Antibodies Neutralizing Hepsin Protease Activity Do Not Impact Cell Growth but Inhibit Invasion of Prostate and Ovarian Tumor Cells in Culture

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Abstract

Hepsin is a type II transmembrane serine protease that is expressed in normal liver, and at lower levels in kidney, pancreas, and testis. Several studies have shown that hepsin mRNA is significantly elevated in most prostate tumors, as well as a significant fraction of ovarian and renal cell carcinomas and hepatomas. Although the overexpression of mRNA in these tumors has been extensively documented, there has been conflicting literature on whether hepsin plays a role in tumor cell growth and progression. Early literature implied a role for hepsin in human tumor cell proliferation, whereas recent studies with a transgenic mouse model for prostate cancer support a role for hepsin in tumor progression and metastases. To evaluate this issue further, we have expressed an activatable form of hepsin, and have generated a set of monoclonal antibodies that neutralize enzyme activity. The neutralizing antibodies inhibit hepsin enzymatic activity in biochemical and cell-based assays. Selected neutralizing and nonneutralizing antibodies were used in cell-based assays with tumor cells to evaluate the effect of antibodies on tumor cell growth and invasion. Neutralizing antibodies failed to inhibit the growth of prostate, ovarian, and hepatoma cell lines in culture. However, potent inhibitory effects of the antibodies were seen on invasion of ovarian and prostate cells in transwell-based invasion assays. These results support a role for hepsin in tumor cell progression but not in primary tumor growth. Consistent with this, immunohistochemical experiments with a mouse monoclonal antibody reveal progressively increased staining of prostate tumors with advanced disease, and in particular, extensive staining of bone metastatic lesions. (Cancer Res 2006; 66(7): 3611-9)

Introduction

Tumor overexpression of mRNA for the membrane protease, hepsin, has been extensively documented (1–5). It is one of the most consistent and extensive changes in gene expression that has been reported in prostate tumors. Hepsin mRNA has been shown to be overexpressed in >90% of cancerous samples, with one study demonstrating 34-fold higher expression in Gleason grades 4 and 5 tumors compared with noncancerous tissues (1). Moreover, hepsin mRNA expression has been correlated with disease aggressiveness in two studies (1, 5). In ovarian tumors, hepsin mRNA is overexpressed in 60% of low grade tumors and in 80% of ovarian carcinomas (6). Overexpression of hepsin in renal cell carcinomas has also been reported (7).

Hepsin is a transmembrane serine protease that was originally identified from a human hepatoma HepG2 cell library using a homology-based cloning strategy (8). The hepsin cDNA encodes a polypeptide of 417 amino acids. Expression and characterization of recombinant hepsin show that the protein is synthesized as a single-chain molecule with an apparent molecular mass of ~51 kDa. It is synthesized as a zymogen and is proteolytically activated by an unknown enzyme. At its NH_2 terminus, hepsin contains a cytoplasmic domain and an integral transmembrane domain. In the extracellular region of hepsin, there is a macrophage scavenger receptor-like domain and a serine protease domain at the COOH terminus (9–12). Biochemical studies indicate that hepsin is an enzyme with trypsin-like substrate specificity that cleaves peptide bonds after basic residues such as arginine and lysine (11, 12). The overall topology of hepsin is similar to those of other type II transmembrane serine proteases of the trypsin superfamily (13), which include corin (14, 15), enterokinase (16), MT-SP1/matriptase (17, 18), human airway trypsin-like protease (19), TMPRSS2 (20), and Stubble-stubbloid (21). Expression of several members of this family is elevated in tumors.

Hepsin mRNA is most abundantly expressed in the liver (10). Low levels of hepsin mRNA expression were also detected in other tissues including kidney, thyroid, pancreas, testis, and prostate (10). To date, the physiologic function of hepsin and its role in tumor development has not been fully elucidated. Hepsin-deficient mice have been shown to be viable, fertile, grow normally, and have no defect in liver function, demonstrating that hepsin is not essential for either embryonic development or maintenance of vital adult functions (22, 23). Early *in vitro* experiments suggest that hepsin may play a role in tumor cell growth. Anti-hepsin antibodies as well as antisense oligonucleotides targeting hepsin significantly blocked hepatoma cell growth (24). In contrast, however, it has been reported that transfection of hepsin cDNA into cultured prostate tumor cells inhibits growth and invasion of the cell (25). More recently, in a mouse transgenic model, it was reported that hepsin overexpression was associated with disorganization of the basement membrane and promotion of metastases (26). It has also been reported recently that hepsin can proteolytically cleave pro–hepatocyte growth factor (HGF), to generate active HFG, the ligand for the c-met receptor (27, 28).

To attempt to resolve these issues and elucidate the functional role of hepsin in tumor development, we generated a modified,

Note: J-A. Xuan and D. Schneider contributed equally to this work.

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soluble and activatable form of hepsin and established an activity assay. We then generated a series of monoclonal antibodies (mAb) that neutralize hepsin protease activity and used these mAbs in cell-based assays for growth and invasion of prostate and ovarian cell lines. It was found that inhibition of hepsin activity failed to affect growth of tumor cells in culture but did block invasion of ovarian and prostate tumor cell lines in Matrigel-based invasion assays. This implies a role for hepsin in invasion and metastases of tumors *in vivo*.

Materials and Methods

Cloning and expression of the hepsin extracellular domain containing an enterokinase activation sequence (hepED/EK). Oligonucleotide primers sense (5′-AGA GGC AGT GAC ATG GCG CAG AAG GAG GGT-3′) and antisense (5′-TGG AGG CTG CGC AGC GAG AAG-3′) were designed based on the published human hepsin cDNA sequence (8). A cDNA fragment spanning the entire coding region of human hepsin was amplified from total RNA derived from human hepatoma HepG2 cells using a reverse transcription-PCR–based method (cDNA cycle kit; Invitrogen, Carlsbad, CA). PCR products were subcloned into pCR vector (Invitrogen) and sequenced. The cDNA fragment was used as a template for the construction of additional plasmid vectors expressing soluble forms of human hepsin.

A construct coding for the hepsin extracellular domain which includes the protease domain, an engineered enterokinase cleavage domain, and scavenger receptor domain, was generated by PCR cloning from the hepsin-pCR construct. Sequences for V5 and His tags were introduced at the 3′-end, and the complete construct cloned into the pIVEX vector (Roche Molecular Biochemicals, Indianapolis, IN) by PCR amplification of the hepsin fragment, subsequent restriction enzyme digestion of the fragment and ligation into the pIVEX vector.

The hepsin EDEK insert in pIVEX was subcloned into pAcgp67 (PharMingen, San Diego, CA) at the *Xma*I/*Not*I sites for expression of the protein in insect cells. The pAcGP67-hepsin EK was transfected into insect cells using Baculogold transfection kit (PharMingen) and virus isolated.

For hepsin ED/EK expression in 293 cells, the cDNA encoding soluble hepsin ED/EK was amplified as a PCR product with the primer pair (CTGATCCGGAcAGGAGTGACCAGGAGCCGC) and (GCCGGGTC CCAGGAAAGGA) using pAcGP67/hepED/EK served as template. The PCR product was digested with *Bsp*EI + *Not*I for cloning into the expression vector and cloned into pCEP4W (Invitrogen), together with an IgK signal sequence in a three-part ligation to generate pCEP4W/hepEK.

Purification of the recombinant hepsin. Conditioned medium from 293 EBNA cells was recovered 3 days after transient transfection by centrifuging the conditioned medium at 5,000 × *g* for 20 minutes followed by a filtration step using a disposable 0.2 μm pore size Opticap capsula filter cartridge (Millipore, Bedford, MA). The conditioned medium was then concentrated 10-fold using an ultrafiltration system with a 10 kDa molecular weight cutoffs (Millipore).

The purification procedure was carried out using an AKTA (Amersham Biosciences, Uppsala, Sweden) purifier with the instrument set to monitor conductivity and UV absorbance at 280 nm. The purification was completed using a 10 mL Ni-NTA chromatography column (Qiagen, Valencia, CA) equilibrated with 50 mmol/L sodium acetate, 150 mmol/L NaCl (pH 6.5). The concentrated conditioned medium was pumped through the column at 4 mL per minute. The column was washed with ~10 column volumes of equilibration buffer. Hepsin was eluted from the column with a step gradient to a buffer containing 50 mmol/L sodium acetate, 150 mmol/L NaCl (pH 4.5). The eluant was collected in a pool corresponding to the increase in absorbance at 280 nm. The eluate pool was ~5-fold concentrated using centrifugal filter devices (Millipore) and then further purified using a size exclusion column (Superdex 200 prep grade, 26 mm × 60 cm by Amersham). The column was equilibrated with 50 mmol/L sodium acetate and 150 mmol/L NaCl (pH 5.0). The concentrated Ni-NTA eluate was applied to the column at 3.5 mL per minute. The hepsin-containing fractions were pooled based on the purity determined in a Coomassie stained gel. The purity of the final product was >90%.

Activation of hepsin using enterokinase. Hepsin was activated using enterokinase provided in the EK max kit by Invitrogen. For activation, the pH of the hepsin-containing sample was adjusted to 7.5 by adding 10× buffer provided in the kit to a final concentration of 1×. Enterokinase was added at 6 units/mL and incubated for 15 hours at room temperature. The activation was analyzed in a Coomassie stained gel. For the removal of enterokinase, hepsin was purified using hydrophobic interaction chromatography. A 7 mL Phenyl-HIC column (Mitsubishi Chemical Corporation, White Plains, NY) was equilibrated with 1 mol/L ammonium sulfate, 50 mmol/L sodium acetate, and 150 mmol/L NaCl (pH 5.5) at 0.5 mL per minute. The activated hepsin sample was diluted 1:2 with a 2 mol/L ammonium sulfate solution and loaded on the HIC column at 0.5 mL per minute. The activated hepsin was eluted from the column with a gradient to the buffer containing 50 mmol/L sodium acetate and 150 mmol/L NaCl (pH 5.5) over three column volumes. The hepsin-containing eluant was collected in a pool corresponding to the increase in absorbance at 280 nm.

Biochemical assay of enzymic activity. Enterokinase-activated human hepsin activity was assayed spectrophotometrically by following the release of *p*-nitroaniline from H-D-Val-Leu-Arg-pNA (Chromogenix S2266, Diapharma, Orangeburg, NY). The assay was done in a total volume of 100 μL containing 100 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 250 pmol/L hepsin, and 40 μmol/L S2266. The assay was done in a clear, flat-bottomed 384-well plate (E&K Scientific, Campbell, CA). Following incubation at room temperature for 90 minutes, the reaction was stopped by the addition of 5 μL of 0.15 N HCl. Control experiments showed that the HCl completely inactivated the enzyme without hydrolyzing unreacted substrate. Product formation was quantitated by measuring the absorbance at 405 nm.

Generation and characterization of mAbs against proteolytically active hepsin. Two recombinant forms of native hepsin were used as immunogen, hepsin EDK-EK, and a Baculovirus expressed form of hepsin. Both male and female hepsin knockout mice (22) were immunized using a "Repetitive Immunization of Multiple Sites" (RIMMS) method (29). On day 0, 20 μg of protein was injected into each animal; this was followed by 10 μg boosts on days 3, 5, 7, and 10. Immune sera were tested by ELISA, FACS, and Western blot for immunoreactivity against native and denatured hepsin-ED-EK. Mice were then given a final boost with denatured hepsin-ED-EK using RIMMS, which resulted in high-serum titers against both native and denatured hepsin.

Two mice were used in hybridoma fusion experiments. Spleens and lymph nodes were harvested and B cells isolated. B cells were fused with P3x63Ag8.653 mouse myeloma cells (American Type Culture Collection, Manassas, VA) using either a polyethylene glycol–mediated fusion method (Sigma, St. Louis, MO) or electrofusion (ECM 200 from BTX, Holliston, MA). Fused cells were grown under HAT selection in 96-well plates at an initial density of 2.4×10^4 cells/well for 10 to 14 days. Hybridomas were screened for immunoreactivity against native and denatured hepsin using ELISA. Positive clones were further screened by Western blot, FACS, and Biacore analyses. Selected hybridoma lines were subcloned twice and then expanded in tissue culture or as ascites. The resulting mAbs were purified using protein A or G affinity chromatography.

Assay of Neutralizing Activity of Antibodies Using Biochemical and Cell-Based Assays

Biochemical assay. The assay was run in 100 mmol/L HEPES and 100 mmol/L NaCl (pH 7.4) using siliconized Eppendorf tubes (VWR International, West Chester, PA). Activated hepsin (2×10^{-13} mol per assay) was preincubated with a molar excess of antibody in a small volume for 1 hour on ice. Assay volume was then increased to 100 μL with buffer at room temperature and transferred to a microtiter plate. Peptide substrate, 100 μL of a 1 mmol/L H-D-Valyl-L-arginine-*p*-nitroaniline dihydrochloride (Chromogenix S-2266), 100 μL of 1 mmol/L stock solution was added immediately before reading the absorbance at 405 nm on a 96-well plate reader. Readings were taken at 2-minute intervals over 30 minutes.

Hepsin live cell assay. This assay monitors the hepsin-based activation of factor VII to form proteolytically active factor VIIa. A subconfluent flask

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of hepsin transfected BHK cells were harvested using Gibco's cell disassociation buffer. The cells were washed, collected by centrifugation (1,000 rpm for 3 minutes), and the final cell concentration adjusted to 1.75×10^7 cells/mL. One hundred microliters of the cell suspension was aliquoted into a 96-well V-bottomed plate and the plate then centrifuged at 1,000 rpm for 4 minutes. The pellets were resuspended in buffer [100 μL; 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 0.1% PEG 6000], 10 μL antibody (or buffer control) was added per well, and the plate was incubated on ice for 1 hour. Ten microliters of factor VII (1 mg/mL; HFVII 2321p ERL) was added per well, the plate was incubated at 37°C for 20 minutes. The plate was then centrifuged at 1,300 rpm for 4 minutes. Fifty microliters of supernatant was transferred to a fresh 96-well flat-bottomed plate, and 50 μL of substrate (3.2 mmol/L in H_2O, Chromogenix S-2266) was added per well. The plate was read at 405 nm. No activity was detectable in parental BHK cells that did not express hepsin, nor in hepsin expressing cells that were incubated without factor VII.

Pro-HGF activation assay. Purified active hepsin (3.4×10^{-13} mol/reaction) was mixed with mAb at a molar ratio of 100:1 (mAb/hepsin) in buffer [50 mmol/L Tris, 150 mmol/L NaCl, 2.5 mmol/L $CaCl_2$ (pH 7.5)], 10 μL final volume, and incubated on ice for 1 hour. Siliconized tubes (National Scientific Supply Co., Inc. Claremont, CA) were necessary to minimize the sticking of hepsin to the tubes. After bringing the samples to room temperature, purified, recombinant human pro-HGF (R&D Systems, Inc., Minneapolis, MN) was added (1 μg/reaction) and the final volume was adjusted to 20 μL/reaction. A 5 μL sample was immediately removed and heated in SDS-PAGE sample buffer containing DTT to stop enzymatic activity. Similar samples were collected at 15 and 30 minutes. Samples were run on 10% NuPAGE Bis/Tris SDS gels (Invitrogen) and stained. The 70 to 80 kDa pro-HGF band was then quantified using a densitometer (Bio-Rad Laboratories, Hercules, CA). Control samples included pro-HGF alone and pro-HGF plus antibody in the absence of hepsin.

Immunohistochemistry

Immunohistochemical detection of hepsin expression on normal prostate, benign prostatic hyperplasia (BPH), cancer tissues, and bone metastasis tissues were done using anti-hepsin antibodies. Four samples of each tissue category, normal, BPH, Gleason grades 2 to 5 prostate cancer tissues, and bone metastasis were used. Slides were deparaffinized and subject to antigen retrieval treatment. Slides were incubated with 3% H_2O_2, washed, and incubated with egg/milk solution to block endogenous activities of biotin. Slides were then blocked with 10% horse serum in PBS. Primary antibodies were applied and incubated overnight at 4°C. Anti-hepsin mouse mAb A15 (generated in-house) and 0.5 μg/mL of anti-hepsin polyclonal peptide antibody (Cayman Chemical, Ann Arbor, MI) were used. Mouse and rabbit IgG was used as negative control antibodies for each tissue. Biotinylated secondary anti-mouse or rabbit antibody was applied to the slides on day 2, followed by the avidin-biotin complex method (Elite ABC kit, Vector Laboratories, Burlingame, CA). The peroxidase activity was then visualized with diaminobenzidine and H_2O_2, followed by counterstaining with hematoxylin. Slides were then dehydrated through graded ethanol and xylene, and then mounted. Slides were scored independently by two investigators and composite scores of hepsin staining were generated.

Cell proliferation assay. Hepsin-expressing cell lines, LNCaP, DU-145, and CAOV-3 (3×10^3 to 6×10^3 per well) were seeded on 96-well plates for 4 to 6 hours before treatment. Then, anti-hepsin neutralizing antibodies 94A7, A6, A24, and A174 (10 or 100 μg/mL final concentration in the well) were added into wells. Cells were treated with antibodies either every day or every other day for 6 days. Nonneutralizing antibodies 85B11, A21, mouse IgG_{2a}, and PBS were used as negative controls. The WST-1 assay (Roche) was done to measure cell proliferation. every other day for 6 days. Absorbance was measured at A_{450} using an ELISA reader (V Max, Molecular Devices, Sunnyvale, CA).

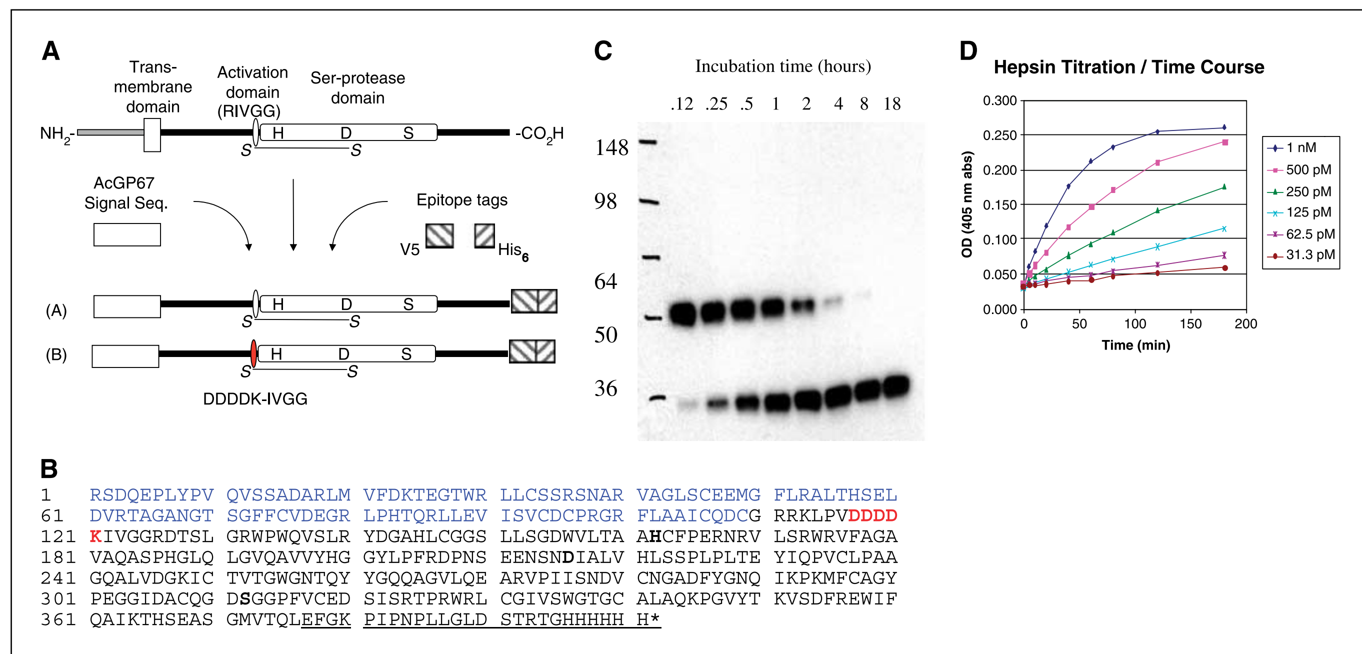

Figure 1. *A,* schematic illustration of the generation of a modified hepsin construct (hepED/EK) used for the studies reported here. The resultant construct lacks the cytoplasmic and transmembrane domains, includes a signal sequence (AcGP67), for expression in bacculovirus, COOH-terminal epitope tags, and an enterokinase activation sequence to permit generation of an active form of the enzyme. *B,* amino acid sequence of secreted hepED/EK, without the signal sequence and with COOH-terminal V5 and His tags. The DDDDK sequence (*red, boldface*) is the enterokinase activation sequence, and the catalytic triad residues, H, D, and S (*boldface*). The scavenger receptor cystein-rich repeat domain located NH_2-terminal of the catalytic domain (*blue*). *Underlined,* the sequence corresponding to V5 and His tags. *C,* activation of expressed hepED/EK using enterokinase. Expressed protein was incubated with enterokinase as described in Materials and Methods, and generation of the putative active form monitored on a SDS-PAGE reducing gel by Western blot analysis using anti-V5 tag antibodies. Enterokinase cleavage leads to the generation of the 36 kDa band from the 52 kDa band. The enterokinase enzyme was removed from the purified hepsin following activation (see Materials and Methods). *D,* assay of proteolytic activity of the activated hepED/EK protein by following the release of *p*-nitroaniline from H-D-Val-Leu-Arg-*p*-nitroaniline. Activated hepED/EK was generated as described in Materials and Methods and the enterokinase enzyme removed from the purified hepsin following activation. The time course of activity and enzyme concentration dependence are shown at a fixed substrate concentration.

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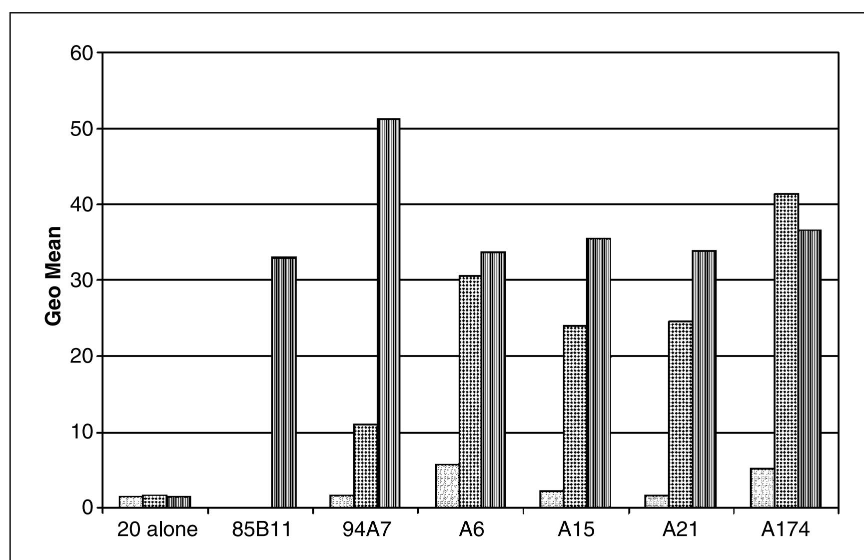

Figure 2. Cell-binding characteristics of mouse mAbs evaluated on hepsin-negative BHK cells (□), LNCaP cells (▩), and BHK cells transfected with a full-length hepsin construct (▥). Significant binding to live LNCaP cells and hepsin-transfected BHK cells was found with minimal binding to nontransfected BHK cells (binding of 85B11 to LNCaP cells was not tested in this experiment but was shown to be positive in subsequent experiments).

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Matrigel invasion assay. CAOV-3 and DU-145 cells were treated with 10 μg/mL of anti-hepsin neutralizing antibodies 94A7, A6, and A174 in test tubes for 30 minutes or 1 hour at room temperature. Untreated cells, or cells treated with $mIgG_{2a}$ or nonneutralizing antibodies, 85B11 or A21, were used as negative controls. Cells (5×10^4 cells) were then added into Matrigel-coated Flourobloc invasion inserts (BD Biosciences, Bedford, MA). Fetal bovine serum (2-5%) plus medium was used as a chemoattractant and included in the lower chamber. The assay was run for 22 to 24 hours at 37°C. Cells which migrated to the lower side of the filter were fixed with Diff quik solution (Dade Behring AG, Deerfield, IL) and stained with Syto-13 reagent. The fluorescent signal of cell nuclei was detected using a Victor II fluorescent plate reader.

Results

Hepsin expression constructs and generation of a modified active enzyme. Hepsin is produced as a membrane-associated zymogen and processed to an active form by proteolytic cleavage. The nature of the activating enzyme, however, is not known. In order to generate an active enzyme in a soluble form, several modifications were made to the sequence (Fig. 1*A*), and the modified enzyme was expressed in insect cells or 293 human kidney cells. The modified hepsin construct includes a deletion of the NH_2-terminal cytoplasmic domain, as well as the *trans*-membrane sequence. Additionally, the enterokinase cleavable peptide sequence, DDDDK, was introduced at the activation site to enable the generation of the active form of hepsin by enterokinase treatment of the modified zymogen. To permit easy purification of the expressed enzyme, 6-His and V5 tags were introduced at the COOH terminus. The structure of this modified hepsin zymogen (referred to as ED-EK hepsin) is shown in Fig. 1*B*. The hepsin constructs generated were suitable for expression in

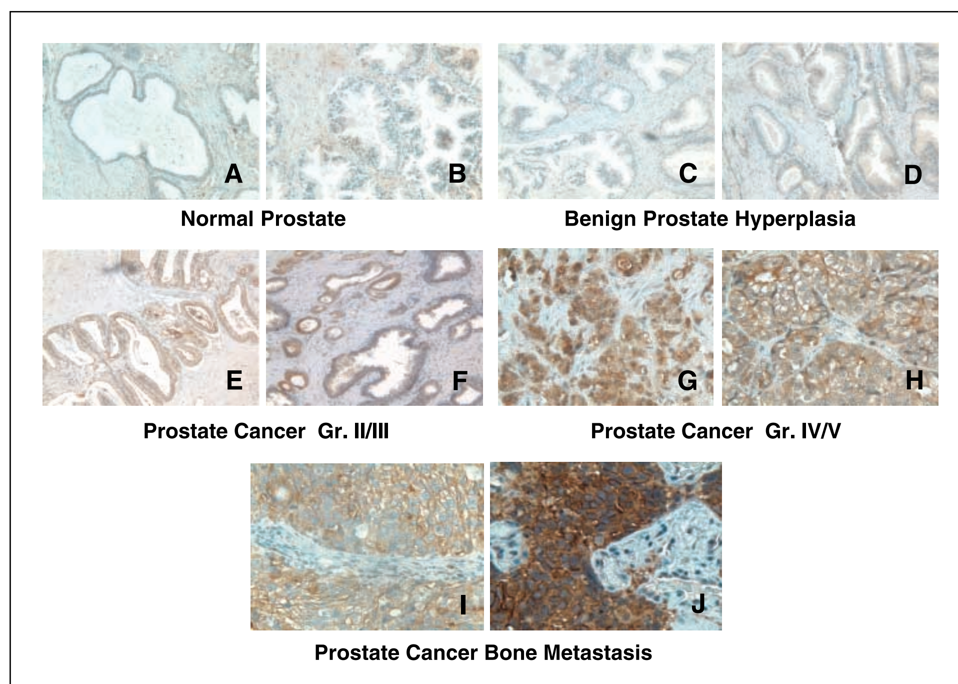

Figure 3. Immunohistochemical detection of hepsin in tissue sections, (done at PhenoPath Laboratories, Seattle, WA) Normal, BPH, grades 2 to 5 prostate cancer tissues, and bone metastases were stained with anti-hepsin mouse mAb A15 (developed in-house). Four samples per tissue were used and mouse IgG was used as a negative control antibody for each tissue. The figure shows pairs of samples each stained with A15. The examples reflect the range of staining seen at each tumor stage. As indicated above, hepsin protein expression was elevated in later stage prostate cancer tissues and prostate cancer bone metastasis. No staining was seen with control mouse IgG. Similar results were obtained with antibody A174 and a rabbit polyclonal anti-hepsin antibody (Cayman Chemical). *A* and *B,* normal prostate; *C* and *D,* BPH; *E* and *F,* Gleason grade 2/3 prostate tumors; *G* and *H,* Gleason grade 4/5 prostate tumors; *I* and *J,* prostate cancer metastases to bone.

either bacculovirus, using a GP64 signal sequence, or 293 cells, using an immunoglobulin signal sequence (see Materials and Methods), and the recombinant protein purified using chromatography procedures. The isolated zymogen was then digested with enterokinase and specific cleavage of the protein to generate active forms of the enzyme was shown by gel electrophoresis and Western blotting using an anti-V5 antibody (Fig. 1*C*).

The activity of this modified hepsin was shown by using tetrapeptide and tripeptide substrates bearing a *p*-nitroaniline tag (Fig. 1*D*). The peptide, H-D-Val-Leu-Arg-pNA (S2266), was cleaved by hepsin with a K_m of 40 μmol/L, and was selected for use in studies of enzyme neutralization.

Generation and characterization of antibodies reactive with native enzyme. Purified, activated ED-EK-hepsin was used to immunize mice to generate hepsin-binding antibodies. To maximize opportunities for obtaining antibodies, we immunized hepsin knockout mice. A series of mAb were made and screened using activated hepsin immobilized to ELISA plates using an antibody targeting the V5 tag on the COOH terminus of the protein. These antibodies were purified, isotyped, and tested for reactivity against active and nonactivated hepsin. The seven antibodies selected were all found to bind to both active and nonactivated hepsin. They were subsequently screened by FACS analysis on BHK cells transfected with a full-length hepsin construct, as well as on the hepsin expressing LNCaP cells. All antibodies bound to both Hep-BHK cells as well as LNCaP cells, demonstrating that the antibodies recognized the native forms of hepsin expressed in transfected cells as well as on tumor cell lines (Fig. 2).

Immunohistochemistry studies of hepsin protein expression in primary prostate tumors and metastases. Two of the seven mAbs, A15 and A174, were found to stain hepsin-expressing tissues including prostate, liver, and kidney. These antibodies were used to monitor hepsin expression in normal prostate tissues and prostate tumors in relation to disease progression. Normal tissues, BPH, low grade tumors (Gleason grades 2 and 3), and higher grade tumors (Gleason grades 4 and 5), as well as metastases to the bone were evaluated. A total of 16 normal and primary tumor tissues were evaluated (4 for each stage), and 6 metastases samples (Fig. 3). Hepsin was expressed at all stages of disease, exhibiting distinct staining of both apical and basal membranes in polarized epithelial cells. No staining of stromal tissues was apparent. Staining generally increased with disease progression (Table 1), and became more diffuse in grade 5 tumors with loss of epithelial cell polarity. Significantly, hepsin expression was maintained in prostate tumor metastases to bone in all six samples evaluated and the staining was stronger than that in early stage primary tumors. Similar data was obtained with a commercially available rabbit antipeptide antibody generated against a hepsin-derived peptide sequence (data not shown). This strong expression of hepsin in bone metastases was consistent with the enzyme playing a role in late stages of disease.

Neutralizing potential of identified antibodies. The mAbs were screened for neutralizing activity in three assays: (*a*) a cell-based assay using BHK cells transfected with a full-length hepsin construct, (*b*) a biochemical assay using the modified, soluble form of hepsin (hepsin ED-EK) together with the peptide substrate H-D-Val-Leu-Arg-pNA (S2266), and (*c*) a biochemical assay monitoring the cleavage of pro-HGF into the activated form of HGF. The cell-based assay monitors membrane-associated hepsin activity by activation of the protein, factor VII to factor VIIa, which is then assayed by its proteolytic activity. In contrast, the biochemical assays use either a small peptide substrate, S2266, or the protein pro-HGF, and soluble enzyme hepsin ED-EK. Of the hepsin-binding antibodies evaluated, antibodies A174 and A6 were shown to be potent neutralizers in the peptide/soluble enzyme assay, but antibody 94A7 was not neutralizing in this assay (Fig. 4*A*). The concentration dependence of the neutralization activity was shown for each of these antibodies in this assay (Fig. 4*A*). Antibodies A6, 94A7 and, to a significantly lesser extent, A174, neutralized enzyme activity when screened using the BHK cell-based assay (Fig. 4*B*). Antibody A174, and to a slightly lesser extent, antibody A6, very effectively blocked hepsin-mediated conversion of pro-HGF into HGF, whereas antibody 94A7 was not neutralizing in this assay (Fig. 4*C*). All other antibodies, including 85B11 and A21, bound to hepsin but did not neutralize enzyme activity in any assay. The assay-dependent neutralizing potential of some of the antibodies may reflect a difference in the binding of antibodies to cell membrane bound hepsin as compared with that to the soluble enzyme. In this respect, it could be speculated that the interaction between 94A7 and hepsin is different in the context of the plasma membrane from that in solution. Alternatively, it is possible that the neutralizing effect of the antibodies could be related to the size or composition of the substrate. Based on the results, with these three distinct assays, we identified antibodies A6, 94A7, and A174 as neutralizing in at least one assay, and 85B11 and A21, as nonneutralizing in all assays evaluated.

Table 1. Hepsin expression in prostate tissue samples as monitored by immunohistochemistry analysis

Case no.	Prostate tissues	Immunohistochemistry score	Staining intensity
1	Normal	++	2
2		++	1-2
3		++	2
4		++	1-2
5	BPH	++	1-2
6		++	2
7		++	2
8		++	2
9	Gleason grades 2/3	+++	2-3
10		+++	2-3
11		++	2
12		++++	3
13	Gleason grades 4/5	++++	3
14		++++	3
15		++++	3
16		++++	3
17	Bone metastasis	++++	3
18		++++	3
19		++++	3
20		++++	3
21		+++	2
22		++++	3

NOTE: Procedures were as described in the Materials and Methods and in the legend to Fig. 3. Immunohistochemistry score, percentage of positively stained cells in tissue section: + <5%; ++ 5% to 30%; +++ 20% to 50%; ++++ >50%. Staining intensity: 1, weak; 2, medium; 3, strong.

Evaluation of the effect of hepsin-neutralizing antibodies on growth and invasion of tumor cells in culture. Experiments

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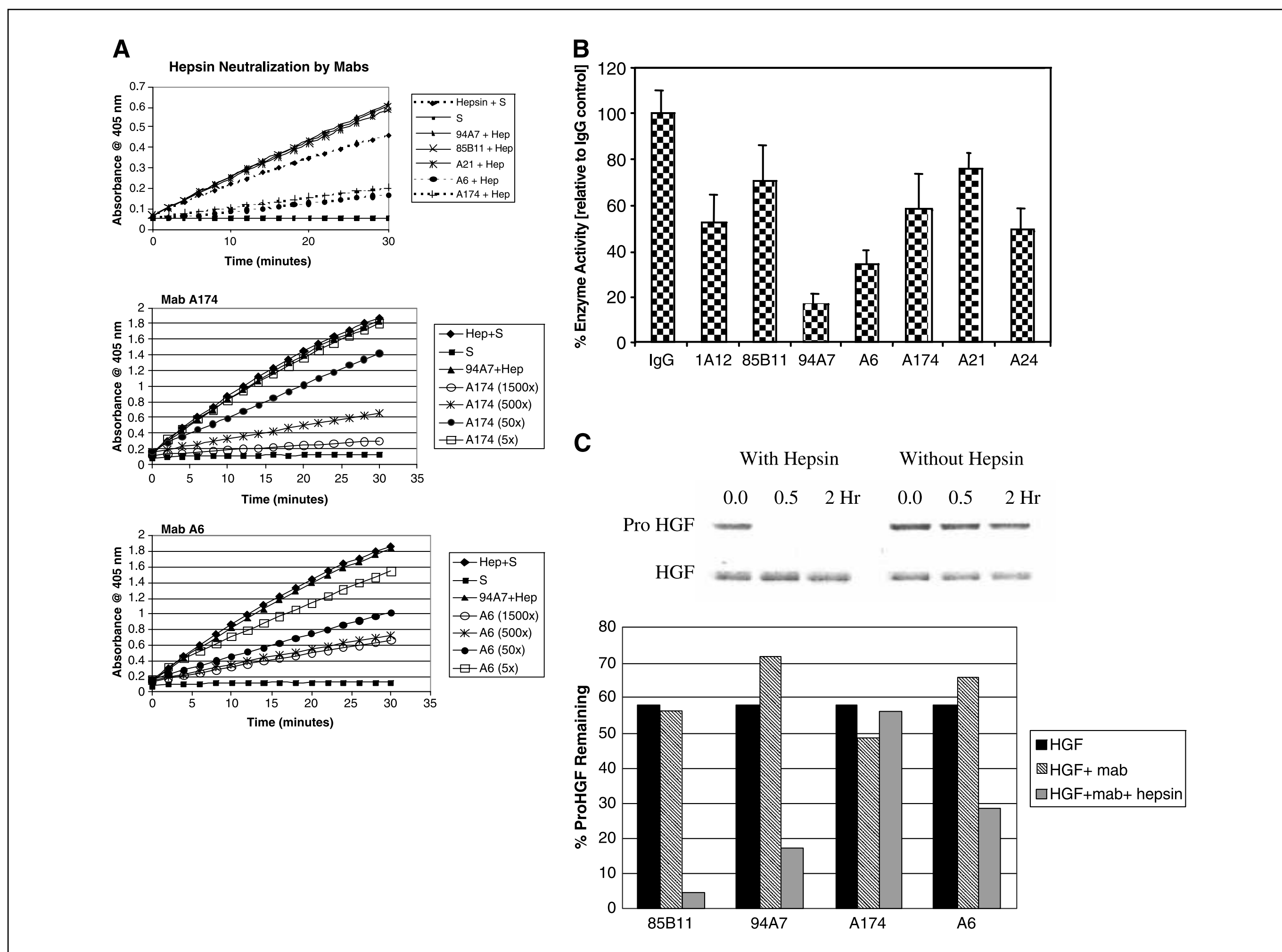

Figure 4. *A,* biochemical screen for neutralizing antibodies (*top*). Hepsin (2×10^{-13} mol) was preincubated with a 500-fold molar excess of mAbs A174, A6, A21, 85B11, and 94A7 for 1 hour on ice. The chromogenic peptide substrate H-D-Valyl-L-Arginine-*p*-nitroaniline dihydrochloride was added to a final concentration of 500 μmol/L immediately before reading the 405 nm absorbance at 2-minute intervals over 30 minutes. mAbs A174 and A6 neutralize hepsin activity whereas antibodies 94A7, A21, and 85B11 failed to neutralize hepsin activity in this assay. Dose-response of mAbs A174 (*middle*) and mAb A6 (*bottom*) neutralization of hepsin activity. The experimental method was similar to the above method (*top*), except that hepsin was preincubated with different molar ratios of mAb/hepsin (1,500:1, 500:1, 50:1, and 5:1) prior to the addition of the chromogenic peptide substrate. Controls included hepsin plus substrate, substrate alone, and the nonneutralizing hepsin mAb 94A7. *B,* antibodies were tested for their ability to neutralize hepsin (full-length unmodified), expressed on BHK cells. Enzyme activity was assayed by monitoring hepsin-mediated conversion of factor VII to factor VIIa (monitored using a chromogenic peptide substrate). BHK parental cells not expressing hepsin, and transfected with BHK cells, in the absence of factor VII, served as controls. Activities were normalized to IgG-treated cells. Antibodies 94A7 and A6 were neutralizing in this cell-based assay. *C,* Coomassie blue–stained SDS polyacrylamide gel (*top*). Pro-HGF protein was incubated either in the presence of hepsin (*left lanes*), or absence (*right lanes*) for 30 minutes or 2 hours. The samples were then resolved by SDS-PAGE and the gels stained. Hepsin digested pro-HGF to HGF within the time frame shown. The identity of the HGF band and the specificity of the cleavage was shown by mass spectrometry-based NH_2-terminal sequencing of the HGF band extracted from the gel. *C,* quantitation of pro-HGF in samples treated with hepsin for 30 minutes in the presence of selected antibodies (*bottom*). Pro-HGF bands were quantified by densitometry in stained gels following hepsin digestion. Enzymatically active hepsin was preincubated for 1 hour, on ice, with a 100-fold molar excess of four anti-hepsin mAbs: 85B11 (nonneutralizing), 94A7, A174, and A6. Purified pro-HGF (1 μg/digest) was then added and incubated for 30 minutes at room temperature. Controls included HGF alone (*black columns*) and HGF plus mAb without hepsin (*striped columns*). Following hepsin digestion, samples were heated in SDS sample buffer containing DTT, run on SDS-PAGE, and stained. After destaining, the remaining pro-HGF band (70 kDa) was quantified using a densitometer and band density was normalized to pro-HGF levels in a sample taken at 0 minutes (i.e., undigested sample). Pro-HGF levels in the presence of neutralizing and nonneutralizing antibodies were then expressed as the percentage of the starting level of pro-HGF. Data shown is representative of three replicate experiments. Antibodies A174, and to a lesser extent A6, inhibited hepsin-based cleavage of pro-HGF, and are considered neutralizing.

were done with the neutralizing antibodies, A6, 94A7, and A174, and the nonneutralizing antibodies, 85B11 and A21, to assess the effect of inhibiting enzyme activity on the growth of hepsin-expressing tumor cells. Growth was monitored over a 6-day period in the presence of antibodies (either at 10 μg or 100 μg/mL), using a WST-1 assay. The prostate tumor cell lines LNCaP and DU145, as well as the ovarian tumor cell line, CAOV-3, were evaluated. No significant effect of any of the neutralizing antibodies was seen on growth of the cells when compared with that of nonneutralizing antibodies, or with the addition of PBS alone (Fig. 5). Similar results were obtained with HepG2 hepatoma cell cultures (data not shown). This implies that hepsin activity is not required for the growth of tumor cells in culture.

A similar set of experiments was done to evaluate the role of hepsin in the invasion of tumor cells. This was done using Matrigel-coated invasion chamber assays with DU145 prostate tumor cell cultures and CAOV-3 ovarian cells (we were unable to monitor the invasion of LNCaP because these cells do not reproducibly migrate

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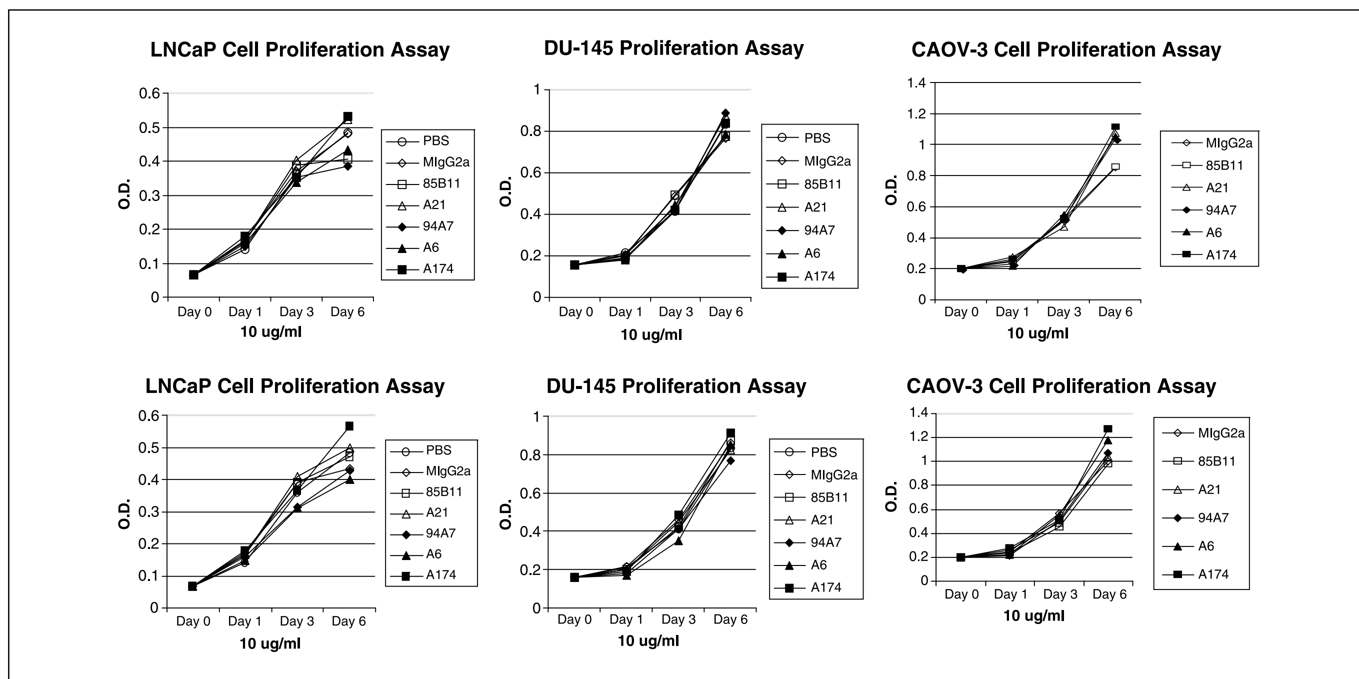

Figure 5. Cell proliferation assay. Hepsin expressing cells, LNCaP, DU-145, and CAOV-3 were treated with anti-hepsin neutralizing antibodies 94A7, A6, and A174 every other day for 6 days. Nonneutralizing antibodies 85B11, A21, mouse IgG_{2a} and PBS were used as negative controls. Cell proliferation was monitored using a WST-1 assay. Treatment of hepsin expressing tumor cells using antibodies did not affect cell proliferation as indicated above.

across Matrigel-coated chambers). In contrast to the lack of effect on cell growth, we found that neutralizing antibodies, 94A7, A6, and A174, all reduced the invasion of both DU145 and CAOV-3 cells, whereas the nonneutralizing antibodies had no significant effect on tumor cell invasion (Fig. 6). Statistical evaluation of the data, comparing the effect of neutralizing antibodies with non-neutralizing antibodies, using Student's t test, showed that the effects were significant at $P < 0.0001$ in DU145 cells and at $P < 0.001$ in CAOV-3 cells. This result then, is consistent with a role for hepsin activity in the invasion of prostate and ovarian tumor cells.

Discussion

Using a modified, activated form of hepsin as an immunogen, we were able to generate a set of mAbs that neutralized hepsin's proteolytic activity in biochemical and cell-based assays. These antibodies provided us with highly specific tools to probe the role of hepsin in the growth and invasion of prostate and ovarian tumor cells in culture. Our experiments showed that inhibiting hepsin's proteolytic activity had no effect on the growth of LNCaP or DU145 prostate tumor cells, or on the growth of CAOV-3 ovarian tumor cells. However, the invasion of the DU145 and CAOV-3 cells across a Matrigel barrier in chamber assays was significantly reduced in the presence of neutralizing antibodies. Nonneutralizing antibodies of similar affinity failed to affect cell invasion. These findings then implicate hepsin in the process of tumor cell invasion. Although it will be important to confirm this conclusion by a different approach, for example, by RNAi-based experiments, the differential effects of neutralizing and nonneutralizing antibodies are striking.

Additionally, these results are consistent with recent studies of transgenic mice overexpressing hepsin in the prostate epithelium, which exhibited prostate cancer progression with the development

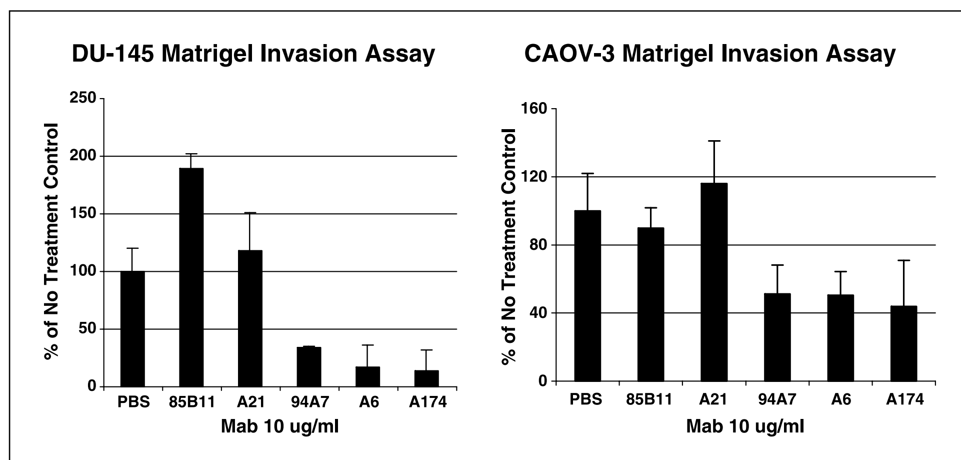

Figure 6. Hepsin neutralizing antibodies 94A7, A6, and A174. Untreated cells, or cells treated with PBS, $mIgG_{2a}$ or nonneutralizing antibodies, 85B11 or A21, were used as negative controls. Cells were treated with PBS or antibodies for 30 to 60 minutes at room temperature, and 5×10^{-4} cells were added into Matrigel-coated inserts (BD Biosciences; 2-5% fetal bovine serum, plus medium was used as a chemoattractant and included in the lower chamber). The assay was run for 22 to 24 hours at 37°C. Cells on the lower side of the filter were fixed and stained with Syto-13. Plates were read using a Victor II fluorescent plate reader. The percentage of no-treatment control was calculated as the fluorescent units of each treated group divided by the fluorescent units of untreated group.

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of metastases to liver, lung, and bone (26). Significantly, primary tumor growth was not changed in transgenic mice. The results, however, are at odds with an early study showing that an anti-hepsin antibody inhibited the growth of tumor cell lines in culture (24). The discrepancy between these findings is not clear but may be due to the different properties (neutralizing versus nonneutralizing) of the antibodies used in the respective studies, and because of the different cells used in these experiments. It has also been reported that hepsin overexpression in metastases-derived prostate tumor lines leads to the inhibition of cell proliferation in culture (25). It is possible that this is a consequence of the high level of hepsin expression seen in transfected cells in culture, which may lead to increased cell apoptosis.

Although the studies presented here do not directly address the mechanism of hepsin action, they are consistent with the mechanism proposed by Klezovich et al. (26), in which hepsin is predicted to act by degradation of basement membranes. By using the set of antibodies reported here, we should be able to address this issue specifically in *in vitro* studies. However, our data do not rule out an additional role for hepsin in activation of factors that promote tumor progression. In this respect, the recent studies demonstrating that active hepsin can catalyze the conversion of pro-HGF to the c-met binding ligand, HGF (27, 28), as confirmed here using the modified activated hepsin protein, point to a possible role for hepsin in the activation of tumor-promoting factors. There is considerable evidence that c-met and HGF are involved in metastatic prostate cancer (30), and development of an autocrine loop for HGF/c-met has been proposed as one of the mechanisms leading to androgen-independent tumors (31). The coexpression of hepsin with HGF and c-met in metastatic cancers may be key in the activation of the HGF/c-met pathway in late stage prostate tumors. The experimental work presented here, implicating a role for hepsin in invasion, does not, and cannot directly address the role of hepsin in the HGF/c-met pathway, as the cell lines used in these studies do not express pro-HGF and an exogenous source of HGF was not present in the invasion assays. However, the set of antibodies we describe may prove to be helpful in elucidating such a role in the future, as well as elucidating the additional importance of extracellular matrix degradation (26–28).

As has been pointed out earlier, numerous studies have documented overexpression of hepsin mRNA in ovarian (6) and, in particular, prostate tumors (1–5). Stamey and colleagues have reported a pattern of increased hepsin mRNA expression with increasing malignant phenotype (1). A large-scale quantitative analysis by Stephan et al. (5) confirmed this and showed significantly elevated levels of hepsin mRNA expression in high-grade tumors as compared with lower grade tumors. In contrast to these results, Dhanasekaran and colleagues (2) reported immunohistochemical studies demonstrating strongest protein expression in high-grade prostate intraepithelial neoplasia lesions, and lower hepsin expression in patients with postoperative PSA failure, and higher Gleason scores, consistent with an inverse correlation of hepsin expression with patient prognosis. Our immunohistochemistry experiments with the prostate tumor tissues reported here show increased hepsin protein expression with increasing tumor grade. Additionally, we show that hepsin expression is maintained in prostate metastases to the bone in all sections examined. Clearly, our conclusions are limited by the small number of samples that were available to us, and it will be important to expand on these results as additional sections of metastases to the bone become available. However, our results suggest that hepsin protein expression tends to follow the reported increases in mRNA expression. To our knowledge, this is the first report of hepsin expression in bone metastases and its expression at this stage of the disease is certainly consistent with it playing a role in disease progression and metastases. In this respect, it is possible that inhibitors of hepsin activity may be valuable drugs to block disease progression in prostate and ovarian cancer. Our previous studies with hepsin-deficient mice (22) have clearly shown that hepsin knockout in normal cells does not have a deleterious effect on cell function, making this a feasible approach for drug development.

Acknowledgments

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