Using FACS analysis, no significant difference in membrane syndecan-1 expression was found between *HPSE^{pos}* HMCLs (median MFI = 656) and *HPSE^{neg}* HMCLs (median MFI = 799) (Figure 2B). The rate of soluble syndecan-1 production in culture supernatant of HMCLs was measured during the exponential growth phase, between day 2 and day 3 of culture. Indeed, soluble syndecan-1 accumulated in culture medium in parallel with cell count increase, as shown for XG-2 and U266 in Figure 2D. Soluble syndecan-1 production was below the detection level (< 8 ng/mL) in 6 of 8 *HPSE^{neg}*

Table 1. SAM-defined overexpressed genes in HMCLs expressing *HPSE*

Probeset	Gene name	Gene description	SAM score
219403_s_at	<i>HPSE</i>	Heparanase	1.82
222881_at	<i>HPSE</i>	Heparanase	1.82
230241_at	<i>IFRG15</i>	Interferon responsive gene 15	1.82
219253_at	<i>FAM11B</i>	Family with sequence similarity 11, member B	1.78
227697_at	<i>SOCS3</i>	Suppressor of cytokine signaling 3	1.78
212037_at	<i>PNN</i>	Pinin, desmosome-associated protein	1.73
HUMISGF3A_MA_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.73
204908_s_at	<i>BCL3</i>	B-cell CLL/lymphoma 3	1.65
221876_at	<i>DKFZp762P2</i>	Hypothetical protein DKFZp762P2111	1.65
218935_at	<i>EHD3</i>	EH-domain containing 3	1.65
201316_at	<i>PSMA2</i>	Proteasome (prosome macropain) subunit, alpha type 2	1.65
HUMISGF3A_3_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.65
212269_s_at	<i>MCM3AP</i>	Minichromosome maintenance deficient 3-associated protein	1.61
201286_at	<i>SDC1</i>	Syndecan-1	1.61
238418_at	<i>SLC35B4</i>	Solute carrier family 35, member B4	1.61
209969_s_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.61
230917_at	—	—	1.57
227749_at	—	—	1.57
202086_at	<i>MX1</i>	Myxovirus resistance 1, interferon-inducible protein p78 (mouse)	1.57
237942_at	<i>SNRK</i>	SNF related kinase	1.57
HUMISGF3A_3_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.57
237299_at	—	—	1.53
221764_at	<i>C19ORF22</i>	Chromosome 19 open reading frame 22	1.53
217925_s_at	<i>C6ORF106</i>	Chromosome 6 open reading frame 106	1.53
201082_s_at	<i>DCTN1</i>	Dynactin 1 (p150, glued homolog, <i>Drosophila</i>)	1.53
206662_at	<i>GLRX</i>	Glutaredoxin (thioltransferase)	1.53
227713_at	<i>KATNAL1</i>	Katanin p60 subunit A-like 1	1.53
225752_at	<i>NIPA1</i>	Nonimprinted in Prader-Willi/Angelman syndrome 1	1.53
201964_at	<i>ALS4</i>	Amyotrophic lateral sclerosis 4; juvenile	1.49
207522_s_at	<i>ATP2A3</i>	ATPase, Ca ⁺⁺ transporting, ubiquitous	1.49
236831_at	<i>C3orf6</i>	Chromosome 3 open reading frame 6	1.49
239629_at	<i>CFLAR</i>	CASP8 and FADD-like apoptosis regulator	1.49
225097_at	<i>HIPK2</i>	Homeodomain interacting protein kinase 2	1.49
211824_x_at	<i>NALP1</i>	NACHT, leucine rich repeat and PYD containing 1	1.49
213241_at	<i>PLXNC1</i>	Plexin C1	1.49
226782_at	<i>SLC25A30</i>	Solute carrier family 25, member 30	1.49
235035_at	<i>SLC35E1</i>	Solute carrier family 35, member E1	1.49
212926_at	<i>SMC5L1</i>	SMC5 structural maintenance of chromosomes 5-like 1 (yeast)	1.49
HUMISGF3A_MA_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.49
200887_s_at	<i>STAT1</i>	Signal transducer and activator of transcription 1, 91 kDa	1.49
209197_at	<i>SYT11</i>	Synaptotagmin XI	1.49

These data are the 41 probesets overexpressed at least 2-fold in the 11 *HPSE^{pos}* HMCLs compared with the 8 *HPSE^{neg}* HMCLs (SAM analysis with a FDR of 7.7%). Genes are ordered based on the SAM score.

— indicates Expressed Sequence Tags (EST).

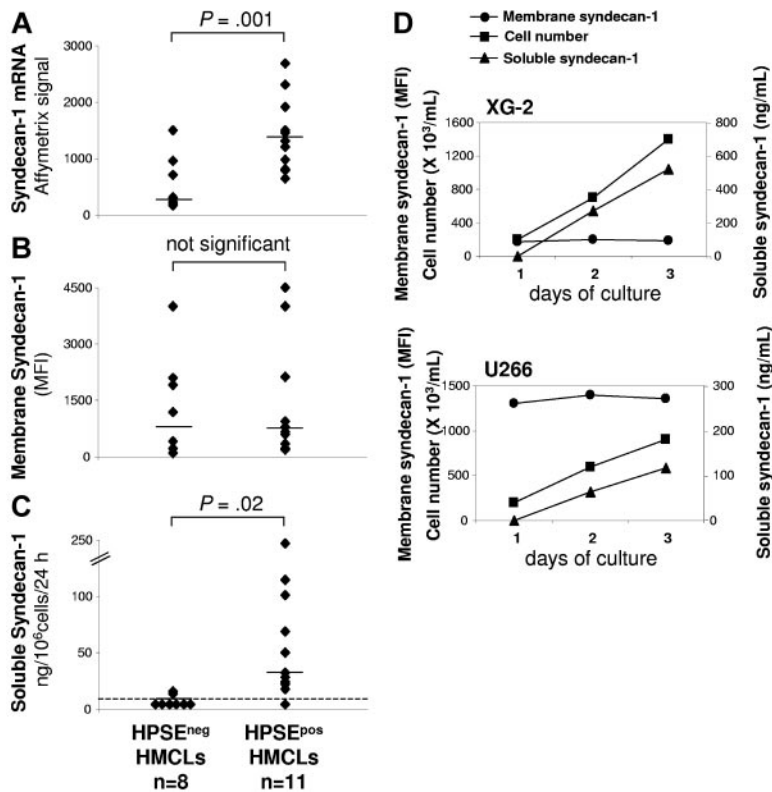


Figure 2. HMCLs that express *HPSE* produce higher amounts of soluble syndecan-1. (A) Syndecan-1 gene expression was determined in the 19 HMCLs using Affymetrix U133 set microarrays. (B) Mean fluorescence intensity of membrane syndecan-1 expression determined with a PE-conjugated anti-CD138 MoAb, a PE-isotype-matched control MoAb, and FACS analysis. For each cell line, the mean fluorescence intensity obtained with the control MoAb was set between 4 and 6. (C) For the 19 HMCLs, the rates of production of soluble syndecan-1 per 10^6 cells and per 24 hours was determined during the second and third days of culture (exponential growth phase) and measured by ELISA. The dotted line indicates the detection limit of the test (< 8 ng/mL). Horizontal bars indicate the median value. (D) For XG-2 and U266 HMCLs, the cell count, the density of membrane syndecan-1, and the concentration of soluble syndecan-1 in the culture supernatant were determined each day for 3 days.

HMCLs, whereas it was detected in 10 of 11 *HPSE*^{pos} HMCLs with a median production of 35 ng/ 10^6 cells/24 hours ($P = .02$; Figure 2C). The rate of soluble syndecan-1 production did not correlate with the density of membrane syndecan-1 in the 19 HMCLs (data not shown). This data suggested that *HPSE* may influence syndecan-1 gene expression and/or the shedding of soluble syndecan-1.

HPSE down-regulation reduces syndecan-1 mRNA expression and soluble syndecan-1 production in HMCLs

To address this question, we used siRNA technology. Electroporation of the U266 HMCL with a *HPSE*-specific siRNA resulted in a

87% inhibition of *HPSE* expression as determined by real-time RT-PCR in 5 independent experiments ($P < .01$), whereas electroporation with a nontargeting control siRNA (siRNA-co) did not affect the mRNA level of *HPSE* (Figure 3A, first panel). Of major interest, *HPSE* down-regulation induced a 38% reduction of syndecan-1 gene mRNA expression ($P < .01$), without affecting *GAPDH* mRNA expression (Figure 3A, second and third panels). The *HPSE* nontargeting siRNA-co did not affect syndecan-1 or *GAPDH* gene expression. We then investigated whether targeting *HPSE* mRNA may influence membrane syndecan-1 expression or soluble syndecan-1 production. Electroporation of *HPSE*-specific

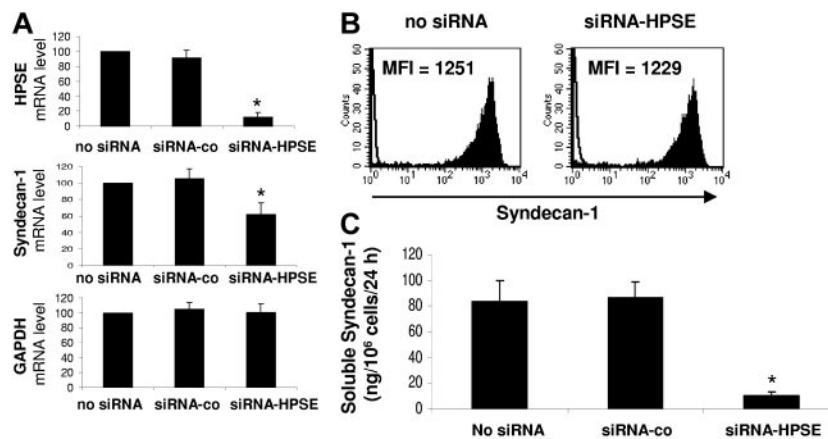


Figure 3. Syndecan-1 gene mRNA and soluble syndecan-1 are down-regulated in *HPSE*-silenced cells. U266 cells were electroporated with no siRNA or with a nontargeting control siRNA (siRNA-co) or with an *HPSE*-specific siRNA (siRNA-*HPSE*) and cultured for 2 days. (A) At day 2, *HPSE*, syndecan-1 gene, and *GAPDH* expression were quantified by real-time RT-PCR and were normalized for each sample to that of B2M. Cells electroporated with no siRNA were used as a reference and were assigned the arbitrary value of 100. Data are means \pm SD of the gene expression levels determined for 5 independent experiments. *Mean value is statistically significantly from that obtained with the control (no siRNA), using a Student *t* test for pairs ($P \leq .05$). (B) Membrane expression of syndecan-1 was determined with a PE-conjugated anti-CD138 MoAb and a PE-isotype-matched control MoAb and FACS analysis. Results shown are those of 1 experiment representative of 5. (C) U266 cells were electroporated with no siRNA or with a nontargeting control siRNA (siRNA-co) or with siRNA-*HPSE*. Data are expressed as the means \pm SD of the rates of production of soluble syndecan-1 per 10^6 cells and per 24 hours determined during the first and second days of culture after electroporation determined in 5 separate experiments. *Mean value is statistically significantly different from that obtained in the control (no siRNA), using a Student *t* test for pairs ($P \leq .05$).

siRNA had no effect on the expression of membrane syndecan-1 (Figure 3B). In contrast, *HPSE* down-regulation resulted in nearly 80% inhibition ($P < .01$) of soluble syndecan-1 production (Figure 3C). No inhibition was found with the control siRNA (Figure 3C). Of note, the siRNA-*HPSE* did not significantly affect the survival and proliferation of U266 cells (data not shown). Those data indicate that targeting *HPSE* mRNA results in a decrease of syndecan-1 gene expression and soluble syndecan-1 production without effecting the expression of membrane syndecan-1.

Heparanase is mainly expressed by cells from the bone marrow environment, in particular monocytes and osteoclasts

To determine whether *HPSE* is expressed in the bone marrow of patients with MM, we evaluated the gene expression level of *HPSE* in the whole bone marrow (WBM) (including MMCs and cells of the microenvironment) of 39 patients with newly diagnosed MM as well as in the MMCs purified from the BM of the same 39 patients, using U133 Plus 2.0 Affymetrix microarrays. A high *HPSE* expression was detected in the WBM samples (Figure 4A). The *HPSE* expression in the WBM samples (median, 76; range, 25-221) was 7.6-fold higher ($P < .001$) than that found in the corresponding MMCs (median, 10; range, 8-26). Furthermore, *HPSE* had an Affymetrix present call in 92% (36 of 39) of the WBM samples, whereas it had a present call in only 10% (4 of 39) of the purified MMC samples (Figure 4A). *HPSE* was also expressed in the WBM of 7 healthy donors (median, 84; range, 70-110; data not shown). To confirm those data, *HPSE* expression was analyzed by real-time RT-PCR in the WBM of 7 additional patients with newly diagnosed MM, as well as in the corresponding purified MMCs and the corresponding microenvironment cells depleted from MMCs ($< 2\%$ MMCs, noted ENV; Figure 4B). For each patient, the WBM sample

was used as a reference and was assigned the arbitrary value of 100. For 4 of 7 patients (P1-P4), *HPSE* was almost exclusively expressed by cells from the environment (ENV). For the other 3 patients (P5-P7), *HPSE* was expressed by both MMCs and cells from the environment (Figure 4B). Because *HPSE* was much more frequently expressed in HMCLs derived from patients with extramedullary myeloma (58% of presence; see Figure 1A) compared with BM primary MMCs, we looked for *HPSE* expression in purified MMCs from 5 patients with plasma cell leukemia (PCL1-5). *HPSE* expression was in the same range than that found in purified MMCs from patients with intramedullary myeloma (Figure 4B). Environment cells depleted from MMCs (ENV) and purified MMCs were also analyzed by Western blot. *HPSE* protein was detected in 5 of 5 environment cell samples. It was barely detectable in 3 of 3 purified MMC samples, but its expression was much lower than that found in the environment cells (Figure 4C), thus confirming the Affymetrix and real-time RT-PCR data.

To identify the cell populations expressing *HPSE* in the myeloma microenvironment, bone marrow T lymphocytes ($CD3^+$), monocytes ($CD14^+$), polymorphonuclear cells ($CD15^+$), and MMCs ($CD138^+$) were purified from the BM of 5 patients with newly diagnosed myeloma. In addition, BM stromal cells (BMSCs, $n = 5$) and osteoclasts ($n = 7$) were generated from cells of patients with MM in vitro. For each population, the gene expression level of *HPSE* was assayed using Affymetrix U133 plus 2.0 microarrays. As shown in Figure 5A, *HPSE* was mainly expressed by $CD14^+$ monocytes and osteoclasts. *HPSE* expression in monocytes (median, 819) and osteoclasts (median, 355) was, respectively, 200-fold and 88-fold higher than that in MMCs (median, 4). *HPSE* was not expressed by BMSCs. All data were validated by real-time RT-PCR (Figure 5B). Of note, no *HPSE* expression could

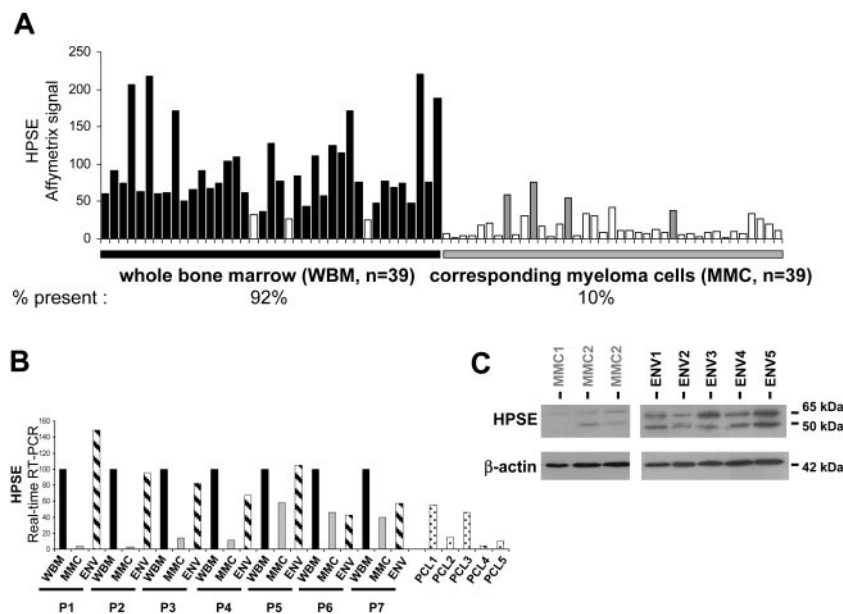


Figure 4. Comparison of *HPSE* expression between WBM and the corresponding purified MMCs of patients. (A) Expression of *HPSE* was determined in the WBM of 39 patients with myeloma as well as in the myeloma cells purified from the bone marrow of the same patients, using Affymetrix U1332 Plus 2.0 microarrays. □ indicates that the Affymetrix call is absent; ■ (WBM) and ▤ (MMC) indicate that it is present. (B) *HPSE* expression was measured by real-time RT-PCR in the whole bone marrow of 7 patients with MM (WBM; ■), in the corresponding MMCs (▤), in the corresponding microenvironment depleted from myeloma cells ($< 2\%$ MMCs, ENV; ▨), and in MMCs from 5 patients with plasma cell leukemia (PCL). *HPSE* expression was normalized to that of *GAPDH*. For each patient, the WBM sample was used as a reference and was assigned the arbitrary value of 100. For the 5 patients with PCL, the WBM sample of patient 1 was used as a reference. The median percentage of plasma cells in bone marrow aspirates from the 7 patients with intramedullary myeloma was 8.5% (range, 6%-40%). (C) *HPSE* protein expression was determined in microenvironment cells depleted from MMCs ($< 2\%$ MMCs, ENV1-5) of 5 patients and in purified MMCs of 3 other patients (MMC1-3). Cells were lysed, and the lysates were separated on a 12% SDS-PAGE and analyzed by Western blot with a polyclonal anti-*HPSE* antibody. Both the 65-kDa MW proenzyme and the 50-kDa MW active form were identified. β -Actin was used as a loading control. Results are from 2 separate Western blots, one with 3 purified MMCs, the other one with the 5 microenvironment cells. The *HPSE*^{pos} XG-2 HMCL was used as a positive *HPSE* protein control in the 2 blots (results not shown). kDa indicates molecular weight in thousands.

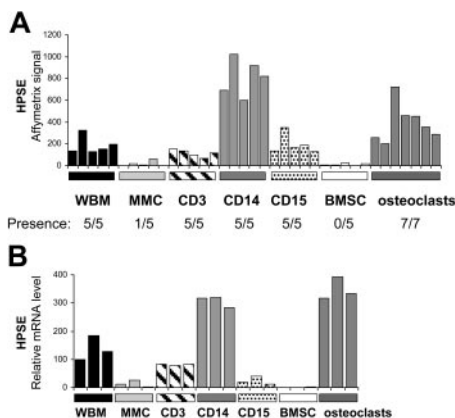


Figure 5. Expression of *HPSE* in subpopulations of the BM environment of patients with MM. (A) Expression of *HPSE* was determined in the WBM of 5 patients with MM as well as in MMCs, CD3⁺ cells, CD14⁺ cells, and CD15⁺ cells purified from the bone marrow of the same patients, using Affymetrix plus 2.0 microarrays. The 5 BMSC and 7 osteoclast samples were obtained by culture in vitro. (B) *HPSE* expression was measured by real-time RT-PCR. *HPSE* expression was normalized to that of *GAPDH*. One WBM sample was used as a reference and was assigned the arbitrary value of 100.

be detected in monocyte-derived immature or mature dendritic cells using real-time RT-PCR (data not shown). Thus, *HPSE* is highly expressed by the BM environment, mainly by monocytes and osteoclasts, and occasionally by primary MMCs.

To further clarify the contribution of MMCs in the *HPSE* expression, bone marrow biopsies from 20 patients with MM were stained using the polyclonal anti-*HPSE* antibody. The anti-*HPSE* antibody efficiently labeled the XG-2 HMCL which has a present Affymetrix detection call (Figure 6Ai-ii), unlike the XG-7 HMCL which has an absent call (Figure 6Aiii). As expected from the Affymetrix data, *HPSE* was expressed by polymorphonuclear cells and myeloid cells within the BM of 20 of 20 patients (as shown for

one patient in Figure 6Biv-v). In addition, a strong staining of megakaryocytes was found (Figure 6Bvi). Regarding MMCs, *HPSE* expression was heterogeneous among patients. Data are summarized in Table 2. *HPSE* expression lacked in the MMCs of 3 of 20 patients (as illustrated for one patient in Figure 6Bvii). In the majority of the patients, a strong heterogeneity was found with few positively stained MMCs close to negative tumor cells (Figure 6Bviii-ix; Table 2). This heterogeneous *HPSE* expression among MMCs is in agreement with previously published data.¹⁹ *HPSE* expression level in the positively stained MMCs was low. In 2 of 20 samples, we found a higher percentage of more intensely labeled MMCs (Figure 6Bx; Table 2). Of note, a strong nuclear staining appeared in the MMCs from those 2 patients.

Taken together, those data indicate that *HPSE* is mainly expressed by cells of the BM environment of patients with MM.

***HPSE* expression in the whole bone marrow of patients with myeloma at diagnosis correlates with survival**

We investigated the connection of clinical parameters with *HPSE* gene expression for 30 of the 39 patients with newly diagnosed MM who were treated with HDC and ASCT. Patients were classified into 2 groups: 15 *HPSE*^{low} patients with the lowest *HPSE* expression in the WBM (median expression, 60) and 15 *HPSE*^{high} patients with the highest *HPSE* expression (median expression, 125) (Figure 7A, left). The *HPSE*^{high} group had an increased percentage of patients with elevated C-reactive protein and β 2-microglobulin ($P \leq .05$; Table 3). Other clinical parameters (lactate dehydrogenase, serum calcium, albumin, hemoglobin, age, presence of bone lesions, and cytogenetic abnormalities) were not significantly different. According to the International Staging System (ISS), the *HPSE*^{high} group included a significantly higher frequency of patients with stage III MM and a lower frequency of patients with stage I MM. The stages according to the Durie-Salmon classification were not significantly different between the 2 groups (Table 3). *HPSE*^{high} patients had a shorter EFS (50% of survival, 6 months; $P = .017$) than *HPSE*^{low} patients (50% of survival not reached) (Figure 7A). The OAS was also significantly

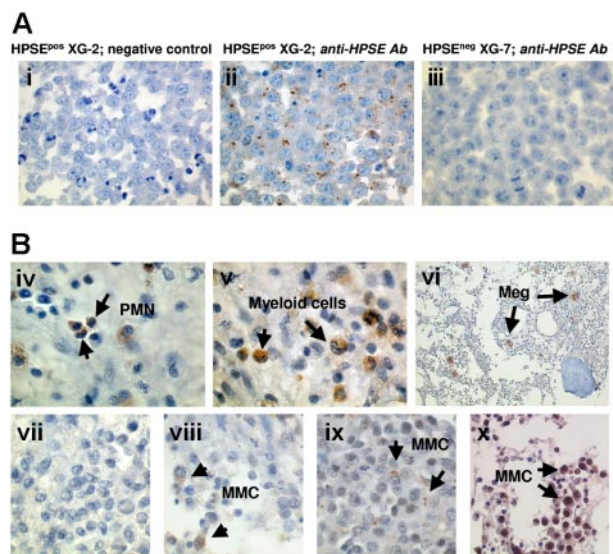


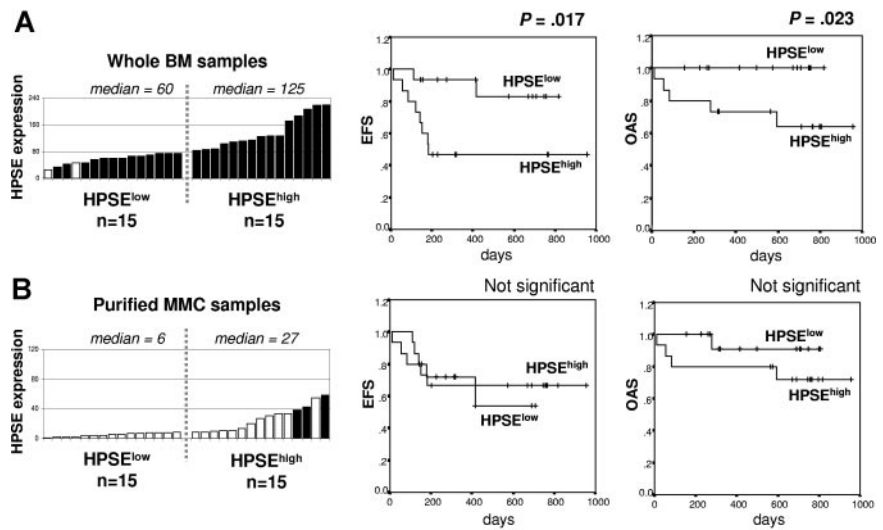
Figure 6. *HPSE* is expressed by BM environment cells and by a minor subpopulation of myeloma cells. (A) XG-2 and XG-7 cells were stained with control rabbit polyclonal antibodies (Abs) (i) or with a polyclonal anti-*HPSE* Ab at 4.5 μ g/mL (ii-iii). Note the dotlike staining signal. (B) BM biopsies from patients with MM were stained with the polyclonal anti-*HPSE* Ab (4.5 μ g/mL). (iv-vi) Environment cells in the BM of 1 patient representative of 20. PMN indicates polymorphonuclear cell; Meg, megakaryocyte. (vii-x) Heterogeneous *HPSE* expression among MMCs in the bone marrow of 4 representative patients (vii, patient 1; viii, patient 4; ix, patient 11; x, patient 20; see Table 2). The brown reaction product indicates the location of polyclonal Ab against *HPSE*. Original magnification, $\times 1000$ ($\times 200$ in vi).

Table 2. Heparanase expression in the MMCs from 20 patients with MM

Patients	<i>HPSE</i> ^{pos} MMC, %
1	0
2	0
3	0
4	1
5	2
6	2
7	3
8	4
9	6
10	7
11	10
12	10
13	12
14	14
15	18
16	20
17	24
18	25
19	73
20	75

BM biopsies from patients with MM were stained with the polyclonal anti-*HPSE* Ab (4.5 μ g/mL) as described in "Materials and methods." Both *HPSE*^{pos} and *HPSE*^{neg} MMCs were counted on each slide, and the results are expressed as the percentage of *HPSE*^{pos} MMC. The median was 8.6%.

Figure 7. Event-free survival and overall survival of patients with newly diagnosed MM according to *HPSE* expression in the WBM and MMCs. (A) Expression of *HPSE* in the WBM of 30 patients with newly diagnosed MM (left) and Kaplan-Meier survival curves for patients with MM with the highest (*HPSE*^{high}; n = 15) or the lowest (*HPSE*^{low}; n = 15) *HPSE* expression in the WBM (right). (B) Expression of *HPSE* in the purified MMCs from 30 patients with newly diagnosed MM and Kaplan-Meier survival curves for patients with MM with the highest (n = 15) or the lowest (n = 15) *HPSE* expression in the MMCs. Open and filled bars indicate that the Affymetrix cell is absent or present, respectively.



shorter in *HPSE*^{high} patients ($P = .023$). In a Cox proportional-hazard model monitoring for the highest or lowest *HPSE* expression in the WBM as well as ISS stage, both parameters were significantly predictive for EFS ($P = .029$ and $P = .002$, respectively). If *HPSE* expression was tested together with ISS staging, ISS staging remained an independent prognostic factor ($P = .02$) unlike *HPSE* expression. Classifying these 30 patients into 2 groups according to *HPSE* expression in MMCs did not result in any significant differences in the EFS or OAS between the 2

groups (Figure 7B). This is in agreement with the low *HPSE* expression in MMCs compared with that found in the microenvironment. Those data indicate that *HPSE* expression in the WBM, but not in the MMCs, is linked to the prognosis of patients with myeloma.

Discussion

Given the major role of the syndecan-1 HSPG in MM, mainly as a coreceptor for numerous MMC growth factors, we investigated the expression and possible function of *HPSE* in myeloma. We found that *HPSE* is expressed in 11 of 19 HMCLs and influences syndecan-1 gene mRNA expression: (1) when comparing the gene expression profile of the 11 *HPSE*^{pos} HMCLs to that of the 8 *HPSE*^{neg} HMCLs, syndecan-1 was one of the genes overexpressed with the highest fold change in *HPSE*^{pos} HMCLs; (2) the silencing of *HPSE* expression with siRNAs induced a down-regulation of syndecan-1 gene mRNA. We used the siRNA method because neutralizing antibodies to *HPSE* are not presently available commercially. Interestingly, although *HPSE* expression does not interfere with membrane syndecan-1 expression, it is strongly correlated with the rate of soluble syndecan-1 production by HMCLs. Seven of 8 *HPSE*^{neg} HMCLs failed to produce detectable soluble syndecan-1, whereas 10 of 11 of *HPSE*^{pos} HMCLs produced large amounts. In addition, targeting *HPSE* mRNA with siRNA induced a strong inhibition of soluble syndecan-1 production. One possible mechanism to explain the effect of *HPSE* on the shedding of syndecan-1 could be that degradation of HS chains by the *HPSE* enzyme would render membrane syndecan-1 more easily accessible to metalloproteinases. This hypothesis is supported by the observation that treatment of MM cells with the bacterial enzyme heparitinase, which completely degrades HS chains, also induced an increase in soluble syndecan-1 production by HMCLs (data not shown). Of note, membrane syndecan-1 expression in the 19 HMCLs did not correlate with the rate of soluble syndecan-1 production, and the *HPSE* inhibition by siRNA did not affect membrane syndecan-1 expression. One explanation is that the down-regulation of *HPSE* yields a decreased mRNA expression and concomitantly reduces shedding of syndecan-1 protein, which taken together does not influence the net amount of syndecan-1 on the surface of HMCLs.

Table 3. Clinical data for patients with *HPSE*^{high} and *HPSE*^{low}

	<i>HPSE</i> ^{low} , %	<i>HPSE</i> ^{high} , %	<i>P</i>
Age at least 65 y	13	27	.36
β2-microglobulin level			
Less than 3.5 mg/L	80*	47*	.05*
Greater than 5.5 mg/L	0*	27*	.03*
C-reactive protein level greater than 5 mg/L	7*	40*	.03*
Lactate dehydrogenase level at least			
240 IU/L	20	27	.66
Albumin level less than 35 g/L	33	40	.7
Hemoglobin level less than 10 g/dL	20	47	.1
Ca ⁺⁺ level less than 2.65 mM	100	100	> .999
κ Light chain	67	53	.45
λ Light chain	33	47	.45
IgA subtype	20	13	.61
Bone lesions, major structural damage (≥3)	15	23	.61
t(4;14)	15	0	.14
del(chr13)	62	46	.39
DS classification			.82
Stage I	13	7	
Stage II	20	20	
Stage III	67	73	
ISS classification			.008*
Stage I	47*	7*	
Stage II	53*	60*	
Stage III	0*	33*	

Thirty patients with newly diagnosed MM were separated in 2 subgroups of 15 patients each (*HPSE*^{high} and *HPSE*^{low}), according to *HPSE* gene expression in the WBM and assayed with Affymetrix U133 microarrays. Data are the percentages of patients within each subgroup with the indicated clinical or biologic parameters. Statistical comparisons were made with the chi-square test.

To convert β2-microglobulin level from milligrams per liter to nanomoles per liter, multiply milligrams per liter by 85. To convert hemoglobin level from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

*The percentages between the 2 groups are significantly different.

HPSE expression has been documented in several solid tumors. In particular, by real-time RT-PCR, increased levels of *HPSE* mRNA have been found in human breast, colon, lung, prostatic, and ovarian tumors, compared with corresponding normal tissues.¹⁶ To demonstrate the biologic significance of HPSE in multiple myeloma, we analyzed HPSE expression in the bone marrow of patients with MM. Here, we show that HPSE is expressed in the BM of patients with MM, both at the mRNA and protein levels, and that cells of the environment are the main source of HPSE. Among the different cell components of the BM, the highest *HPSE* expression was found in monocytes. We detected no *HPSE* expression in monocyte-derived immature or mature dendritic cells that were generated in vitro. Our findings are in agreement with recently published data showing that HPSE protein is present both in monocytes and dendritic cells unlike *HPSE* mRNA, which is exclusively expressed in monocytes. This indicates that *HPSE* gene expression and synthesis mainly occur in monocytes or in the very early steps of differentiation toward immature dendritic cells.²⁶ *HPSE* was also expressed in T cells and polymorphonuclear cells at a lower level. Because MMCs survive and proliferate in a tumor niche involving BMSCs and osteoclasts which cannot be harvested in vivo, we produced them in vitro and found that osteoclasts highly expressed *HPSE* unlike BMSCs. It is likely that the MMCs themselves can also be a source of HPSE, at least in some patients. However, overall, HPSE expression in MMCs was much lower than that found in the environment cells. First, the *HPSE* Affymetrix signal was 7.6-fold lower in MMCs compared with WBM samples, and it was reliably detectable in tumor cells of only 10% of the patients (4 of 39). Second, HPSE protein expression measured by Western blot was barely detectable in MMCs compared with environment cells. Third, using immunohistochemistry, we found that HPSE was expressed by the environment cells of 20 of 20 patients, whereas most MMCs were negative or expressed low levels of HPSE. In the majority of the patients, few positively stained MMCs were surrounded by HPSE^{neg} MMCs. This heterogeneity, which is in agreement with the previous report by Kelly et al,¹⁹ might explain why we found a low *HPSE* expression in the majority of the purified MMC samples compared with the BM environment samples. Note that in 2 of 20 patients (patients 19 and 20) we found groups of intensely labeled myeloma cells. This is in agreement with the Affymetrix data showing that 10% of the patients expressed reliable amounts of *HPSE* mRNA in the MMCs. Contrary to primary MMCs, *HPSE* expression was detectable in 58% (11 of 19) of the HMCLs. We already pointed out that HMCLs may express some genes (*HB-EGF*, *IL-6*, and *APRIL*) that are originally expressed by the BM environment but not by primary MMCs themselves.^{6,27} Of note, *HPSE* expression in MMCs from patients with plasma cell leukemia was in the same range than that found in MMCs from patients with intramedullary myeloma.

A major finding of this study is that *HPSE* expression in the WBM of patients with MM is an indicator of poor prognosis. Thus, *HPSE* mRNA expression in the WBM holds prognostic value even though its median expression in patients with MM did not differ from that of healthy donors. In agreement with this observation, it was previously reported that healthy persons and MM patients have similar amounts of IGF-1 in the circulation, whereas MM patients with low serum IGF-1 levels have a favorable prognosis.²⁸ Despite the low number of patients whose *HPSE* expression and clinical data were available and the short follow-up (median, 2 years), the difference in EFS and OAS between *HPSE*^{high} and *HPSE*^{low} patients was highly significant with *HPSE* expression predicting a

less favorable disease outcome. *HPSE*^{high} patients had higher CRP and β 2-microglobulin levels than did *HPSE*^{low} patients. *HPSE* expression was also linked to ISS staging, the *HPSE*^{low} group having a higher percentage of stage 1 and a lower percentage of stage 3 patients than the *HPSE*^{high} group. The prognostic value of *HPSE* expression in the WBM was not independent of ISS staging. Of note, the percentage of MMCs in the WBM in the *HPSE*^{low} group (median 4.5; range, 0.03–45) was not statistically significantly different from that in the *HPSE*^{high} group (median, 2.9; range, 0.5–12). Classifying patients in 2 groups according to *HPSE* expression in purified MMCs did not yield any difference in EFS or OAS, neither in this set of 30 patients (Figure 6B) nor in a larger set of MMCs from 104 patients with newly diagnosed MM (data not shown). This again emphasizes that *HPSE* is mainly expressed by the BM environment and that this expression is biologically relevant. This is the first study showing that a gene measured in the bone marrow microenvironment has prognostic value in MM.

The fact that HPSE controls syndecan-1 expression and soluble syndecan-1 production may explain at least in part the poor prognosis of *HPSE*^{high} patients, as increased levels of soluble syndecan-1 have been described as a bad prognostic factor in patients with MM.^{10,11} Soluble syndecan-1 can promote tumor growth in a murine model,¹³ presumably because it can sequester heparin-binding growth factors that support MMC growth as well as angiogenic factors such as VEGF,^{5,29,30} thus providing MMCs with an increased amount of growth factors. Consistently, Kelly et al¹⁹ have shown HPSE to increase microvessel density, which in turn correlates with poor prognosis in MM.^{31,32} In an independent series of 20 patients with newly diagnosed MM, we did not find any significant correlation between *HPSE* expression in the WBM and soluble syndecan-1 concentration in the plasma (data not shown), likely because soluble syndecan-1 can be retained by the BM matrix and hence only partially circulates.³³ The impact of *HPSE* expression on survival may further be linked to the modification of syndecan-1 HS chains by the endoglycosidase enzymatic activity of HPSE. The ability of HPSE to degrade HS chains into small fragments is critical to promote tumor growth in epithelial tumors,^{16,34} and HPSE enzymatic activity is present in the BM aspirates of patients with myeloma.¹⁹ HPSE may promote tumor growth by releasing heparin-binding MMC growth factors or angiogenic factors that are trapped within the environment through the HS chains of syndecan-1. Accordingly, it has been shown that HPSE can release HS-bound growth factors such as FGF2 from the extracellular matrix.³⁵ Alternatively, HPSE may improve the HS-mediated effect of heparin-binding growth factors. Indeed, small fragments resulting from the HPSE cleavage were reported to be more biologically active than the native HS chains from which they are derived.¹⁷ Finally, besides its endoglycosidase activity, it is now becoming clear that HPSE might have other nonenzymatic functions which may also have an impact on tumor growth. It was not within the scope of our study to elucidate the mechanism involved in the regulation of syndecan-1 gene expression and shedding by HPSE, but one can speculate that it might involve such nonenzymatic functions. These nonenzymatic functions include stimulation of endothelial cell invasion via AKT pathway activation,³⁶ enhanced cell adhesion of glioma,³⁷ lymphoma,³⁸ and T cells mediated by β 1-integrin,³⁹ and induction of VEGF expression.⁴⁰ These mechanisms are poorly understood but might involve a yet undefined receptor that could lead to activation of transduction pathways and mediate diverse biologic effects.

Altogether, these data provide evidence that HPSE can modulate syndecan-1 activity through the cleavage of its HS chains but also through the regulation of syndecan-1 mRNA expression and shedding. HPSE, mainly produced by the environment, is an indicator of poor prognosis for patients with MM. Therefore, HPSE inhibitors such as PI-88, which is currently in clinical trials,⁴¹ will be of interest for the treatment of patients with MM.

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References

- Wijdenes J, Vooijs WC, Clement C, et al. A plasmo-cyte selective monoclonal antibody (B-B4) recognizes syndecan-1. *Br J Haematol*. 1996;94:318-323.
- Costes V, Magen V, Legouffe E, et al. The Mi15 monoclonal antibody (anti-syndecan-1) is a reliable marker for quantifying plasma cells in paraffin-embedded bone marrow biopsy specimens. *Hum Pathol*. 1999;30:1405-1411.
- Mahtouk K, Cremer FW, Reme T, et al. Heparan sulfate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma. *Oncogene*. 2006;25:7180-7191.
- Couchman JR. Syndecans: proteoglycan regulators of cell-surface microdomains? *Nat Rev Mol Cell Biol*. 2003;4:926-937.
- Derksen PW, Keehnen RM, Evers LM, van Oers MH, Spaargaren M, Pals ST. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. *Blood*. 2002;99:1405-1410.
- Mahtouk K, Jourdan M, De Vos J, et al. An inhibitor of the EGF receptor family blocks myeloma cell growth factor activity of HB-EGF and potentiates dexamethasone or anti-IL-6 antibody-induced apoptosis. *Blood*. 2004;103:1829-1837.
- Mahtouk K, Hose D, Reme T, et al. Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells. *Oncogene*. 2005;24:3512-3524.
- Dhodapkar MV, Kelly T, Theus A, Athota AB, Barlogie B, Sanderson RD. Elevated levels of shed syndecan-1 correlate with tumour mass and decreased matrix metalloproteinase-9 activity in the serum of patients with multiple myeloma [published erratum appears in *Br J Haematol*. 1998; 101:398]. *Br J Haematol*. 1997;99:368-371.
- Dhodapkar MV, Abe E, Theus A, et al. Syndecan-1 is a multifunctional regulator of myeloma pathobiology: control of tumor cell survival, growth, and bone cell differentiation. *Blood*. 1998; 91:2679-2688.
- Klein B, Li XY, Lu ZY, et al. Activation molecules on human myeloma cells. *Curr Top Microbiol Immunol*. 1999;246:335-341.
- Seidel C, Sundan A, Hjorth M, et al. Serum syndecan-1: a new independent prognostic marker in multiple myeloma [published erratum appears in *Blood*. 2000;95:2197]. *Blood*. 2000;95:388-392.
- Sanderson RD, Yang Y, Suva LJ, Kelly T. Heparan sulfate proteoglycans and heparanase—partners in osteolytic tumor growth and metastasis. *Matrix Biol*. 2004;23:341-352.
- Yang Y, Yaccoby S, Liu W, et al. Soluble syndecan-1 promotes growth of myeloma tumors in vivo. *Blood*. 2002;100:610-617.
- Vlodavsky I, Friedmann Y, Elkin M, et al. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat Med*. 1999;5:793-802.
- Kussie PH, Hulmes JD, Ludwig DL, et al. Cloning and functional expression of a human heparanase gene. *Biochem Biophys Res Commun*. 1999;261:183-187.
- Vlodavsky I, Goldshmidt O, Zcharia E, et al. Mammalian heparanase: involvement in cancer metastasis, angiogenesis and normal development. *Semin Cancer Biol*. 2002;12:121-129.
- Kato M, Wang H, Kainulainen V, et al. Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. *1998;4:691-697*.
- Vlodavsky I, Friedmann Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J Clin Invest*. 2001;108:341-347.
- Kelly T, Miao HQ, Yang Y, et al. High heparanase activity in multiple myeloma is associated with elevated microvessel density. *Cancer Res*. 2003; 63:8749-8756.
- Yang Y, Macleod V, Bendre M, et al. Heparanase promotes the spontaneous metastasis of myeloma cells to bone. *Blood*. 2005;105:1303-1309.
- Moreaux J, Cremer FW, Reme T, et al. The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. *Blood*. 2005;106:1021-1030.
- Tarte K, Fiol G, Rossi JF, Klein B. Extensive characterization of dendritic cells generated in serum-free conditions: regulation of soluble antigen uptake, apoptotic tumor cell phagocytosis, chemotaxis and T cell activation during maturation in vitro. *Leukemia*. 2000;14:2182-2192.
- Zhang XG, Gaillard JP, Robillard N, et al. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. *Blood*. 1994;83:3654-3663.
- Rebouissou C, Wijdenes J, Autissier P, et al. A gp130 interleukin-6 transducer-dependent SCID model of human multiple myeloma. *Blood*. 1998; 91:4727-4737.
- Liu WM, Mei R, Di X, et al. Analysis of high density expression microarrays with signed-rank call algorithms. *Bioinformatics*. 2002;18:1593-1599.
- Benhamron S, Nechushtan H, Verbovetski I, et al. Translocation of active heparanase to cell surface regulates degradation of extracellular matrix heparan sulfate upon transmigration of mature monocyte-derived dendritic cells. *J Immunol*. 2006;176:6417-6424.
- Klein B, Tarte K, Jourdan M, et al. Survival and proliferation factors of normal and malignant plasma cells. *Int J Hematol*. 2003;78:106-113.
- Standal T, Borset M, Lenhoff S, et al. Serum insulinlike growth factor is not elevated in patients with multiple myeloma but is still a prognostic factor. *Blood*. 2002;100:3925-3929.
- De Vos J, Hose D, Reme T, et al. Microarray-based understanding of normal and malignant plasma cells. *Immunol Rev*. 2006;210:86-104.
- Mahtouk K, Hose D, Reme T, et al. Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma. *Blood*. 2006;108:999a. Abstract 3502.
- Munshi NC, Wilson C. Increased bone marrow microvessel density in newly diagnosed multiple myeloma carries a poor prognosis. *Semin Oncol*. 2001;28:565-569.
- Pruneri G, Ponzoni M, Ferreri AJ, et al. Microvessel density, a surrogate marker of angiogenesis, is significantly related to survival in multiple myeloma patients. *Br J Haematol*. 2002;118:817-820.
- Bayer-Garner IB, Sanderson RD, Dhodapkar MV, Owens RB, Wilson CS. Syndecan-1 (CD138) immunoreactivity in bone marrow biopsies of multiple myeloma: shed syndecan-1 accumulates in fibrotic regions. *Mod Pathol*. 2001;14:1052-1058.
- Parish CR, Freeman C, Hulett MQ. Heparanase: a key enzyme involved in cell invasion. *Biochim Biophys Acta*. 2001;1471:M99-M108.
- Whitelock JM, Murdoch AD, Iozzo RV, Underwood PA. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. *J Biol Chem*. 1996;271:10079-10086.
- Gingis-Velitski S, Zetser A, Flugelman MY, Vlodavsky I, Ilan N. Heparanase induces endothelial cell migration via protein kinase B/Akt activation. *J Biol Chem*. 2004;279:23536-23541.
- Zetser A, Bashenko Y, Miao HQ, Vlodavsky I, Ilan N. Heparanase affects adhesive and tumorigenic potential of human glioma cells. *Cancer Res*. 2003;63:7733-7741.
- Goldshmidt O, Zcharia E, Cohen M, et al. Heparanase mediates cell adhesion independent of its enzymatic activity. *FASEB J*. 2003;17:1015-1025.
- Sotnikov I, Hershkoviz R, Grabovsky V, et al. Enzymatically quiescent heparanase augments T cell interactions with VCAM-1 and extracellular matrix components under versatile dynamic contexts. *J Immunol*. 2004;172:5185-5193.
- Zetser A, Bashenko Y, Edovitsky E, Levy-Adam F, Vlodavsky I, Ilan N. Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation. *Cancer Res*. 2006;66:1455-1463.
- Parish CR, Freeman C, Brown KJ, Francis DJ, Cowden WB. Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. *Cancer Res*. 1999; 59:3433-3441.