Heparanase stimulation of protease expression implicates it as a master regulator of the aggressive tumor phenotype in myeloma*

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High levels of heparanase are an indicator of poor prognosis in myeloma patients, and upregulation of the enzyme enhances tumor growth, angiogenesis and metastasis in animal models. At least part of the impact of heparanase in driving the aggressive tumor phenotype is due to its effect on increasing the expression and shedding of the heparan sulfate proteoglycan syndecan-1, a molecule known to promote myeloma progression. The present work demonstrates that elevation in heparanase expression in myeloma cells stimulates sustained ERK1 phosphorylation which in turn drives MMP-9 expression. In addition, uPA/uPAR expression levels increase and blocking the proteolytic activation of either MMP-9 or uPA inhibits the heparanase-induced increase in syndecan-1 shedding. Together these data provide a mechanism for heparanase-induced syndecan-1 shedding and, more importantly, demonstrate that heparanase activity in myeloma cells can lead to increased levels of proteases that are known to play important roles in the aggressive behavior of myeloma tumors. This, in addition to its other known biological roles, indicates that heparanase acts as a master regulator of the aggressive tumor phenotype by upregulating protease expression and activity within the tumor microenvironment.
invasion in vitro as well as the pleural dissemination of cells implanted into nude mice (9). The effects of heparanase in cancer may not solely be due to its enzymatic activity because mutated, non-enzymatically active heparanase, retains some biological functions. For example, heparanase enhances Akt signaling and stimulates PI3K and p38-dependent migration and invasion of endothelial cells and this can occur via mutated heparanase that lacks enzymatic activity (10). In addition, VEGF expression is upregulated in several tumor cell lines following their transfection with mutated heparanase, and downregulated in melanoma cells transfected with heparanase-specific siRNA (11). Overall, the data from cancer patients coupled with experimental data from animal models strongly point to heparanase as a potent pro-tumorigenic, pro-angiogenic and pro-metastatic enzyme.

Multiple myeloma is a devastating cancer that resides predominantly within the bone marrow microenvironment and is characterized by fatigue, intractable bone pain, renal failure and recurrent infections. These effects result from widespread tumor dissemination with accompanying high tumor burden, cytokine dysregulation, osteolytic bone disease and deposition of high levels of immunoglobulin light chain (12). The syndecan-1 heparan sulfate proteoglycan is expressed by almost all myeloma tumors and when present at high levels in the serum of patients, is an indicator of poor prognosis (13-15). Moreover, the shed form of syndecan-1 promotes tumor growth and metastasis in vivo (16). Because heparanase modulates the structure and function of syndecan-1 by cleaving its heparan sulfate chains, in previous studies we investigated its expression and function in myeloma. We discovered that enzymatically active heparanase is present at high levels in the bone marrow plasma of many myeloma patients and this correlates with high microvessel density, suggesting that high heparanase is associated with poor prognosis (17,18). Using in vivo models we also demonstrated that heparanase promotes the growth and spontaneous metastasis of myeloma tumors to bone and that an inhibitor of heparanase potently blocks tumor growth (19,20). Interestingly, heparanase also regulates both the level and location of syndecan-1 within the myeloma microenvironment by enhancing syndecan-1 expression and shedding (18,21). This suggests an important functional link between heparanase expression, syndecan-1 shedding and an aggressive tumor phenotype.

The present study was undertaken to examine the mechanism by which heparanase promotes syndecan-1 shedding. We find that elevation of heparanase expression stimulates a prolonged ERK signaling by the myeloma cells. This signaling upregulates expression of MMP-9 and uPA/uPAR which appear to work in concert to enhance syndecan-1 shedding. This enhanced shedding of syndecan-1 likely has an impact on tumor behavior but, more importantly, the upregulated proteases are known to promote tumor growth, angiogenesis, metastasis and bone disease. Together these data imply that heparanase acts as a master regulator of the aggressive tumor phenotype in myeloma.

EXPERIMENTAL PROCEDURES

Cells and transfections - CAG cells were established from a bone marrow aspirate of a myeloma patient at the Arkansas Cancer Research Center as previously described (22). ARH-77 cells were obtained from the American Type Culture Collection (Manassas, VA). CAG and ARH-77 cells were grown in RPMI medium supplemented with 10% FBS. As described previously, the cells were transfected with either empty vector or vector containing the human heparanase cDNA to generate heparanase-low and heparanase-high cells, respectively (17,19). For construction of vectors carrying mutations in the enzyme active site of heparanase, mutations were generated at Glu-225 or Glu-343 as described previously (21).

Gelatin zymography - After cells were incubated with serum-free medium for 48 hour, supernatants were collected and concentrated in centriplus columns with a 30 kDa cutoff value (Millipore Corp., Bedford, MA). Protein in the concentrated media were quantified by BCA protein assay reagent kit (Pierce, Rockford, IL) and an equal amount of protein (50µg) was mixed with non-reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) and analyzed by SDS-PAGE using 10% polyacrylamide gels co-polymerized with gelatin (BioRad Laboratories, Hercules, CA). Electrophoresis was carried out at 10mA for 2 hours. The SDS in the acrylamide gel was...
extracted by incubation with 2.5% Triton X100 solution for 2 hours at room temperature and gelatinolytic activities were developed in a buffer containing 50mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂ and 0.02% Brij 35 at 37°C, overnight. The gel was then stained with Coomassie Blue-250. Following destaining, sites of proteolytic activities were visualized as clear bands against the blue background of stained gelatin. For some experiments, cells were treated with recombinant human heparanase (kindly provided by Dr. Israel Vlodavsky and prepared as described (23)) or MAPK inhibitor PD98059 (Calbiochem, LaJolla, CA) (50 µM) and incubated at 37ºC, overnight. NIH Image (NIH, Bethesda, MD) was used to quantify the bands.

Western Blotting - For MMP-9 and uPA detection, serum-free media conditioned for 48 h were concentrated using Centriplus YM30 (Millipore) and proteins (20µg for MMP-9 blots and 80µg for uPA blots) were subjected to 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and probed with either rabbit anti-human MMP-9 antibody (Chemicon, Inc., Temecula, CA) or rabbit anti-human uPA (H-140) (Santa Cruz Biotech, Inc., Santa Cruz, CA) followed by HRP-conjugated donkey anti-rabbit IgG. Bands were quantified using NIH Image. MMP-9 within tumors was detected by western blotting following their homogenization in lysis buffer (1:4 weight/volume) containing 50mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate and 0.1% SDS. uPAR expression was detected in the cell lysate using a mouse anti-human uPAR antibody (CD87) (R&D System Inc., Minneapolis, MN) and heparanase expression was detected using a rabbit anti-human heparanase polyclonal antibody (kindly provided by Hua-Quan Miao, Imclone, Inc.) (24). Equal loading of protein was confirmed by staining membranes for human β-actin (Sigma, Saint Louis, Missouri).

For analysis of signaling pathways, equal amounts of cell lysate protein from heparanase high or heparanase low cells were run on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with either of the following antibodies; mouse monoclonal phospho-ERK (Cell Signaling Tech., Inc., Beverly, MA), rabbit polyclonal phospho-p38 (Cell Signaling Tech., Inc.) or rabbit polyclonal phospho-Src (Cell Signaling Tech., Inc.), followed by the corresponding HRP-labeled secondary antibody. Immunoactive bands were detected using the ECL detection reagent (Amersham Sciences, Buckinghamshire, UK). After stripping, the same blots were reprobed with antibodies to total ERK, p-38 or Src (all from Cell Signaling Tech., Inc.).

**Knockdown of heparanase by shRNA** – Using the nucleotide target sequence (423GGAATCAACCTTTGAAGAG441) (6), a double-strand oligonucleotide was synthesized (IDT, Coralville, IA) to knockdown heparanase. Oligonucleotide1: CGCGTCCCCGCGGAATCAACCTTTGAA GAGTTCAAGAGAC TCTTCAAAGGTTGA TTCTTTTTTTGGAAT; oligonucleotide 2: CGATTTCCAAAAAAGGAATCAACCTTTGA AGAGTCTTTGAACCTTCAAAAGGTTGATT CCGGGGA. The control shRNA includes a scrambled sequence that does not match any sequence of human genes. Scramble oligonucleotide 1:CGCGTCCCCCGTGCTCCGAAC GTGTCAGTTTTCAAGAAGGACGACGT TCAGGACTTTTGGAAT; scramble oligonucleotide 2:CGATTTCCAAAAAAGTGCTCCCGA ACGTGTCAGTTTTCAAGAAGGACGACG ACATCGGAGACGGGA. The complementary oligo-nucleotides were annealed and the double stranded oligonucleotides were ligated between MluI and ClaI restriction sites on pLVTHM vector (containing the H1 promoter), and lentivirus was packaged by transfection of the pLVTHM vector and plasmids pMD2G and pCMV-dR8.91 (vectors and plasmids kindly provided by Dr. Didier Trono, University of Geneva, Switzerland) into 293FT cells (Invitrogen, Carlsbad, CA). The conditioned medium of the transfected 293FT cells was filtered (0.45 µm), and the titer of virus was determined by FACS and calculated by the percentage of green fluorescent protein (GFP)–positive cells after 48 hours post-infection of CAG cells. CAG cells were infected at a multiplicity of infection (MOI) of 50, followed by cell sorting by GFP expression. The reduction of heparanase expression was confirmed by reverse transcription (RT)–PCR and immunoblotting. For PCR, the forward and reverse primers were 5’-CGCGTAGTGATGCCCATCTTGGAAGAG-3’ (forward) and 5’-CGCTTCGATCCGAAGAAGG AATCAA-3’ (reverse) for heparanase and 5’-
ACCACAGTCCATGCCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR was performed as an initial denaturation at 95°C for 2 minutes, followed by 32 cycles of 95°C for 45 seconds, 60 °C for 1 minute; and 72°C for 1 minute, and ended by extension at 72°C for 10 minutes. PCR products were separated by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunohistochemistry - Sections from tumors formed from heparanase low and heparanase high cells were deparaffinized with xylene and then rehydrated through graded concentrations of ethanol and distilled water. Epitope retrieval was performed by steaming the slides for 20 minutes in citrate buffer solution (pH 6.0). Slides were washed and incubated with 2.5% H₂O₂ for 30 minutes to quench endogenous peroxides activities and then were blocked with 1% BSA in PBS for 1 hour at room temperature. The slides were then stained overnight with rabbit anti-human MMP-9 antibody (10 µg/ml in 1% BSA/PBS) at 4°C. The sections were washed with PBS and stained with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature followed by Vectastain ABC reagent (Vector Laboratories) for another 1 hour at room temperature. Detection was accomplished using a 3,3’ diaminobenzidine substrate kit (Vector Laboratories). Photographs were taken using an Olympus BX60 System Microscope.

Quantification of Syndecan-1 - Equal numbers of cells (10⁶ cells/ml) were plated in wells of 12-well plates in complete RPMI medium. MMP-9 neutralizing antibody 6-6B (Calbiochem) or MMP-9 inhibitor 1 (Calbiochem) were added to some wells. In some experiments the cells were incubated with monoclonal anti-human uPAR antibody which blocks uPA activation (R&D System, Inc.). After 48 hours of incubation at 37°C in 5% CO₂, the cell culture media were collected and the levels of shed syndecan-1 assessed by ELISA using an Eli-pair kit from Diaclone (Cell Sciences Inc., Norwood, MA). The standard curve was linear between 8 and 256 ng/ml and all samples were diluted to concentrations within that range.

Matrigel Invasion Assay - The invasiveness of myeloma cells was analyzed using Biocoat matrigel invasion chambers (BD Biosciences, San Diego, CA). The matrigel invasion chamber consists of cell culture inserts containing an 8 micron pore size polyethylene membrane precoated with a thin layer of matrigel basement membrane matrix. 500 µl of warm RPMI medium was added to the interior of the inserts and to each well and the matrigel allowed to rehydrate for two hours in humidified tissue culture incubator at 37°C and 5% CO₂. After rehydration the medium was carefully removed without disturbing the layer of matrigel matrix on the membrane. Cells (2x10⁵) suspended in 500 µl serum free RPMI medium were seeded in the upper compartment of chambers. To examine the role of uPA/uPAR in invasion, cells together with mouse anti-human uPAR antibody were loaded into the upper compartment of the matrigel invasion chamber. 750 µl of RPMI medium with 10% FBS which served as the chemo attractant was added to the lower compartment of the invasion chamber and incubated for 22 hours. Cells that invaded the matrigel-coated filters were recovered from the lower compartment and counted using Coulter Z1 Particle counter. Each assay was carried out in triplicate.

RESULTS

Heparanase enhances the expression of MMP-9 in myeloma cells - Previously we demonstrated that upregulated expression of heparanase enhances the synthesis and shedding of syndecan-1 from the surface of myeloma cells, thereby contributing to tumor progression by elevating levels of syndecan-1 in the tumor microenvironment (16,21). Work by others has shown that several matrix metalloproteinases can mediate syndecan-1 shedding (25-29), suggesting that heparanase may enhance syndecan-1 shedding by mediating the upregulation of one or more of these proteases. Because MMP-9 is known to be present in myeloma tumors we examined its level of expression by CAG myeloma cells expressing high or low levels of the enzyme. Western blotting revealed a significant increase in MMP-9 protein levels in medium conditioned by the cells having high heparanase expression as compared to those having low expression of the enzyme (Fig. 1A). RT-PCR analysis demonstrated a 3-fold increase in MMP-9 mRNA expressed in the heparanase high vs. low cells, thus confirming the western blot data. As we have shown previously, it is important to note that the level of heparanase
expression and enzymatic activity in the heparanase high CAG cells is similar to that found in some myeloma patient tumors (17,19,21). Thus, the increase in MMP-9 expression by these cells is not due to an enhancement of heparanase expression beyond that likely to be found in the human myeloma microenvironment.

To further explore MMP-9 expression in these cells, serum free conditioned medium from myeloma cells was subjected to zymography. Heparanase high cells exhibited high gelatinolytic activity corresponding to pro-MMP-9 (92 kDa gelatinase) as compared to heparanase low cells (Fig. 1A). Analysis of heparanase transfected ARH-77 human lymphoblastoid (myeloma-like) cells also demonstrates a similar increase in MMP-9 enzyme activity as compared to heparanase low cells, suggesting that the upregulation of MMP-9 is not cell-line specific. However, in contrast to what was observed in myeloma cells, zymogram analysis of heparanase transfected MDA-MET breast cancer cells (a highly metastatic variant of MDA-MB-231 cells) (30) demonstrated a decrease in MMP-9 expression as compared to controls. Thus, the effect of heparanase on MMP-9 expression may vary depending on the tumor type.

To determine whether the enhanced expression of MMP-9 by heparanase high myeloma cells requires the enzymatic (heparan sulfate degrading) activity of heparanase, we examined MMP-9 activity levels in media from CAG cells expressing heparanase mutated at the active site of the enzyme (mutated at amino acid 225 (M225) or 343 (M343)) (31). In previous work we demonstrated that these cells express high levels of heparanase protein, but exhibit low heparan sulfate degrading activity as compared to cells expressing high levels of wild-type heparanase (21). When the conditioned medium from cells expressing these heparanase mutants was analyzed by zymogram, the gelatinase activity corresponding to MMP-9 was not elevated as compared to cells expressing the active enzyme (heparanase high cells) (Fig. 1B). This indicates that heparanase enzymatic activity is required to enhance MMP-9 expression in these cells.

We previously found that addition of recombinant heparanase to cells would enhance their shedding of syndecan-1 (21). To determine if recombinant heparanase would affect MMP-9 expression, recombinant heparanase was added to wild-type CAG cells growing in culture. Over a 24-hour time period, the activity of MMP-9 in the medium of cells treated with 10 ng/ml of recombinant heparanase was almost 3-fold higher than that from cells not exposed to exogenous heparanase (Fig.1C). This indicates that the upregulation of MMP-9 in the heparanase high CAG myeloma cells is not simply an artifact related to their transfection with the cDNA for heparanase and that heparanase can stimulate a relatively rapid increase in MMP-9 levels. Moreover, this result provides further evidence that extracellular heparanase can influence behavior of cells that are not actually expressing the enzyme (32,33).

Heparanase down regulation correlates with reduced MMP-9 expression levels - As a final confirmation that heparanase regulates MMP-9 expression in myeloma cells, we used lentiviral vectors coding for an shRNA designed to block heparanase expression. We hypothesized that a reduction in heparanase expression in CAG wild-type cells would reduce their relatively low endogenous level of MMP-9 expression. PCR and western blotting results confirm that cells infected with the heparanase shRNA have dramatically reduced expression of heparanase as compared to cells infected with control shRNA (Fig.2A). This reduction in heparanase expression correlates with a decrease in MMP-9 protein and activity levels (Fig. 2B) and message level as determined by RT-PCR (not shown). These results support the findings in Fig. 1 and the conclusion that heparanase regulates expression of MMP-9 in these myeloma cells.

Elevation of MMP-9 is regulated by ERK phosphorylation - To identify the intracellular signaling pathway underlying MMP-9 upregulation in heparanase transfected cells, we examined the activation status of several signaling mediators known to be involved in regulating MMP-9 expression including Src, p38 MAPK and ERK1/2 (34,35). Lysates from cells expressing low or high levels of heparanase were subjected to immunoblotting with antibodies directed against phosphorylated Src, p38 and ERK. Levels of phospho-Src and phospho-p38 were not affected by an elevation in heparanase expression (Fig. 3A). In contrast, ERK activation was significantly enhanced in the heparanase high cells as compared to the heparanase low cells. Interestingly, levels of
ERK phosphorylation were not elevated in cells expressing the mutated form of heparanase that lacks enzymatic activity (M343), suggesting that the elevation in ERK signaling is dependent on heparanase-mediated degradation of heparan sulfate chains. We next examined the involvement of these signaling molecules in MMP-9 expression by using inhibitors that block the activation of ERK, Src and p38. By zymogram analysis of conditioned medium from heparanase high cells, we noted a significant inhibition in the levels of MMP-9 in cells treated with the MAPK/ERK inhibitor (PD98059) (Fig.3B) but not by a Src inhibitor (PP2) or by a p38 MAPK inhibitor (SB203580)2. This indicates that activation of the ERK signaling is crucial for enhancement of MMP-9 expression.

Heparanase enhances MMP-9 expression in vivo - Previously we demonstrated that elevation of heparanase expression in CAG myeloma cells enhances their growth and metastasis in vivo as compared to control cells (19). Because MMP-9 plays an important role in tumor growth, angiogenesis and metastasis, we investigated whether the heparanase-mediated upregulation of MMP-9 expression that we see in vitro is also present within the tumor microenvironment when these cells are injected into SCID mice. Immunostaining of tumors formed from heparanase high CAG cells revealed that they have high levels of MMP-9 (Fig. 4A). In contrast, tumors formed from heparanase low CAG cells contain very low levels of the enzyme. Importantly, western blotting of tumor lysates revealed that most of the MMP-9 present is in the enzymatically active 82 kDa form (Fig. 4B). This dramatic increase in active MMP-9 correlates with the aggressive phenotype seen in these tumors formed from cells expressing high levels of heparanase (19).

MMP-9 mediates enhanced shedding of syndecan-1 in heparanase expressing cells - To determine if MMP-9 mediates the shedding of syndecan-1, we measured the accumulation of the syndecan-1 ectodomain in cell culture media in the presence or absence of 6-6B, an antibody that blocks activation of MMP-9 (36). As expected based on our previous work (21), analysis of conditioned media 48 h after plating cells reveals that the level of shed syndecan-1 is significantly higher in heparanase high as compared to heparanase low cells (Fig. 5A). Antibody 6-6B significantly blocked shedding of syndecan-1 by the heparanase high cells indicating that elevation of MMP-9 is the mechanism by which heparanase expression enhances syndecan-1 shedding. Addition of the antibody to heparanase low expressing cells had no significant effect on levels of syndecan-1 shedding2, indicating that MMP-9 is not responsible for constitutive low-level shedding that occurs when heparanase expression is low. As another confirmation of the role of MMP-9 in syndecan-1 shedding, MMP-9 Inhibitor 1, a selective inhibitor of MMP-9, was introduced into cells growing in vitro. This also significantly and in a dose-dependent manner inhibited the shedding of syndecan-1 by cells expressing high levels of heparanase (Fig. 5B).

Heparanase upregulates the expression of uPA and uPAR - It has been demonstrated that pro-MMP-9 is activated by a protease cascade initiated by activated uPA (37). Importantly, this functional coupling between the two proteases is required for intravasation of tumor cells into the vasculature, a rate limiting step for metastasis of cancer cells (38). Thus, we sought to determine whether the upregulation of MMP-9 by heparanase is coupled with the upregulation of uPA/uPAR. CAG cells expressing low or high levels of heparanase were incubated in serum-free medium for 24 h and the conditioned media examined for levels of uPA. While uPA was absent from the medium of cells expressing low levels of heparanase, it was clearly present in significant amounts in medium from cells expressing high levels of heparanase, it was clearly present in significant amounts in medium from cells expressing high levels of heparanase (Fig 6A). In addition, western blots of cell extracts show that the uPA receptor, uPAR, is also upregulated. Because uPA/uPAR are known to be involved in proteolysis and cell invasion (39), we explored their effects on syndecan-1 shedding and myeloma cell invasion. Addition to heparanase high cells of an antibody that blocks the activation of uPA significantly inhibits syndecan-1 accumulation in the culture medium (Fig. 6B). This implies that uPA participates in regulating syndecan-1 shedding. The blocking antibody had no significant effect on the levels of syndecan-1 shedding by the heparanase low cells2. To further test uPA/uPAR function, cells were plated on the surface of matrigel coated chambers and the invasion of tumor cells was quantified. The number of invasive cells was almost 3 times higher when cells expressed high levels of heparanase as
compared to those expressing low levels of the enzyme (Fig. 6C). Moreover, the antibody that blocks uPA activation inhibited the aggressive invasive behavior of the heparanase high cells indicating that activation of uPA is important for enhancing the invasive phenotype of these cells.

DISCUSSION

The present work reveals that heparanase upregulates the expression of two proteases known to drive an aggressive tumor phenotype. Transfection of heparanase into myeloma cells or addition of recombinant heparanase to wild-type myeloma cells enhances their expression of pro-MMP-9 in vitro and knockdown of heparanase expression in wild-type myeloma cells reduces levels of MMP-9 expression. When injected into mice, cells expressing high levels of heparanase retain their high level of MMP-9 expression and the protease becomes activated. Upregulation of heparanase enhances ERK phosphorylation and inhibition of ERK phosphorylation by addition of the MAPK/ERK pathway inhibitor PD98059 blocks MMP-9 expression. Moreover, we also found that uPA/uPAR, which is known to be functionally coupled to MMP-9, is also upregulated in response to an increase in heparanase expression. uPA/uPAR participated together with MMP-9 to enhance syndecan-1 shedding and also elevated tumor cell invasion. We also provided evidence that the effects of heparanase on ERK activation and protease expression are dependent on the presence of enzymatically active heparanase. Thus, these biological effects of heparanase occur downstream of heparan sulfate degradation. This is consistent with our previous finding that enzymatically active heparanase is required to enhance shedding of syndecan-1 (21). Together these findings reveal a novel biological pathway in which expression of heparanase increases ERK phosphorylation leading to upregulation of proteases that act to enhance syndecan-1 shedding. In addition, these proteases when present in the tumor microenvironment are known to be important in promoting tumor growth, angiogenesis and metastasis.

To our knowledge this is the first report linking heparanase expression with enhanced ERK signaling. Activation of the ERK pathway plays a major role in regulating cell growth, proliferation, differentiation and angiogenesis and provides a protective effect against apoptosis (40,41). It has been shown that ERK regulates these processes through modulation of strength or duration of ERK activation (40). Moreover, subtle difference in ERK phosphorylation can result in different biological outcomes (42). Exposure of some melanoma cells to exogenous heparanase enhances FGF2 binding to cells with a resulting upregulation of ERK phosphorylation (43). It is possible that by enhancing expression of heparanase in our myeloma cells, signaling via growth factors such as HGF is stimulated. HGF is a likely candidate because myeloma cells express HGF, and HGF is known to bind to syndecan-1 heparan sulfate and stimulate the ERK signaling pathway via the met receptor (44,45). ERK signaling appears to be critical for growth of myeloma tumors because inhibitors of the MAPK pathway can inhibit myeloma cell growth and osteoclast differentiation (46,47). It is interesting that we find that the activation of ERK requires the enzyme activity of heparanase. This suggests that stimulation of signaling occurs as the result of the clipping of heparan sulfate chains by heparanase. We know this clipping occurs in the myeloma cells expressing high levels of heparanase because the syndecan-1 has shorter heparan sulfate chains than those from cells expressing low levels of heparanase (19). Such clipping could “activate” the syndecan-1 by exposing cryptic epitopes within the heparan sulfate chains or the ectodomain core protein to facilitate interactions with ligands (48).

MMP-9 and uPA/uPAR are potent promoters of tumor growth and angiogenesis (39,49-51) and can work in concert to enhance tumor cell invasation, a rate limiting step for metastatic diffusion of cancer cells (38). In myeloma, there is evidence that MMP-9 and uPA/uPAR contribute to disease severity. High MMP-9 levels in myeloma are associated with disease recurrence and poor patient survival (52,53) and expression of uPA/uPAR is an independent factor predicting poor prognosis (54,55). Evidence suggests that MMP-9 and uPA/uPAR help promote the widespread dissemination of myeloma, a hallmark of this cancer. For example, MMP-9 produced by myeloma cells promotes their invasion across basement membranes in vitro (56,57) and there is evidence to suggest that inhibition of MMPs
including MMP-9 may have anti-myeloma effects (58). Inhibition of uPA inhibits invasion of myeloma cells (59) and uPA and MMP-9 have been shown to mediate invasion of the bone marrow extravascular compartment once cells have exited the marrow endothelium (60).

Enhanced MMP-9 and uPA/uPAR expression by myeloma cells may also facilitate the osteolytic phenotype that is responsible for much of the morbidity of this cancer. MMP-9 participates in the recruitment of osteoclasts to sites of bone resorption (61) and high levels of MMP-9 correlate well with bone turnover rate and is a useful prognostic index of bone disease (52,53). High levels of soluble uPAR are associated with poor prognosis and bone disease in myeloma patients (55) and interactions between osteoclasts and myeloma cells stimulate expression of MMP-9 and uPA possibly creating a microenvironment conducive to bone degradation (62).

In addition to the multiple effects that MMP-9 and uPA/uPAR have in myeloma tumor progression, the present work reveals that the mechanism for the heparanase-induced shedding of syndecan-1 is via upregulation of proteases. Shedding of syndecan-1 in myeloma is an important disease feature because the shed proteoglycan promotes tumor growth and metastasis (16,21). Similarly, like shed syndecan-1, heparanase also promotes tumor growth and metastasis and at least part of its pro-tumor effect may be due to its positive effect on syndecan-1 shedding. Also, consistent with these findings is the fact that high levels of shed syndecan-1 and heparanase in myeloma patients are indicators of poor prognosis (15,18). Shedding of syndecan-1 has been attributed to a number of metalloproteinase enzymes which include MMP-1, MMP-7, MMP-9, MMP-14 (MT1-MMP) and MMP-16 (MT3-MMP) (25-29). A role for MMP-9 as a sheddase has been demonstrated in several cancer types, where it can participate in shedding of various cell surface proteins such as E-cadherin and HB-EGF as well as syndecan-1 (24, 34, 35). However, to our knowledge, our data are the first to link uPA/uPAR to shedding of syndecan-1. The blocking antibody we used for these studies blocks uPA binding to uPAR, and thus blocks the proteolytic activation of uPA by uPAR. Activation of uPA initiates a cascade of proteolytic events where uPA activates plasmin, plasmin activates MMP-3 and then MMP-3 activates MMP-9 (37). Thus, it is likely that in the myeloma cells, uPA does not directly cleave the syndecan-1 ectodomain at the cell surface, rather it activates the cascade that results in MMP-9 activation and subsequent syndecan-1 shedding. This scenario is consistent with our finding that when both MMP-9 and uPAR blocking antibodies are added together to cells, the level of inhibition of shedding of syndecan-1 is the same as when antibodies are added singly (i.e., there is no additive effect when both antibodies are present).

Also it is interesting that addition of MMP-9 or uPA/uPAR inhibiting antibodies do not alter the constitutive level of shedding of syndecan-1 in cells that express low levels of heparanase. This suggests that upregulation of heparanase activates a shedding mechanism that is distinct from that mediating constitutive shedding. A number of factors (e.g., chemokines, EGF family growth factors) have been shown to enhance or activate shedding of syndecans via action on specific intracellular signaling pathways (63). For example, EGF-accelerated shedding of syndecans can be inhibited by the ERK inhibitor PD98059 (28). Similarly, we find that this inhibitor blocks the heparanase-mediated increase in MMP-9 levels in the myeloma cells, thus linking receptor activation to the downstream shedding that occurs in these cells.

Our data support a model in which upregulation of heparanase stimulates enhanced ERK signaling, thereby upregulating MMP-9 and uPA/uPAR expression which then catalyze shedding of syndecan-1 from the cell surface. But perhaps more importantly, the increase within the tumor microenvironment of these proteases can dramatically stimulate aggressive behavior of the tumors. This effect on protease expression adds to a growing list of functions of heparanase that includes the release of tumor promoting growth factors (64,65), promotion of signaling via the Akt pathway (10), stimulation of expression of VEGF and tissue factor and regulation of cell adhesion (2). Based on these data and the overwhelming correlation between heparanase expression and poor prognosis in many cancers, we propose that heparanase is a master regulator of the aggressive tumor phenotype and the onset of its expression marks a key defining event in tumor progression.
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FOOTNOTES

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1The abbreviations used are: ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; ELISA, enzyme-linked immunoabsorbant assay; FBS, fetal bovine serum; FGF2, fibroblast growth factor-2; FACS, fluorescence-activated cell sorting; HGF, hepatocyte growth factor; HPSE, heparanase; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PI3K, phosphoinositide-3 kinase; SCID, severe combined immunodeficient; shRNA, small hairpin RNA; siRNA, small interfering RNA; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor.

2A. Purushothaman and R. Sanderson, unpublished observation.

FIGURE LEGENDS

FIGURE 1. Heparanase enhances MMP-9 expression and activity in myeloma cells. A, Western blot of MMP-9 in serum-free conditioned media from CAG cells demonstrates that pro-MMP-9 protein levels are upregulated in CAG cells expressing high levels of heparanase as compared to cells expressing low levels of heparanase. (Heparanase high cells were transfected with vector containing the cDNA for human heparanase, heparanase low cells were transfected with empty vector (21)). Gelatin zymography of conditioned medium from either CAG or ARH-77 cells confirms western blotting data by showing an elevation in levels of MMP-9 activity in cells expressing high levels of heparanase. (Note: The MMP-9 seen in both the western blot and zymogram is the 92 kDa inactive pro-form of the enzyme rather than the cleaved 82 kDa active form. The gelatinolytic activity of MMP-9 routinely seen in zymograms is thought to be due to activation of the uncleaved enzyme resulting from the gel electrophoresis procedure.) B, CAG cells expressing heparanase that is mutated at amino acids 225 or 343 (M225, M343) and thus lacking heparan sulfate degrading activity do not show enhanced MMP-9 levels in zymograms as compared to cells expressing enzymatically active heparanase. C, Addition of recombinant heparanase to CAG cells enhances levels of MMP-9. Recombinant heparanase (rHPSE) was added to wild-type CAG cells at the indicated concentrations and conditioned medium was collected after 24 h and subjected to gelatin zymography. Quantification of MMP-9 gelatinolytic activity in these zymograms revealed an almost 3-fold higher level in cells treated with 10 ng/ml of recombinant heparanase vs. untreated cells. Shown are results of densitometric analysis of a single, representative gel.
FIGURE 2. Downregulation of heparanase reduces MMP-9 expression levels. A, Wild-type CAG myeloma cells were infected with lentiviral vectors coding for control or heparanase knockdown shRNAs. Analysis by both RT-PCR and western blotting from extracts of stably infected cells demonstrates effective knockdown of heparanase (HPSE) expression. B, Densitometric quantification of western blots and gelatin zymograms of MMP-9 levels in conditioned medium of control or heparanase knockdown cells indicates that MMP-9 protein and activity levels are reduced when heparanase is knocked down as compared to controls. Shown are results from single, representative gels.

FIGURE 3. Expression of enzymatically active heparanase enhances ERK phosphorylation and upregulation of MMP-9. A, Cell lysates from CAG cells expressing low or high levels of heparanase were subjected to immunoblotting with antibodies against the phosphorylated forms of Src, P-38 or ERK. Cell lysates from cells expressing the mutated, enzymatically inactive form of heparanase (M343) were also probed for ERK. Blots were subsequently stripped and probed for total Src, p38 or ERK. B, CAG cells expressing high levels of heparanase were treated with MAPK/ERK pathway inhibitor PD98059 (50 µM) and MMP-9 activity in the serum-free conditioned medium was assessed by gelatin zymography.

FIGURE 4. Heparanase induces expression of MMP-9 that becomes activated in vivo. A, CAG myeloma cells expressing low or high levels of heparanase were injected subcutaneously into SCID mice as previously described (19). After tumors formed, they were removed and immunostained for MMP-9 (original magnification, X 200). B, Western blot of an extract from a tumor formed by heparanase high cells demonstrates that MMP-9 is present predominantly in its enzymatically active (82 kDa) form.

FIGURE 5. MMP-9 mediates enhanced syndecan-1 shedding by heparanase high cells. A, CAG cells expressing low or high levels of heparanase were plated at equal density and 0.5 µg/ml of MMP-9 function blocking antibody 6-6B were added. After 48 hours, conditioned media were harvested and the level of shed syndecan-1 was determined by ELISA assay (values represents means of triplicate determination ± standard deviation). *, P<0.001 vs. HPSE high cells. B, Cells expressing high levels of heparanase were grown in the presence of varying levels of MMP-9 inhibitor 1 and syndecan-1 shedding measured by ELISA assay. *, P<0.05 vs. 0 nM; ** P<0.01 vs. 0 nM.

FIGURE 6. Enhanced expression of heparanase upregulates uPA and uPAR. A, Immunoblotting of serum-free conditioned media with an antibody to uPA or cell lysates with an antibody to uPAR or actin reveal upregulation of uPA/uPAR by cells expressing high levels of heparanase. B, Heparanase mediated upregulation of syndecan-1 shedding is inhibited by an antibody that blocks activation of uPA. Cells were plated at equal density and 5 µg/ml of antibody was added to cells expressing high levels of heparanase. After 48 hours conditioned media was harvested and the level of syndecan-1 was determined by ELISA assay (values represents means of triplicate determination ± standard deviation). *, P<0.001 vs. HPSE high cells. C, Myeloma invasion is enhanced by expression of heparanase and is blocked by the antibody that inhibits uPA activation. An equal number of CAG cells were seeded on invasion chambers coated with matrigel and cells allowed to migrate in the presence or absence of antibody. Cell invasion data represent the mean ± SD of three independent experiments. *, P<0.01 vs. HPSE high cells.
Figure 1

A

| Cell Line | Western blot | Zymogram |
|-----------|--------------|----------|
| CAG       | MMP-9 Low    | MMP-9 Low |
|           | High         | High     |


B

Heparanase Expression

MMP-9

C

MMP-9 Activity (% of control)

rHPSE (ng/ml): 0 1 10
Figure 2

A

HPSE
GAPDH

RT-PCR

Western blot

B

MMP-9 protein (% of control)

Control

HPSE Knockdown

MMP-9 activity (% of control)

Control

HPSE Knockdown
Figure 3

A

| Level of Heparanase Expression |
|-------------------------------|
| Low High | Low High | Low High M343 |
| Phospho | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| Total   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| Src     | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| p38     | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| ERK     | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |

B

| Inhibitor (PD98059) | + | − |
|---------------------|---|---|
| MMP-9               | ![Image](image16.png) | ![Image](image17.png) |
Figure 4

A

Heparanase Expression

|   | Low | High |
|---|-----|------|

Immunostain of tumors for MMP-9

B

Pro-MMP-9 (92kDa)  
Active MMP-9 (82 kDa)
Figure 5

A

Shed syndecan-1 (% of control)

HPSE Expression: Low, High, High + Anti-MMP-9

B

Shed syndecan-1 (% of control)

MMP-9 Inhibitor: 0nM, 5nM, 10nM

* *
**Figure 6**

A. HPSE Expression

| HPSE Expression | Low | High |
|-----------------|-----|------|
| uPA             | 52 kDa |     |
| uPAR            | 55 kDa |     |
| Actin           |      |     |

B. Shed syndecan-1 (% of control)

- HPSE Low
- HPSE High
- HPSE High + Anti-uPAR

C. Cells Invaded (x10^4)

- HPSE Low
- HPSE High
- HPSE High + Anti-uPAR
Heparanase stimulation of protease expression implicates it as a master regulator of the aggressive tumor phenotype in myeloma

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