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Heparanase enhances local and systemic osteolysis in multiple myeloma by upregulating the expression and secretion of RANKL

Yang Yang^{1,2}, Yongsheng Ren¹, Vishnu C Ramani¹, Li Nan¹, Larry J. Suva³, and Ralph D. Sanderson^{1,2}

¹Department of Pathology, University of Alabama at Birmingham, Birmingham, AL

²Comprehensive Cancer Center and the Center for Metabolic Bone Disease, University of Alabama at Birmingham, Birmingham, AL

³Department of Orthopaedic Surgery, Center for Orthopaedic Research, University of Arkansas for Medical Sciences, Little Rock, AR

Abstract

Excessive bone destruction is a major cause of morbidity in myeloma patients. However, the biologic mechanisms involved in the pathogenesis of myeloma-induced bone disease are not fully understood. Heparanase, an enzyme that cleaves the heparan sulfate chains of proteoglycans, is upregulated in a variety of human tumors, including multiple myeloma. We previously demonstrated that heparanase promotes robust myeloma tumor growth and supports spontaneous metastasis of tumor cells to bone. In the present study, we demonstrate, for the first time, that the expression of heparanase by myeloma tumor cells remarkably enhances bone destruction locally within the tumor microenvironment. In addition, enhanced heparanase expression in the primary tumor also stimulated systemic osteoclastogenesis and osteolysis, thus mimicking the systemic osteoporosis often seen in myeloma patients. These effects occur, at least in part, as the result of a significant elevation in the expression and secretion of RANKL by heparanase-expressing myeloma cells. Moreover, analysis of bone marrow biopsies from myeloma patients reveals a positive correlation between the level of expression of heparanase and RANKL. Together these discoveries reveal a novel and key role for heparanase in promoting tumor osteolysis and demonstrate that RANKL is central to the mechanism of heparanase-mediated osteolysis in myeloma.

Keywords

Heparanase; osteolysis; multiple myeloma; RANKL; tumor microenvironment

Introduction

Multiple myeloma is a hematologic B cell malignancy and is the most frequent cancer to involve the skeleton, with up to 90% of patients developing destructive bone lesions (1). Myeloma-induced bone disease is associated with bone pain, fractures, and hypercalcemia which affect quality of life and lead to increased morbidity in myeloma patients. Numerous studies have shown that bone disease in myeloma is caused by elevated bone resorption, which is stimulated by the activation of osteoclasts and the inhibition of osteoblasts (2-4). However,

Correspondence: Yang Yang, Department of Pathology, University of Alabama at Birmingham, 1530 Third Avenue South, SHEL 816, Birmingham, Alabama 35294, Telephone (205) 996-6228, Fax (205) 996-6119, yangyang@uab.edu.

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the specific components and mechanisms responsible for driving the elevated bone resorption have not been fully determined.

Heparanase-1 (heparanase, HPSE) is the only known enzymatically active endoglycosidase that degrades heparan sulfate at specific intrachain sites (5,6). Expression of heparanase is rare in normal tissues, but it is upregulated in many human tumors, including myeloma (7-13). Upregulation of heparanase has been correlated with increases in lymph node metastasis, distant metastasis, microvessel density, and reductions in post-operation survival of several types of cancer – all of which provide strong clinical support for the pro-metastatic and pro-angiogenic features of the enzyme (11,14-17). Upregulation of heparanase expression is associated with poor prognosis in myeloma (18) and we previously demonstrated that elevated expression of heparanase in myeloma cells dramatically enhances their growth, angiogenesis, and metastasis to bone (19-22). However, regarding the role of heparanase in regulating bone turnover, there are conflicting reports in the literature. Expression of heparanase by breast cancer cells implanted in the mammary fat pad of mice enhances systemic bone turnover (23,24). A limited descriptive study using a murine model of prostate cancer suggested a role for heparanase in enhancing osteolysis (25). However, in this study heparanase also increased tumor growth and it was not determined if the increased osteolysis was due to heparanase or simply due to an increase in the tumor size. In contrast to the proposed role of heparanase in osteolysis, it was discovered that mice transgenic for heparanase exhibited a marked increase in bone density as compared to controls, suggesting that heparanase may promote bone formation (26). To address the role of heparanase in tumor osteolysis we utilized two murine models of myeloma as well as biopsies from myeloma patients. We discovered that the expression of heparanase by tumor cells induces significant osteolysis both locally and systemically and that this was not solely a function of increased tumor size. Furthermore, we found that elevated levels of heparanase lead to the upregulation of the expression of receptor activator of nuclear factor κ B ligand (RANKL) in myeloma cells with a resulting increase in osteoclastogenesis, thus defining a mechanism whereby heparanase expression can drive the destruction of bone in myeloma. These novel findings point to heparanase as a key regulator of myeloma bone disease that is upstream of RANKL and further validate heparanase as a viable target for osteolytic cancers.

Methods and materials

Cells and reagents

CAG myeloma cell line was established at the Myeloma Institute for Research and Therapy (Little Rock, AR) as described previously (27). CAG cells with modified levels of heparanase expression have been previously extensively characterized (19,20,28) and include i) heparanase-low (HPSE-low) cells prepared by transfection with empty vector; ii) heparanase high (HPSE-high) cells prepared by transfection with vector containing the cDNA for human heparanase (Although HPSE-high cells express a 4 fold-higher level of heparanase than do the HPSE-low cells, the elevated levels of enzyme activity present in HPSE-high cells is in the same range as that present in the bone marrow of many myeloma patients (21). This indicates that HPSE-high cells very closely mimic the level of heparanase activity present in many myeloma patients and thus represent an appropriate model for examining the effects of heparanase on myeloma tumors); iii) cells transduced with viral vectors containing a control shRNA sequence; and iv) HPSE k/d cells transduced with viral vectors containing a shRNA sequence to knockdown heparanase expression. U266 cells were obtained from ATCC, (Manassas, VA) and MM.1S human myeloma cells were generously provided by Dr. Steve T. Rosen and Dr. Nancy Krett (Northwestern University). During the course of this study the CAG cells were confirmed as myeloma cells by their expression of CD138 and kappa immunoglobulin light chain.

Recombinant heparanase (rHPSE) and heparanase antibodies were kindly provided by Dr. Israel Vlodavsky (Technion, Haifa, Israel). Human kappa antibody and kappa ELISA kit were purchased from Bethyl Laboratories (Montgomery, TX); TRAP staining kit and β -actin antibody were from Sigma (St Louis, MO); Human TRAP 5b ELISA kit was from Immunodiagnostic Systems (Fountain Hills, AZ); Human RANKL and OPG antibodies were from R&D Systems (Minneapolis, MN); and human RANKL and OPG ELISA kits were from ALPCO (Salem, NH).

***In vivo* models of myeloma**

5- to 6-week-old male CB.17 scid/scid mice obtained from Harlan Sprague Dawley (Indianapolis, IN) were used in the studies. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee.

SCID-hu model—The SCID-hu model has been previously described (19,29,30). Briefly, human femora (Advanced Bioscience Resources, Inc., Alameda, CA) were cut into halves and implanted subcutaneously, one on each side of the dorsum of the SCID mice. 6-8 weeks after implantation, 10^5 CAG HPSE-low or HPSE-high cells were injected directly into the cut end of one human bone graft in each SCID-hu mouse. Murine sera were collected bi-weekly. After mice were euthanized, tumor-injected human bones and non-injected contralateral human bones were collected and evaluated for the extent of bone resorption. Final tumor burden in each mouse was determined by measurement of human kappa light chain in mouse serum harvested just prior to euthanasia.

SCID-tibia model— 10^5 CAG HPSE-low or HPSE-high cells were injected into the right-side tibia of SCID mice. Murine sera were collected every two weeks. After mice were euthanized, tumor-injected tibia and non-injected contralateral tibia were evaluated for the extent of bone resorption.

Evaluation of bone resorption and osteoclast number

Implanted human bones (SCID-hu model) or murine tibia (SCID-tibia model) were fixed in 10% neutral-buffered formalin, bone microarchitecture and density was assessed by microCT (Scanco Medical AG, Bassersdorf, Switzerland) as described previously (31). After imaging, the bones were decalcified with 10% EDTA, embedded in paraffin and sectioned. Bone sections were stained using a TRAP staining kit (19). Osteoclast number was determined via counting of TRAP positive multinucleated osteoclasts (3 or more nuclei per cell) adjacent to the bone surface as described previously (23). In addition, the levels of human TRAP 5b in murine sera of SCID-hu mice were measured using a human TRAP 5b ELISA kit.

Immunohistochemistry

Human kappa, heparanase, RANKL and OPG were stained on paraffin-embedded tissue sections harvested from animal models following the manufacturer's recommendation (19, 28).

Paraffin-embedded bone marrow core biopsy specimens of myeloma patients, obtained from the Department of Pathology at UAB, were also stained for heparanase, RANKL and OPG. The experimental procedures and protocols were approved by the UAB Institutional Review Board. Scoring for staining densities was determined in a blinded fashion by two different readers including a board certified hematopathologist as described previously (21).

Real-Time PCR

RNA was extracted from cultured cells using Qiagen RNeasy columns (Qiagen, Valencia, CA) and equal amounts were reverse transcribed using RevertAid First Strand cDNA synthesis kit (Fermentas, Hanover, MD). For real-time PCR 2X IQ™ SYBR® green supermix (Bio-Rad), along with 25ng of cDNA from each sample and gene specific primers were used. The primers and the cycle parameters for real-time PCR analysis of human RANKL (32) and the internal control 18S ribosomal RNA (33) have been published previously. The PCR cycle at which the fluorescence exceeded a set threshold (C_T), was determined by the iCycler software and data were analyzed according to the comparative C_T method (34). Semiquantitative results represent the n-fold difference of normalized human RANKL transcript levels.

Western blotting

Equal amounts of protein from cell or tumor tissue extract were subjected to 4-12% gradient SDS-PAGE (BioRad) and transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) (19). They were then probed with anti-human RANKL or OPG or β -actin antibodies and were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Enzyme-linked immunosorbent assay (ELISA)

Human immunoglobulin kappa light chain in murine sera was measured using human kappa ELISA kit (29). RANKL levels in conditioned media of HPSE-low and HPSE-high cells, as well as U266 and MM.1S myeloma cells treated with rHPSE were measured using human RANKL ELISA kit. Though heparanase transfection does not affect cell proliferation in vitro (19,28) we seeded an equal number of cells at the beginning of each experiment and counted them again when the conditioned medium was collected. RANKL levels in the media were then normalized to cell number.

Osteoclast differentiation assay

The effect of medium conditioned by HPSE-low and HPSE-high CAG cells on osteoclastogenesis was determined as previously described (23). Medium was harvested 48 hours after cell plating, diluted 50% with α MEM, and added to the cultures of human osteoclast precursors. Macrophage colony-stimulating factor (mCSF; 25 ng/mL) was present in all groups. rRANKL (25 ng/mL) was used as a positive control. Cell cultures were maintained at 37°C and half of the medium in each well was replaced with fresh medium thrice weekly. After 10 days of culture, the cells were fixed with 10% formalin. TRAP staining was utilized for quantification of multinucleated osteoclasts (23).

Statistical analysis

Statistical significance between each two experimental groups was analyzed by Student's t test, and the correlations between heparanase and RANKL expression and between heparanase and OPG expression in MM patients' samples were assessed using Spearman correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Heparanase dramatically enhances osteolysis locally and systemically in animal models of multiple myeloma

To explore the effect of heparanase expression in myeloma related bone disease, two animal models were utilized: the SCID-hu model in which myeloma tumor cells are grown within human bone implanted in mice, and the SCID-tibia model in which tumor cells are injected directly into the tibia of SCID mice. In SCID-hu mice, although tumors were allowed to grow

to a larger size in the HPSE-low group than in the HPSE-high group (as shown on x-ray image and as determined by human kappa light chain analysis, Fig. 1A, left panel), bones injected with HPSE-low cells retained a normal spongy appearance with only minor osteolysis (arrows mark osteolytic areas, Fig. 1A, middle panel). In contrast, bones injected with HPSE-high cells showed massive destruction. Thus, the elevated bone destruction in the animals bearing tumors formed from HPSE-high cells was not simply due to high tumor burden. Rather, it points to a key role for heparanase in the osteolytic phenotype. In addition to the human bone that was injected with tumor cells, there was a second human bone implanted in each animal that did not receive injection of tumor cells. Importantly, in the animals bearing tumors formed by HPSE-high cells, extensive osteolysis also appeared in this uninjected bone. In contrast, uninjected bones from animals bearing HPSE-low tumor cells exhibited no evidence of osteolysis (Fig. 1A, right panel). Immunohistochemical staining for human kappa light chain, which specifically detects myeloma cells, revealed no detectable tumor cells in these uninjected contralateral bones (data not shown). Together these results indicate that heparanase dramatically enhances osteolysis within the tumors growing in bone and can also stimulate osteolysis in distal bones prior to the arrival of tumor cells at those sites.

To confirm these findings in a different animal model, we implanted tumor cells directly within the tibia of mice (SCID-tibia model). HPSE-high cells again induced severe bone destruction, whereas little damage was observed in bones injected with HPSE-low cells (Fig. 1B, left panel). MicroCT analyses of the contralateral non-injected tibia demonstrated that in mice bearing HPSE-high tumors, the bone volume, trabecular number and connectivity density were all significantly decreased, while trabecular spacing was increased compared to mice bearing HPSE-low tumors (Fig. 1B, right panel). Immunostaining for human kappa light chain confirmed the absence of metastasized tumor cells in the distal tibias.

The enhanced bone destruction in both injected and contralateral bones in two animal models suggest that heparanase activates both local and systemic upregulation of osteoclastogenesis *in vivo*. To test this idea, the number of osteoclasts in bones harvested from SCID-hu animals was assessed by TRAP staining. In addition, we also measured levels of human TRAP 5b (a soluble marker that reflects the level of human bone resorption (23)) in the serum of SCID-hu mice bearing tumors. The number of TRAP positive cells (osteoclasts) in both the injected bone and the contralateral bone were significantly higher in animals injected with HPSE-high cells compared to animals with HPSE-low cells (Fig. 2A and 2B). Similarly, levels of human TRAP 5b were also markedly elevated in the serum of SCID-hu mice bearing HPSE-high tumors (Fig. 2C), whereas murine soluble TRAP levels were unchanged. These results demonstrate that the expression of heparanase by the myeloma tumor cells promotes bone resorption by driving an increase in both local and systemic osteoclastogenesis.

RANKL expression is enhanced in myeloma tumors formed by cells expressing high levels of heparanase

Because RANKL is a primary stimulator of osteoclastogenesis, we compared the level of RANKL expression in HPSE-low and HPSE-high tumors. Immunostaining of human bones injected with tumor cells (SCID-hu mice) revealed that the HPSE-high tumors had much higher RANKL levels than the tumors formed by HPSE-low cells (Fig. 3A). In addition, western blots confirmed a higher level of RANKL protein was present in subcutaneous tumors formed by the HPSE-high cells as compared to HPSE-low cells (Fig. 3B and 3C). This demonstrates that RANKL expression is enhanced in HPSE-high tumors even if they are not growing within the bone marrow microenvironment. Interestingly, levels of osteoprotegerin (OPG, the RANKL competitive inhibitor) were not significantly altered in HPSE-high tumors (Fig. 3B and 3C).

Heparanase enhances RANKL expression and secretion in myeloma cells

Real-time PCR and western blots of the extracts from HPSE-low and HPSE-high cells growing *in vitro* confirmed that RANKL expression was increased in HPSE-high cells at both mRNA and protein levels, compared to HPSE-low cells (Fig. 4). Furthermore, RANKL expression was decreased in CAG cells following knockdown of heparanase. Note that levels of RANKL expressed by the HPSE-high CAG cells are even higher than those expressed by Saos-2 cells. This is important since Saos-2 cells are known to express high levels of RANKL and often serve as a positive control for RANKL assays (35). Thus, the heparanase-expressing CAG cells are making substantial amounts of RANKL as indicated by the immunohistochemistry results shown in Fig. 3.

Analysis of medium conditioned by CAG cells demonstrated that RANKL secretion was elevated in the medium of HPSE-high cells and reduced in the medium of HPSE-knockdown cells, compared to the corresponding controls (Fig. 4D). OPG was undetectable in either the cells or the conditioned medium, confirming that myeloma cells do not express OPG, which is consistent with previously published data (2,36). In addition, IL-8, a chemokine known to promote osteoclastogenesis, was not detectable by ELISA of cell extracts or condition medium of either CAG HPSE-low or HPSE-high cells (data not shown).

To ensure that the heparanase induced upregulation of RANKL in CAG cells was not cell type-specific or a non-specific consequence of cell transfections or viral infection, we also determined the effects of heparanase on myeloma cell lines U266 and MM.1S by treating these cells with recombinant human heparanase (rHPSE). Previous studies have shown that rHPSE when added exogenously to cells is taken up by the cells and remains biologically active (20, 28,37). As shown in Fig. 5, after the addition of rHPSE, the expression and secretion of RANKL was significantly increased in both U266 and MM.1S cells, confirming findings with the CAG myeloma cells.

Conditioned medium from HPSE-high cells induces osteoclastogenesis via RANKL

The finding that osteolysis is enhanced in tumor free bones that are contralateral to bones injected with tumor cells in both SCID-hu and SCID-tibia animals (Fig. 1) suggests that osteoclastogenesis is being enhanced by a factor or factors released into the systemic circulation by HPSE-high cells. To test this idea, *in vitro* osteoclastogenesis assays were utilized to measure the osteoclastogenic activity of conditioned medium from HPSE-high and HPSE-low CAG myeloma cells. The results revealed that the medium conditioned by HPSE-high cells significantly enhanced osteoclastogenesis *in vitro* as compared to medium from HPSE-low cells (Fig. 6). Moreover this enhancement was significantly inhibited by addition of OPG, thus confirming that the enhanced osteoclastogenic activity in the medium is mediated by RANKL (Fig. 6).

The level of RANKL expression correlates with the level of heparanase expression in tumor cells growing in the bone marrow of myeloma patients

Immunohistochemical staining of heparanase and RANKL was performed on 20 bone marrow core biopsy specimens of myeloma patients. A significant positive correlation ($p < 0.0001$) between expression of heparanase and RANKL by the myeloma tumor cells was observed (Fig 7 and table 1 shown in Supplemental material). Interestingly, the subcellular localization of heparanase and RANKL within the bone marrow myeloma cells also correlated significantly ($p < 0.0001$). Variable expression of heparanase and RANKL was also noted on bone marrow cells including osteoblasts (data not shown). As expected, the expression of OPG was detected in bone marrow cells but not in myeloma cells. However, there was no significant correlation between the expression of heparanase and OPG within the bone marrow ($p = 0.22$).

Discussion

Although several studies have implicated heparanase in playing a role in bone turnover, definitive studies examining the role of heparanase in osteolytic cancers have not been reported (23-25). We demonstrate in the present work that when tumors are growing in bone and expressing high levels of heparanase, there is a marked increase in both systemic and local osteolysis as compared to controls. This increased osteolysis is not the result of an increase in the tumor size mediated by the heparanase. Rather, it is due, at least in part, to an increase in expression and secretion of RANKL leading to enhanced osteoclastogenesis. Evidence of a role for RANKL in heparanase driven osteolysis includes the detection of high levels of RANKL within tumors formed by heparanase-high CAG myeloma cells and the fact that elevated RANKL in medium conditioned by heparanase-high CAG cells drives osteoclastogenesis *in vitro*. Enhanced osteoclastogenesis in animals bearing heparanase-high tumors was confirmed by the presence of elevated levels of TRAP5b and by the presence of elevated numbers of osteoclasts both at the injected tumor site (primary tumor) and in distal bones where no tumor cells were injected or detected. In fact, the measurements of osteoclast number per bone surface are likely underrepresented, because in the heparanase-high CAG tumors, bone destruction is rampant and little bone (with resident osteoclasts) remain, thus contributing to the wide variation in osteoclast numbers observed (although the difference is still significant).

Multiple signaling pathways are involved in RANKL expression and we do not yet know how the expression of heparanase upregulates RANKL levels. We have previously examined the activation status of several signaling mediators—including Src, p38 MAPK, and ERK—in HPSE-low and HPSE-high cells. We found that levels of phosphorylated Src and phosphorylated p38 were not affected by an elevation in heparanase expression; but ERK activation was significantly greater in the HPSE-high cells (28). Thus, this upregulation of ERK signaling may be involved in heparanase-induced RANKL expression in myeloma cells. This and other possibilities await further investigation.

Furthermore, the analysis of primary human myeloma bone marrow biopsies confirmed that patients having high levels of heparanase expression also express higher levels of RANKL than do patients expressing low levels of heparanase. These clinical correlations are important since osteolytic bone disease is found in over 80% of newly diagnosed myeloma patients, is the most debilitating manifestation of this cancer and is a strong indicator of poor overall survival in myeloma (38,39). Moreover, as a consequence of tumor osteolysis, factors may be released that drive tumor growth and further stimulate disease progression (3). The discovery of a role for heparanase in the cascade of events leading to osteolysis underscores its important role in regulating the tumor microenvironment and contributes to our understanding of the contribution of heparanase with poor prognosis in myeloma (18).

Our finding that heparanase enhances RANKL expression is consistent with the elevation of RANKL and systemic osteolysis observed in many myeloma patients. The hyperstimulation of osteoclastogenesis that occurs in myeloma is primarily mediated via RANKL. In myeloma, both the myeloma tumor cells and the bone marrow stroma of patients exhibit elevated levels of RANKL expression (40-45). The high levels of membrane-associated RANKL by myeloma cells have been correlated with the presence of multiple bone lesions in myeloma patients (44,45). In addition, a soluble form of RANKL, released from tumor cells by protease activity, can diffuse away from the local tumor microenvironment to promote widespread osteoclast activation, thereby likely contributing to the systemic bone loss that is also a feature of myeloma (46). This idea is consistent with the present study where both local and systemic osteolysis is observed following upregulation of RANKL by the tumors formed from heparanase-high cells. Interestingly, we observed that in heparanase-high tumors growing in bone (SCID-hu model)

there were much higher levels of RANKL than in heparanase-high tumors growing subcutaneously (Figure 3). This finding indicates that the increased expression of heparanase by myeloma cells may also affect bone marrow stromal cells and osteoblasts, resulting in increased RANKL expression from these cells. These possibilities remain to be explored.

The relationship between elevated heparanase expression and increased myeloma osteolysis is of significance because most patients express active heparanase enzyme within their bone marrow (21). Expression of heparanase within the myeloma bone marrow cells has been demonstrated by Affymetrix gene arrays where 92% of the 39 patients tested expressed heparanase mRNA in whole bone marrow samples (18). This study confirmed the presence of heparanase expression by myeloma tumor cells and demonstrated that heparanase was also expressed by monocytes and osteoclasts within the bone marrow-tumor microenvironment. As demonstrated in Fig. 5, heparanase released by cells can be taken up and utilized by neighboring cells. Thus, it is conceivable that bone marrow-derived heparanase in patients may be taken up by neighboring myeloma tumor cells where it stimulates RANKL expression. Interestingly, in a previous study, upregulated heparanase expression was also detected in osteoclasts in a murine model of bone fracture repair (47). Thus, during osteolysis that is characteristic of myeloma bone disease, heparanase produced directly by osteoclasts may contribute to events leading to bone resorption.

Our finding that heparanase stimulates RANKL expression, leading to both local and systemic osteolysis adds another important function of heparanase in regulating the myeloma microenvironment and promoting the aggressive disease phenotype. It will be important to determine if inhibitors of heparanase which have been shown to block myeloma growth (30) can also interfere with myeloma-induced osteolysis *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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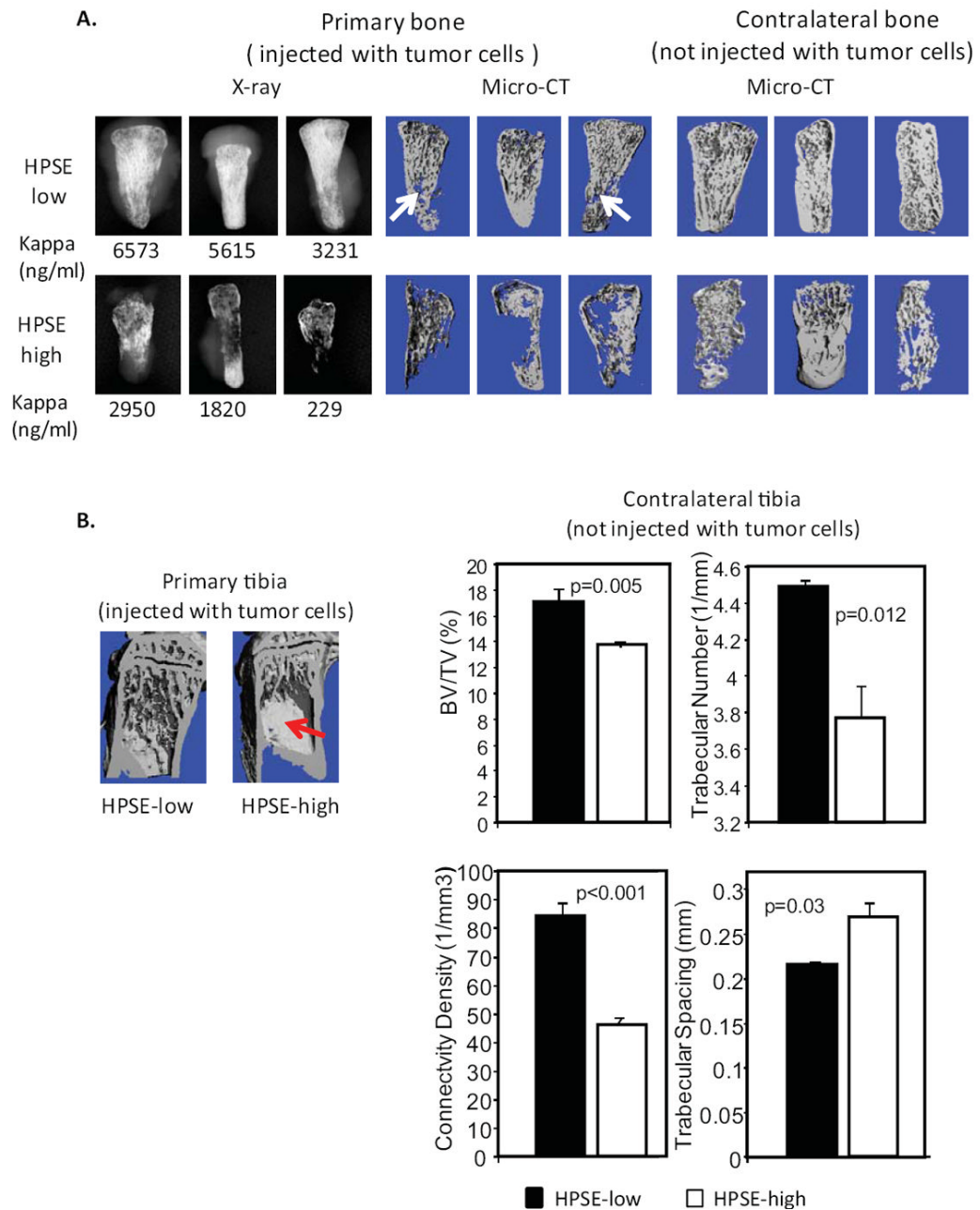


Figure 1. Elevated expression of heparanase by myeloma cells dramatically enhances osteolysis locally and systemically in animal models

A. Imaging of human bones harvested from the SCID-hu model (7 mice per group). Left panel: X-ray image showing three most highly degraded bones from each group that were injected with either HPSE-low or HPSE-high cells. Numbers below the images are the ng/ml of human kappa light chain present in the murine serum, which reflects the whole animal tumor burden. Middle panel: MicroCT imaging of the same bones shown in the x-ray. Right panel: human bones contralateral to those shown in the left panel. **B.** Left panel: MicroCT imaging of a primary murine tibiae injected with tumor cells (HPSE-low and HPSE-high) Significant bone destruction is observed only in the tibia bearing HPSE-high tumor cells (red arrow, note loss of trabecular architecture) (5 mice per group). Right panel: MicroCT microarchitectural

analyses of contralateral tibiae of mice injected with either HPSE-low or HPSE-high tumor cells. Significant decreases in bone volume/total volume (BV/TV), trabecular number, connectivity density, and increased trabecular spacing are observed in animals bearing HPSE-high tumors, as compared to animals bearing HPSE-low tumors.

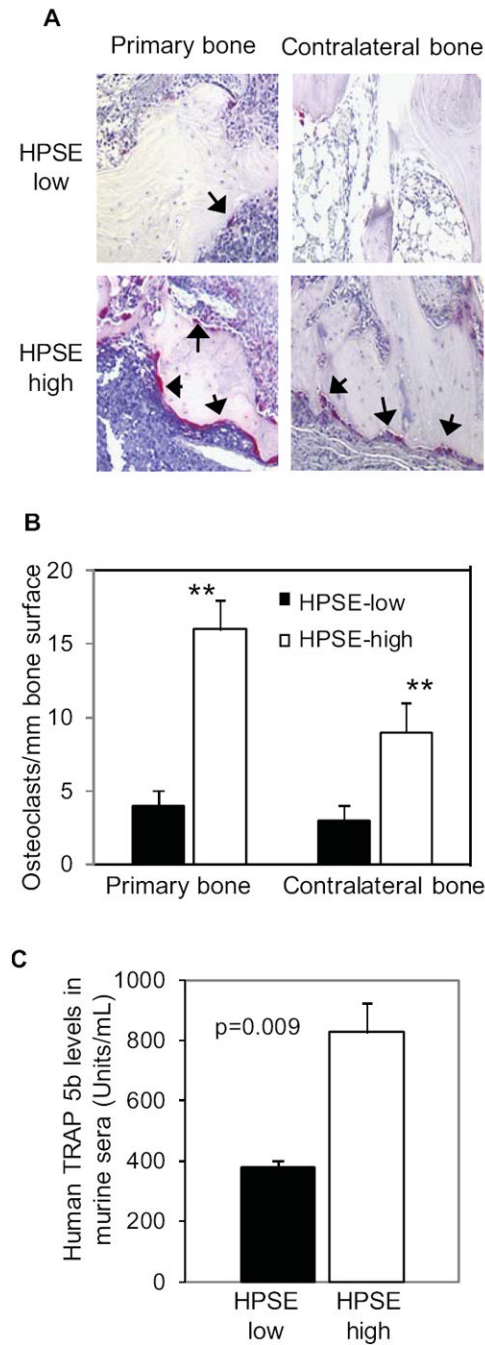


Figure 2. Osteoclastogenesis is enhanced in mice bearing HPSE-high tumors

A. TRAP staining was performed on the primary and contralateral bones harvested from SCID-hu mice. The purple-red stained cells are osteoclasts (indicated by arrows; original magnification 200x). **B.** Numbers of TRAP-positive osteoclasts in human bones harvest from the SCID-hu model were determined. ** $p < 0.01$ for HPSE-high compared with HPSE-low. **C.** TRAP 5b present in the murine sera is significantly higher in animals bearing HPSE-high tumors compared with HPSE-low tumors.

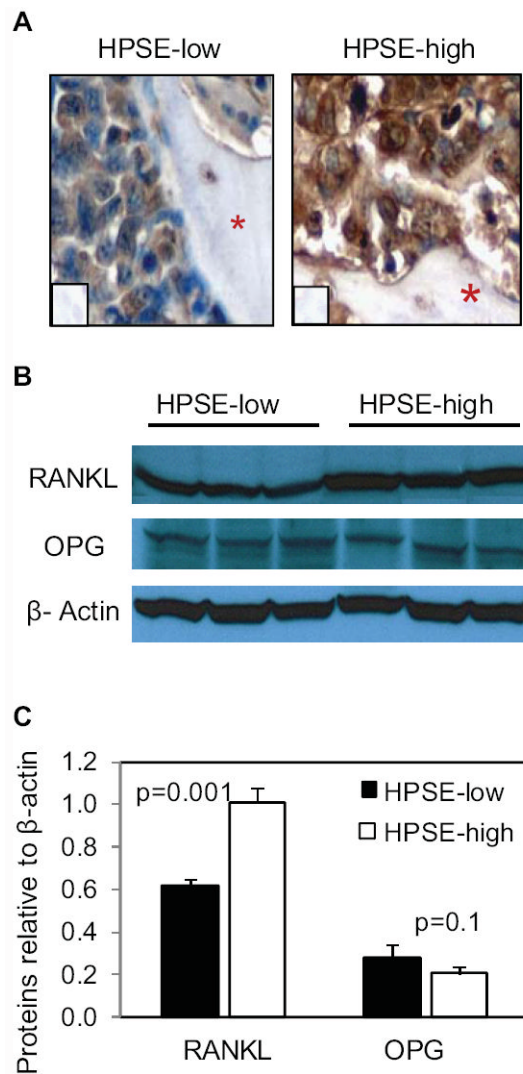


Figure 3. RANKL expression is elevated in tumors formed by HPSE-high cells

A. Human RANKL staining within human bones from SCID-hu mice bearing tumors formed by HPSE-low or HPSE-high cells. The red asterisks denote bone and inserts are controls lacking addition of primary antibody (original magnification 400x). **B.** Primary myeloma tumors formed by HPSE-low or HPSE-high cells growing subcutaneously in SCID mice were extracted with detergent and western blotting was performed for detection of RANKL, OPG, and β -actin. Each lane of the blot represents tumor from a different animal. **C.** The bands from western blots were quantified relative to β -actin using ImageJ software. The bars represent means \pm SD.

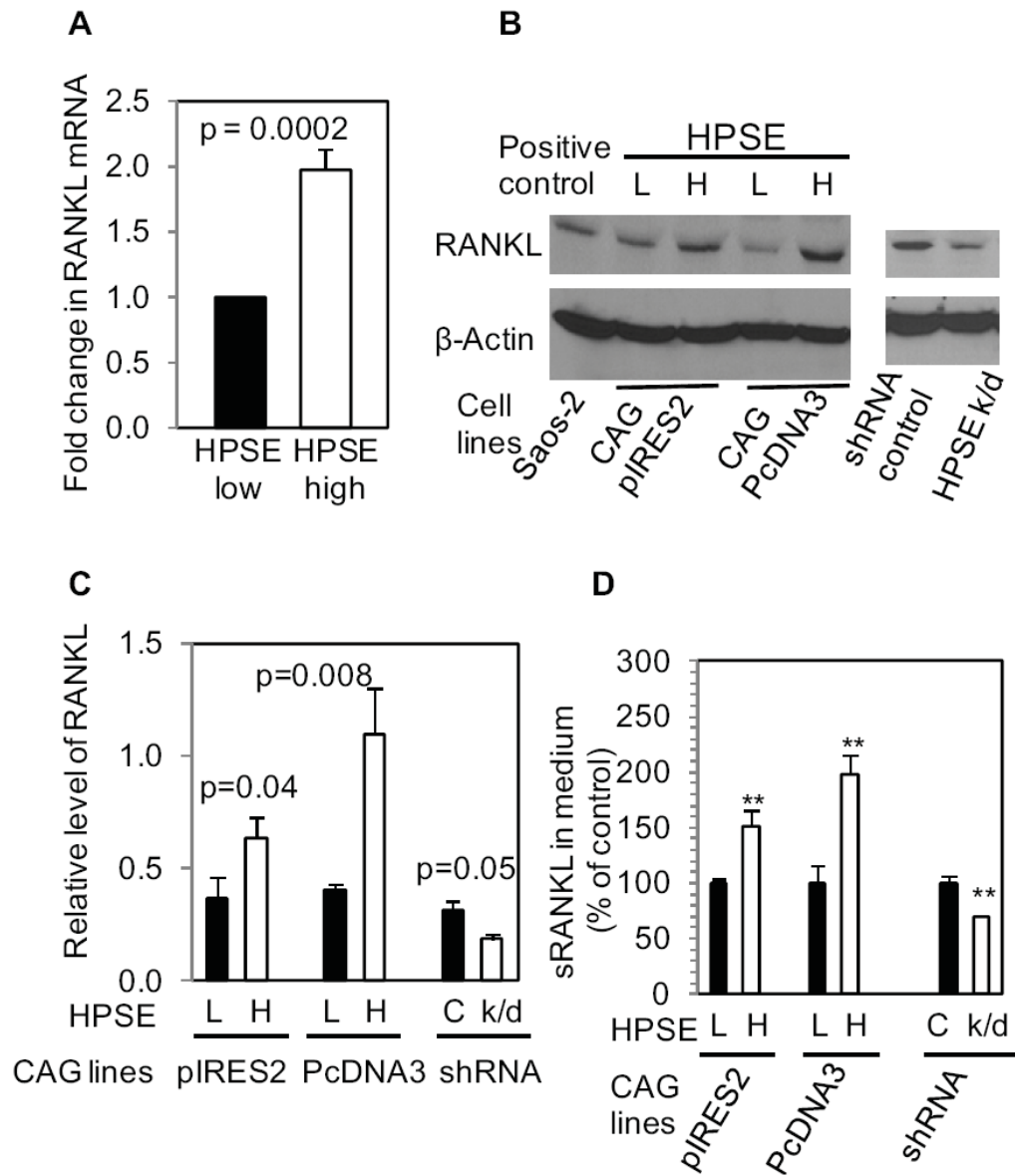


Figure 4. Heparanase upregulates RANKL expression and secretion in myeloma cells
A. Real-time PCR for human RANKL. The graph is representative of three individual experiments performed in triplicate. **B.** CAG cells from two different heparanase transfections using different vectors (pIRES2 or pcDNA3) or from heparanase knock-down (HPSE k/d) cells were lysed and probed for human RANKL by Western blotting((HPSE-low (L), HPSE-high (H), HPSE knock-down (k/d)). **C.** Quantification of the RANKL from Western blots. Each bar graph represents an average reading number of RANKL/β-actin from 3 independent experiments. **D.** RANKL levels in conditioned medium were determined by ELISA. The bar graph shows the changes in RANKL levels in HPSE-high cells or HPSE k/d cells as the percentage of control. The results (representative of 3 independent experiments) are means ± SD, **p < 0.01 compared to controls.

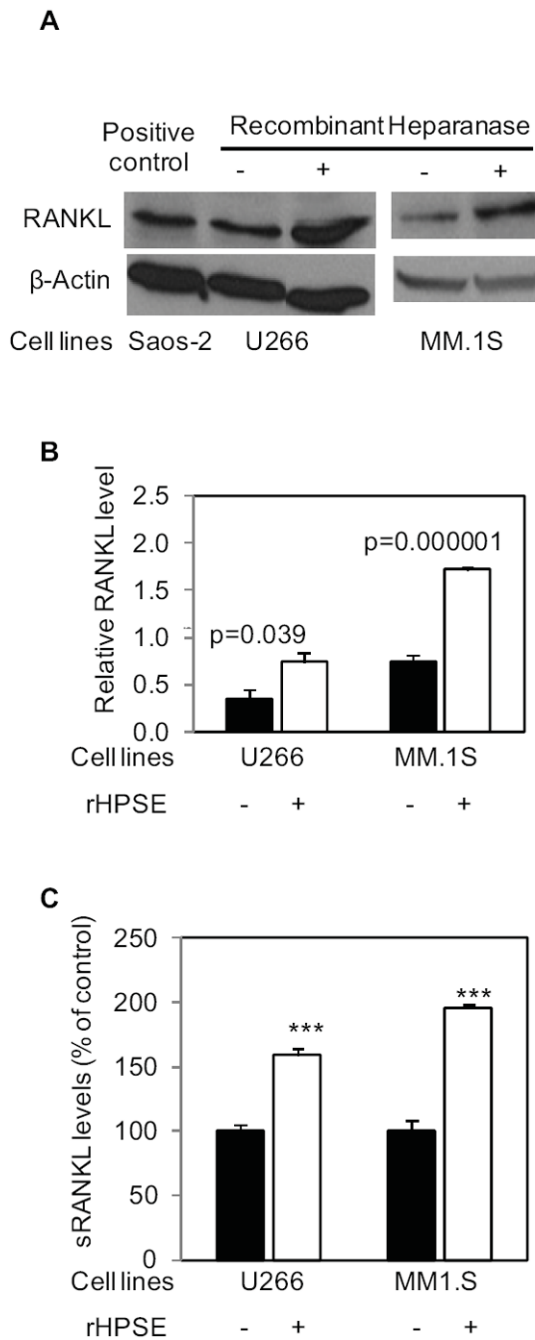


Figure 5. Recombinant heparanase (rHPSE) induces RANKL expression and secretion in myeloma cells

Equal numbers of human myeloma U266 or MM.1S cells were grown in complete medium in the absence or presence of rHPSE (100 ng/ml, added every 12 hours) for 48 h. The cells were lysed for western blotting, and the conditioned media were harvested for ELISA. **A.** Western blots were probed with antibody to human RANKL or β -actin. Saos-2 cell lysate was used as a positive control for human RANKL. **B.** RANKL bands from western blots were quantified using ImageJ and normalized to the corresponding β -actin bands. Each bar graph represents an average quantitative result of two independent experiments with two western blots. **C.** RANKL ELISA of conditioned media. The graph shows the percentage increase in RANKL

present in medium conditioned by cells treated with rHPSE compared to control treated cells. The results are means \pm SD from 2 independent experiments, *** $p < 0.001$ to the controls.

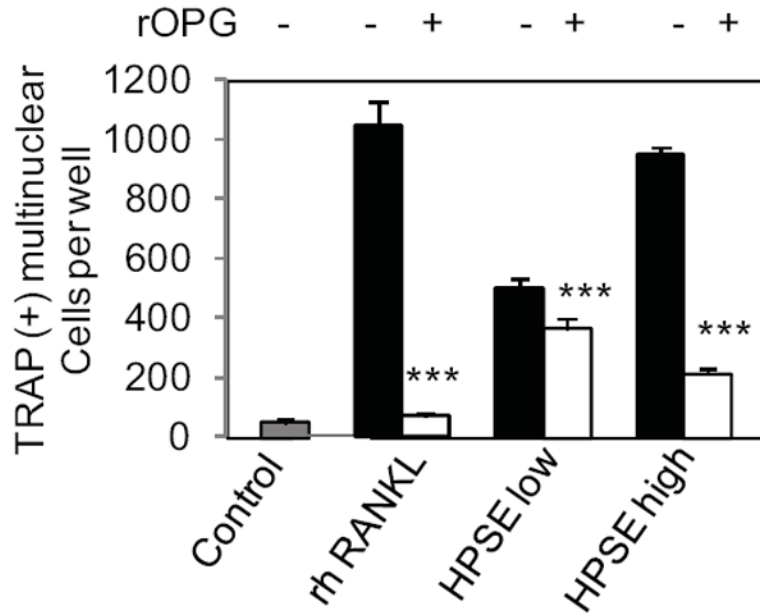


Figure 6. Medium conditioned by CAG HPSE-high cells induces osteoclastogenesis via increased RANKL

Human PBMCs were cultured in conditioned medium from CAG cells expressing either low or high levels of heparanase (HPSE-low or HPSE-high) in the presence or absence of OPG. Addition of recombinant human (rh) RANKL was used as a positive control. The negative control was cells cultivated only with DMEM and mCSF. *** p<0.001 compared to “no OPG” group.

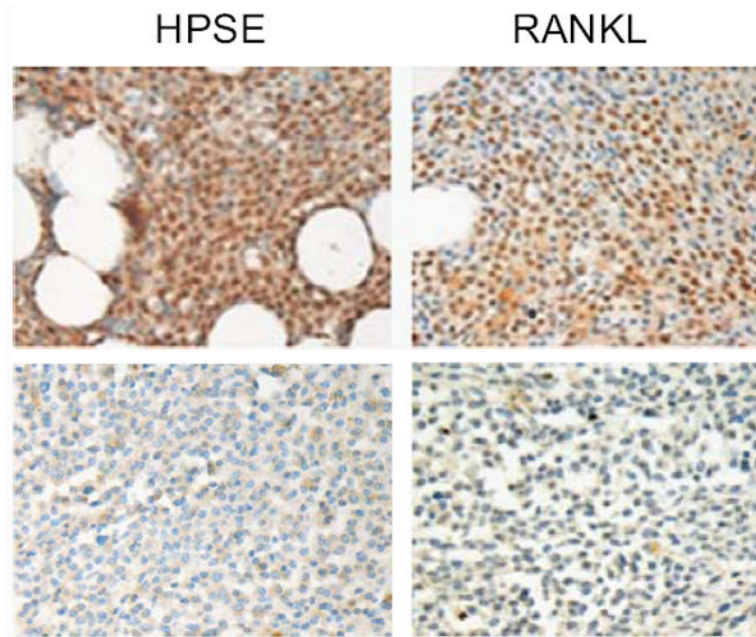


Figure 7. Myeloma tumor cell expression of heparanase correlates with the level of RANKL in the bone marrow of myeloma patients

Heparanase and RANKL staining were performed on 20 of bone marrow biopsy specimens from myeloma patients. Myeloma cells expressing high level of heparanase show elevated RANKL expression (original magnification 400x).