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Human kallikrein 13 involvement in extracellular matrix degradation ☆

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Abstract

The human kallikrein family is a group of 15 serine protease genes clustered on chromosome 19q13.4 and shares a high degree of homology. These proteolytic enzymes have diverse physiological functions in many different tissues. Growing evidence suggests that many kallikreins are differentially expressed in cancer and may play a role in metastasis. Human kallikrein gene 13 (*KLK13*) is a member of this family and codes for a trypsin-like, secreted serine protease (hK13) that is overexpressed in ovarian cancer patients. The aim of this study was to determine if hK13 can degrade extracellular matrix components. Recombinant hK13 was produced in yeast and purified using cation exchange and reverse-phase chromatography. The protein was used as an immunogen to generate mouse monoclonal antibodies. Enzymatic activity of hK13 was verified by using synthetic tri-peptide fluorogenic substrates and gelatin zymography. Active hK13 was incubated with biotinylated extracellular matrix (ECM) proteins and degradation was evaluated by Western blot analysis. hK13-secreting cancer cell lines were treated in a chemotaxis invasion chamber that was coated with various ECM proteins, to determine if hK13 plays a role in tumor cell migration and invasion. Assay with the synthetic substrates and zymography have shown that recombinant hK13 was enzymatically active. The Western blot results showed that hK13 was able to cleave the major components of the extracellular matrix. In the chemotaxis invasion chamber experiment, it was found that ovarian cancer cell lines that secreted hK13 and were treated with an hK13 neutralizing antibody migrated less than untreated cells. Human kallikrein13 may play a role in tissue remodeling and/or tumor invasion and metastasis. Targeting hK13 activity with neutralizing antibodies may have therapeutic applications.

Keywords: Human kallikreins; Human kallikrein 13; Tumor markers; Serine proteases; Metastasis; Extracellular matrix protein degradation; Inhibition assay; Cell migration; Invasion assays; Zymogram

The most lethal gynaecological malignancy is epithelial ovarian cancer, constituting approximately 90% of all ovarian cancer cases [1,2]. The American Cancer Society estimates that in 2003, 25,400 new cases of epithelial ovarian cancer were diagnosed and approximately 14,300 women will die from this disease, despite therapeutic advances over the last decade [1]. The high mortality rate is attributed to late diagnosis, since epithelial ovarian tumors commonly lack early warning symptoms. In addition, ovarian carcinomas often lack definite precursor lesions, are quite heterogeneous, and the molecular pathways underlying their progression remain unknown. Therefore, numerous attempts have been made to predict the biology of ovarian tumors in order to determine prognosis and develop individualized treatment strategies [3]. Until reliable screening and diagnostic strategies are available, the identification of new prognostic and predictive biomarkers can contribute positivity to optimal ovarian cancer patient management.

☆ *Abbreviations: KLK*, kallikrein gene; hK, kallikrein protein; hK13, human kallikrein 13; mAb, monoclonal antibody; ECM, extracellular matrix; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AMC, 7-amino-4-methylcoumarin; DMSO, dimethyl sulfoxide; serpins, serine protease inhibitors.

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Proteolytic enzymes have emerged as important prognostic factors in ovarian cancer. One requirement for invasion and metastasis of ovarian cancer cells is the expression of proteases of several catalytic types, which degrade components of the basement membrane and extracellular matrix [4–8]. Numerous reports indicate that aberrant protease expression is often associated with a poor prognosis in ovarian cancer patients [8–12]. The prognostic relevance of proteases has also proved important in the implementation of new treatment modalities specifically targeted at metastasis formation, including the use of protease inhibitors as anti-cancer agents [13–17].

Among the newly identified prognostic factors for ovarian and other cancers are the human tissue kallikreins that all co-localize to chromosome 19q13.4 [17]. The entire human kallikrein gene locus includes 15 genes, designated *KLK1* to *KLK15*; their respective proteins are known as hK1 to hK15. Accumulating evidence indicates that at least 11 kallikrein family members are differentially expressed in ovarian cancer at both the mRNA and protein levels, and several demonstrate clinical utility as prognostic biomarkers [18,19]. More specifically, the serum levels of hK6, hK10, and hK11 are elevated in a proportion of ovarian cancer patients and as such, they may represent putative serological screening and/or diagnostic biomarkers for ovarian cancer [20–24].

KLK13, previously known as *KLK-L4*, is one of the newly identified kallikrein genes [25], cloned by the positional candidate approach. The gene spans approximately 10 kb of genomic DNA sequence, and has five coding exons and four introns. The protein has 47% and 45% sequence identity with hK3 (PSA) and hK2, respectively. Predictions made from the primary structure suggest that hK13 has trypsin-like substrate specificity [25]. However, the function of the protein is still unknown.

Our initial findings indicated that *KLK13* is downregulated, at the mRNA level, in breast cancer tissues and cell lines. Subsequent studies suggested that *KLK13* expression is an independent and favorable prognostic marker for breast carcinoma [26]. We have also shown that the expression of this gene is regulated by steroid hormones, mainly androgens and progestins, and to a lesser extent by estrogens, in the breast cancer cell line BT-474 [26]. Taken together, these data suggest that human kallikrein 13 may be involved in the pathogenesis and/or progression of some endocrine-related cancers.

Immunohistochemical studies using polyclonal and monoclonal antibodies demonstrated that hK13 is expressed by many glandular epithelia, some endocrine organs, and some specialized epithelia and cells [27]. Recently, we developed an immunofluorometric assay for hK13 and found that this protease is expressed in various tissues including esophagus, tonsil, trachea, lung, cervix, and prostate [28]. hK13 is also found in seminal plasma, amniotic fluid, follicular fluid, and breast milk. In addition, hK13 was found in the cytosolic extracts of ovarian cancer tissues and in ascites fluid of ovarian cancer patients [28]. hK13 was also shown to form complexes with α_2-macroglobulin and two members of the serine protease inhibitor family; α_2-antiplasmin and α_1-antichymotrypsin [29]. Accumulating evidence suggests that hK13 is involved in endocrine-related malignancies and may play a role in metastasis [25,28,29]. Our objective was to determine whether hK13 is involved in ECM degradation and metastasis.

Materials and methods

hK13 enzymatic activity assay. The trypsin-like synthetic substrate Val-Pro-Arg-AMC was obtained from Bachem, King of Prussia, PA, USA [29]. The substrate was diluted in DMSO to a final concentration of 80 mM and stored at −20 °C. The optimal buffer for the analysis of hK13 enzymatic activity was 50 mM Tris, 0.1 M NaCl, and 0.1% BSA, pH 7.4. All measurements were performed in 100 μL of the standard kinetic analysis buffer at 37 °C using 8.2 nM of recombinant hK13. BSA was added to the reaction mixture in order to minimize adsorption of the enzyme to the walls of the microtiter wells. The measurements were performed with the Wallac Victor Fluorometer using a 355-nm excitation filter and a 460-nm emission filter. The fluorescence of standard 7-amino-4-methylcoumarin (AMC) concentrations was also measured and used for conversion of fluorescence per unit time to pmol of AMC per unit time.

SDS–PAGE zymography for detection of hk13 activity. hK13 was run on a gelatin gel according to a standard protocol [30]. The 1% gelatin gel, along with the running, renaturation, and developing buffers, was purchased from Invitrogen, Carlsbad, CA. hK13 was run on the gel according to the manufacturers' instructions. After running the gel, it was incubated in renaturation buffer for 30 min at room temperature followed by incubation in developing buffer for 12 h in order to renature the enzyme. The gel was then incubated in Simply Blue Safestain (Invitrogen) for 1 h and then de-stained in water. White bands against a blue background indicate presence of enzyme activity.

Biotinylation of extracellular matrix proteins. Collagen I, collagen II, collagen III, fibronectin, and laminin comprise the majority of the extracellular matrix (ECM) and basement membrane proteins. Hence, they were selected for use in the experiments to test the ability of hK13 to cleave the ECM and basement membrane. Biotinylation of the ECM proteins was performed as previously described [31]. The proteins were dialyzed at 4 °C overnight in 100 mM sodium phosphate buffer (pH 7.4) in order to remove any salts. NHS-LC-biotin (Pierce Chemical, Rockford, IL) was then added at a 100-fold molar ratio and the mixtures were kept for 1 h at room temperature. The biotinylated proteins were then dialyzed for 72 h in 5 L of sodium phosphate buffer (pH 7.4) at 4 °C with 3 buffer changes per day in order to remove any unbound biotin. These biotinylated proteins were then tested as substrates for hK13.

Incubation of biotinylated ECM proteins with hK13. Reaction mixtures were prepared where 30 μg of each of the ECM proteins was incubated with 1–2 μg purified hK13 in 100 mM sodium phosphate buffer (pH 7.4) at 100 μL reaction volume. These mixtures were placed on a shaker in a 37 °C incubator. Twenty microliters was taken out of the reaction mixture after 5 min, 2, 4, and 8 h. These 20 L aliquots were frozen immediately to stop further enzyme activity. For the negative control, the same mixture was prepared but without hK13.

The frozen aliquots were run on 4–12% SDS–PAGE gels (Nupage, Invitrogen) under reducing conditions and transferred to nitrocellulose membranes. The nitrocellulose membranes were then blocked overnight in a 5% non-fat dried milk solution. After the blocking step, the membranes were incubated for 1 h in a streptavidin–alkaline phosphatase conjugate solution (1:40,000 dilution; Jackson Immunoresearch, West Grove, PA). The membranes were then rinsed in washing buffer (50 mM Tris, 50 mM NaCl, and 0.1% Tween 20, pH 7.4) and exposed to a dioxetane-based chemiluminescence substrate for 1 min. The membranes were then developed by exposure to X-ray films.

Production and characterization of anti-hk13 monoclonal antibodies. Female BALB/c mice were immunized with recombinant hK13 protein. The immune splenocytes were fused with murine myeloma cells by standard hybridoma technology. Briefly, 100 μg (200 μL) of hK13 was diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for subsequent injections and administered subcutaneously three times at three-week intervals. Two weeks after the third injection, the mouse received an intraperitoneal injection of aqueous hK13, and 3 days later it was sacrificed and its spleen was removed. To generate monoclonal anti-hK13 antibodies, the splenocytes were fused with the Sp2/0 myeloma cells with polyethylene glycol 1500. The fused cells were cultured in 96-well plates in DMEM (Invitrogen) containing 200 mL/L fetal calf serum, 200 mmol/L glutamine, 10 g/L OPI (oxaloacetic acid, pyruvic acid, insulin), and 20 g/L HAT (hypoxanthine, aminopterin, thymidine; Sigma) at 37 °C in a 5% CO_2 atmosphere for 10–14 days. The supernatants were collected and screened for positive clones by the following immunoassay. Sheep anti-mouse IgG, Fc fragment-specific antibody (Jackson ImmunoResearch) was immobilized on 96-well white ELISA plates. Tissue culture supernatants diluted 10-fold in a general diluent [containing 60 g/L BSA, 50 mmol/L Tris (pH 7.80), and 0.5 g/L sodium azide] were applied to the plates and incubated for 1 h; the wells were then washed six times. Biotinylated recombinant hK13 was then added (5–10 ng/well) and incubated for 1 h, and the plates were washed. Finally, alkaline phosphatase-conjugated streptavidin was added and incubated for 30 min, the plates were washed, and the alkaline phosphatase activity was detected with time-resolved fluorescence, as described elsewhere [32]. The positive clones were expanded sequentially in 24- and 6-well plates in complete medium (reducing the fetal calf serum to 150 mL/L and changing the HAT to HT). The supernatants were further tested by performing IgG isotyping and were subjected to limiting dilution. The clones were then expanded in flasks to generate large amounts of supernatants in serum-free medium (CD-1 medium; Invitrogen) containing 200 mmol/L glutamine.

Purification of monoclonal anti-hk13 antibodies. Tissue culture supernatants containing monoclonal antibodies were diluted 2-fold in 20 mmol/L sodium phosphate buffer (pH 7.0) and injected into a Hi-Trap Protein G column (Pharmacia). After the column was washed with 20 mmol/L sodium phosphate, the antibodies were eluted with 0.1 mol/L glycine buffer (pH 2.7). The eluted antibody solutions were neutralized and then dialyzed overnight in 0.1 mol/L sodium bicarbonate solution.

Inhibition of hK13 catalytic activity by monoclonal antibodies. Four monoclonal antibodies (clones 11C1, 27-1, 2-2A, and 33-1) specific for hK13 were tested, to identify the optimal hK13-neutralizing antibody. In each of the 100 μL reaction mixtures, 8.2 nM of hK13 was used and the monoclonal antibodies were added in 6 different amounts (0.1, 0.2, 0.5, 1, 2, and 5 μg). A control mixture with hK13 but without any antibody was also prepared. The antigen–antibody mixtures were incubated for 1 h at 4 °C. After incubation the mixtures were pipetted in triplicates into a standard 96-well white polystyrene microtiter plate. The activity of hK13 was monitored using the substrate Val-Pro-Arg-AMC (250 μM). Substrate hydrolysis was measured for 30 min after adding the substrate to the reaction mixtures. The inhibitory effects were evaluated by comparison with inhibitor-free controls.

ELISA of tumor cell supernatants for secreted hK13. Several cancer cell lines were tested to determine if any of them secrete significant amounts of hK13 into the supernatant using the hK13 ELISA previously developed in our laboratory [28].

Boyden chamber assay. A 96-well reusable Boyden Chamber with a polycarbonate filter with pores covered with collagen I, collagen II, collagen III, fibronectin, and laminin was used to study the effect of hK13 in the migration of tumor cells [33]. An ovarian cancer cell line (OV90) that secretes hK13 was utilized for the invasion assays. OV90 cells were cultured in serum-free media and grown to confluence in 250 mL culture flasks. The filter was soaked overnight in the five different ECM proteins at a concentration of 10 μg/cm^2. Briefly, a suspension of the cells was added in triplicate with and without the hK13 neutralizing antibody (clone 33-1) to the tissue culture wells containing the filter coated with ECM protein. The optimum concentration of clone 33-1 required to neutralize hK13 enzymatic activity was determined from the inhibition assay. Fifty microliters of culture media with 10% FBS was used in the lower chamber as a chemo-attractant. The chamber was placed in a CO_2 incubator at 37 °C with 5% humidified air for 48 h. At the end of the incubation period, the cells remaining attached to the upper surface of the filter were wiped off with cotton swabs and the cells on the under surface (migrated cells) of the filter were fixed and stained [34]. Finally, pictures of the migrated cells were taken by an inverted microscope (Olympus, Japan) at 200× magnification with random selection of at least four fields. The mean values were calculated as a percentage of the antibody-free control, assuming untreated invaded cells as 100%. A combined analysis of the cell counts was used to calculate the percentage of the total population of cells that had invaded through the matrix layer.

Results

hK13 enzymatic activity

Incubation of hK13 with Val-Pro-Arg-AMC showed that it is enzymatically active (please see later sections for more details). When 0.25 μg and 0.5 μg of hK13 were run on a zymogram, we obtained white bands on a blue background at the expected molecular weight of highly glycosylated hK13 [28], indicating lysis of gelatin (Fig. 1).

Digestion of extracellular matrix components

Analysis of the biotinylated extracellular matrix components (ECM) proteins treated with hK13 by Western blot showed generation of many new bands of different molecular weights, in comparison to the enzyme-free control reactions (Fig. 2). The number of new bands gradually increased as a function of incubation time indicating further cleavage.

Inhibition of hK13 catalytic activity by monoclonal antibodies

In the hK13 inhibition assays, four different monoclonal antibodies were tested for their ability to neutralize the enzyme activity of hK13. Clone 33-1 was the most effective. 0.5 μg of clone 33-1 was able to inhibit more than 90% of hK13 enzyme activity while 1 μg neutralized the enzyme completely (Fig. 3). This concentra-

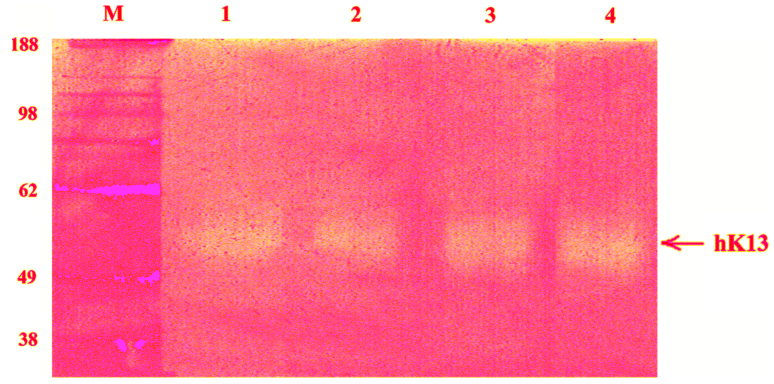

Fig. 1. Gelatin zymograph of hK13. Lane M: molecular mass standards. Lanes 1, 2: 0.2 μg of hK13. Lanes 3, 4: 0.5 μg hK13.

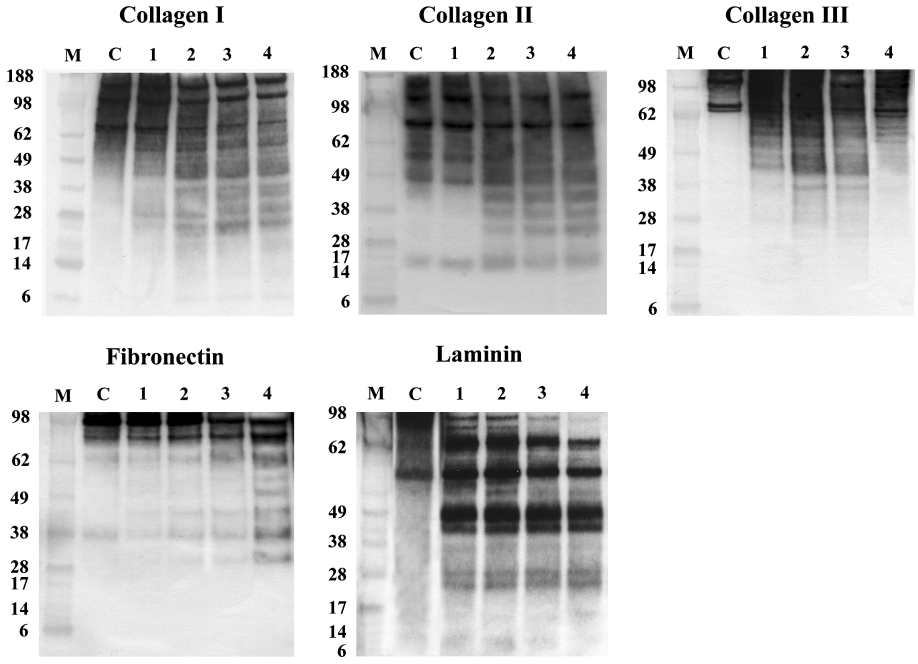

Fig. 2. Western blot of 10 μg of biotinylated collagen I, collagen II, collagen III, fibronectin, and laminin incubated with 200 ng of hK13. Lane M: molecular mass standards with masses in kDa, shown on the left of panels. Lane C: biotinylated extracellular matrix (ECM) protein. Lane 1: biotinylated ECM protein incubated with hK13 for 5 min. Lane 2: biotinylated ECM protein incubated with hK13 for 2 h. Lane 3: biotinylated ECM protein incubated with hK13 for 4 h. Lane 4: biotinylated ECM protein incubated with hK13 for 8 h. Note fragmentation of ECM proteins by hK13 with generation of multiple fragments, especially after 8 h incubation.

tion was used in the invasion assays performed with the Boyden chamber.

Cell lines, secreting hK13

The hK13 ELISA results revealed that the ovarian cancer cell line OV90 secreted the highest amount of hK13 (8 μg/L), compared to other cell lines (data not shown). This cell line was used for the Boyden Chamber assays.

Boyden chamber assays

In the wells treated with the hK13-neutralizing antibody, significantly fewer cells migrated through the matrices, in comparison to the antibody-free control wells. By cell counting, the difference between antibody-treated and not treated cells was approximately 40% ($p < 0.05$, Student's t test).

Discussion

The extracellular matrix is a highly specialized structure composed of different high molecular weight proteins that surround normal as well as malignant cells. Collagen is one of the main components of the extracellular matrix and represents 60% of the total protein in our body. There are several different types of collagen

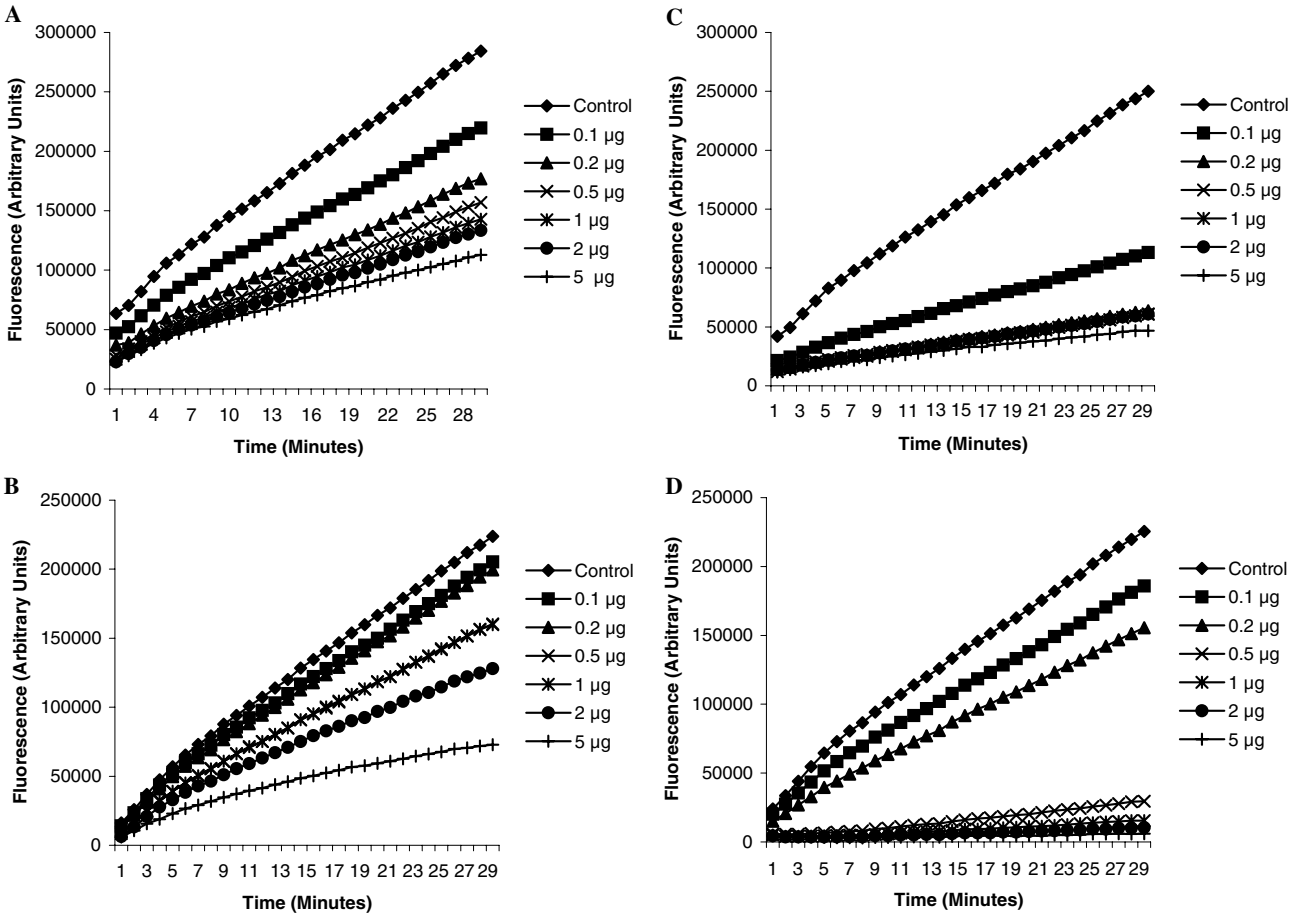

Fig. 3. Inhibition of hK13 with mouse monoclonal antibodies. (A) Clone 11C1. (B) Clone 2-2A. (C) Clone 27-1. (D) Clone 33-1 antibody. Note complete inhibition of hK13 enzymatic activity with $\geqslant 1$ µg of clone 33-1 antibody.

with different structures and molecular weights. Type I collagen is the major structural component of extracellular matrix, found in connective tissue and internal organs, but is most prevalent in the dermis, tendons, and bone [35]. Collagen II is abundant in cartilage and forms a network of fibrils extended by proteoglycans that serve as a platform for the attachment of collagen IX, growth factors, and cells [35]. Collagen III is reported to be an extracellular matrix protein that interacts with Maspin (mammary serine protease inhibitor), a tumor suppressor protein in human breast epithelial cells. A direct interaction between Maspin and extracellular matrix collagen may contribute to cell adhesion, which plays a role in the prevention of tumor cell migration and angiogenesis [36]. Among the non-collagen components of the extracellular matrix, fibronectin and laminin are the most important. Collagen is associated with these proteins to form the ECM that regulates cell migration, differentiation, proliferation, metastasis, and invasion [37–39]. Fibronectin interacts with integrin molecules during migration of tumor cells [40–43]. Degradation of fibronectin influences cell migration, attachment, and signal transduction. Hence, degradation of this protein may have multiple effects in metastasis. Laminin is another member of the ECM and basement membrane that forms a network with the collagens [44]. Integrins can bind to laminin and fibronectin during cell migration.

In this paper, we have provided evidence for the role of hK13, a secreted trypsin-like serine protease, in tumor invasion and metastasis in ovarian cancer patients. First, we demonstrated the ability of hK13 to breakdown gelatin (Fig. 1), which is derived from collagens. We also demonstrated the ability of hK13 to cleave collagen I, collagen II, collagen III, fibronectin, and laminin (Fig. 2).

To further support our hypothesis, we used the Boyden chamber, which is now considered a powerful in vitro tool for testing cell migration [33]. We screened several cancer cell lines using the hK13 ELISA and found an epithelial ovarian cancer cell line (OV90) that secretes hK13 in relatively large amounts. We cultured the cells in media that were protein and serum- free, to eliminate the effects of other proteins in the migration assays. Tumor cells cleave ECM proteins through the action of their secreted proteases and the metastatic cells

move to the other side, through the dissolved matrix, and ultimately, through the pores in the polycarbonate membrane. The possible role of hK13 in invasion is further suggested by results which show that in the presence of hK13-specific neutralizing antibody, the ability of OV-90 cells to migrate through the filter coated with the various ECMs is reduced by approximately 40%, in comparison to the controls in which either a non-specific antibody was used or no antibody was added.

Strong evidence suggests that invasion and metastasis in solid tumors require the action of tumor-associated proteases, which promote dissolution of the surrounding matrix and basement membranes [6]. A well-studied pathway is the urokinase-type plasminogen activator (uPA) system, which, upon activation, leads to degradation of components of the tumor stroma [45]. Recently, it was shown that hK6, which is also overexpressed in ovarian cancer patients, degrades fibronectin, laminin, collagen type I, and collagen type IV, all of which are major constituents of the extracellular matrix (ECM) and basement membrane [46,47]. More recently, we have also shown that hK6 degrades collagen II and collagen III [48].

The ability of hK13 to cleave ECM proteins, along with its over expression in ovarian cancer, classifies it in the spectrum of serine proteases that may play a role in tumor metastasis. These findings raise the possibility that hK13 may be an important factor in pericellular proteolysis and tumor invasion. Lysis of certain components of the ECM disrupts its dynamic interaction with the cells and is linked to altered regulation of cell proliferation that leads to tumor cell growth and malignant transformation. However, we have recently shown that hK13 levels in ovarian cancer cytosols are associated with good patient prognosis [49]. These apparently discordant data are difficult to interpret due to our incomplete knowledge of hK13 physiological role. In the future, it is possible that serine proteases, including members of the human kallikrein family, may become valuable therapeutic targets when the biological pathways that they are involved in are further delineated.

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