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Human Kallikrein 6 Degrades Extracellular Matrix Proteins and May Enhance the Metastatic Potential of Tumour Cells

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Key Words
Human kallikrein 6 · Serine proteases · Extracellular matrix proteins · Invasion · Metastasis

Abstract
Human kallikrein 6 (hK6), a trypsin-like serine protease, is a newly identified member of the kallikrein gene family. Its involvement in inflammatory CNS lesions and in demyelination has been reported. Recent work has suggested that expression of this enzyme is significantly elevated in patients with ovarian cancer. We have identified many tumour cell lines that secrete hK6, but its physiological role is unknown. Here, we try to unveil the role of this kallikrein in the metastasis and invasion of tumour cells. We demonstrate that purified human recombinant hK6 can cleave gelatin in zymography and can efficiently degrade high-molecular-weight extracellular matrix proteins such as fibronectin, laminin, vitronectin and collagen. In Boyden chamber assays, we found that tumour cells treated with a neutralizing hK6 antibody migrate less than control cells. We conclude that hK6 might play a role in the invasion and metastasis of tumour cells and may be a candidate therapeutic target.

Introduction

The human kallikrein family consists of 15 closely related serine proteases, encoded by genes that are tandemly localized on chromosome 19q13.4 [1]. Recent work has suggested that the expression of different kallikreins varies in different pathophysiological conditions. Several kallikreins are regarded as potential diagnostic markers of cancer and Alzheimer's disease [2–7]. One of the newly identified members of the human kallikrein gene family is kallikrein 6 (hK6), also known as protease M [8], neurosin [9] or zyme [10]. This kallikrein is a 223-amino acid residue serine protease with trypsin-like activity. Its expression at the mRNA level has been confirmed in several tissues such as kidney, endometrium, breast, pancreas, prostate and the CNS (brain, cerebellum and spinal cord) [8, 9, 11]. By ELISA assays, this protein has been found in different biological fluids such as milk, nipple aspirate fluid, breast cyst fluid, male and female serum, seminal plasma, cerebrospinal fluid and amniotic fluid [6, 12, 13]. Immunohistochemistry has shown abundant hK6 expression in various other normal tissues and confirmed its epithelial localization [14]. The potential involvement of this protein in the development and progression of Alzheimer's disease has been reported [6, 9, 10, 13]. It was also found that ovarian cancer patients have significantly elevated levels of hK6 in the circulation [5]. This finding is supported at both the mRNA and pro-

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tein levels in ovarian tumours [15, 16]. Although the presence of hK6 in various pathological conditions has been reported, its physiological role is still unknown. Scarisbrick et al. [13] reported the presence of myelencephalon-specific protease, which is identical to hK6, in inflammatory CNS lesions and suggested that this enzyme might promote demyelination. Also, it has been reported that this protein might play a role in the degradation of β-amyloid or turnover of amyloid precursor protein [17]. Magklara et al. [18] showed that hK6 can cleave collagen I, collagen IV, fibrinogen and casein. The kinetic properties of hK6 have been studied, and hK6 is regarded as a degradative protease with broad specificity for cleavage after an arginine residue in the P_1 position of the substrate [18, 19].

In the present investigation, we examine the role of hK6 in the degradation of extracellular matrix (ECM) proteins. ELISA assays of tumour cell culture supernatants have shown that many tumour cells secrete hK6 in significant amounts, along with other enzymes such as metalloproteinases. The crystallographic work on hK6 has suggested that this protein has low affinity for low-molecular-weight peptide substrates, whereas it may function very efficiently with larger peptide substrates with full potential for extended-contact interaction such as myelin basic protein, fibronectin or laminin [19]. We thus decided to explore the role of hK6 in degrading ECM proteins, including basement membrane proteins such as collagen, fibronectin, laminin and vitronectin. Basement membrane acts as a regulator of specific biological functions such as cellular growth, differentiation, repair and migration as well as a modulator of pathological events like tumour cell differentiation, invasion and metastasis [20–29].

Materials and Methods

Production and Purification of Recombinant hK6

Human embryonic kidney 293 cells, stably transfected with a plasmid containing the 1.4-kb pro-hK6 cDNA, were grown as previously described [10]. The expressed pro-hK6 was purified from the culture supernatant following the method of Magklara et al. [18]. Briefly, the supernatant was concentrated by passing through an ultrafiltration membrane and added to an FPLC cation-exchange column (CM Sepharose, Amersham Biosciences, N.J., USA) mixed with running buffer in a 1:2 ratio (50 m*M* sodium acetate buffer, pH 5.3). Repeated washing of the column with the running buffer ensured removal of unbound proteins. Then, hK6 was eluted using a linear gradient of elution buffer (0–1 *M* NaCl with 50 m*M* sodium acetate buffer, pH 5.3) at a flow rate of 1 ml/min. The presence of pro-hK6 in the fractions was tested by ELISA and SDS-PAGE. The fractions enriched with hK6 were pooled and concentrated by ultrafiltration and used in HPLC for further purification. The concentrated mixture was diluted in a running buffer (i.e. mobile phase water with 0.1% trifluoroacetic acid) and loaded onto the pre-equilibrated reversed-phase C8 column with the equilibration buffer at a flow rate of 0.5 ml/min. The column was washed thoroughly and hK6 was eluted using a gradient system (acetonitrile with 0.1% trifluoroacetic acid). The presence of pro-hK6 in the fractions was tested by ELISA, as well as by SDS-PAGE. The fractions containing purified pro-hK6 were pooled for further work. Traces of organic solvents were removed by nitrogen bubbling. Purified pro-hK6 was stored in aliquots at –70°C.

Assay of hK6 Enzymatic Activity with Synthetic Substrates

The following synthetic peptides were purchased from Bachem (King of Prussia, Pa., USA): Pro-Phe-Arg-aminomethylcoumarin (AMC), Val-Pro-Arg-AMC (VPR-AMC), Phe-Ser-Arg-AMC (FSR-AMC), Glu-Gly-Arg-AMC, Asp-Pro-Arg-AMC, Gly-Gly-Arg-AMC, Val-Leu-Lys-AMC, Glu-Lys-Lys-AMC and Ala-Ala-Pro-Phe-AMC. All substrates were diluted in DMSO at a final concentration of 80 m*M* and stored at –20°C. The optimum buffer system for the analysis of the activity of hK6 was 50 m*M* Tris, 0.1 *M* NaCl and 0.01% Tween-20, pH 7.3. In 100 μl of reaction buffer, 1 μ*M* substrate and 0.5–1 μg of purified pro-hK6 were added in microtiter plates and equilibrated at 37°C in a spectrofluorometer (Wallac-Victor, Perkin-Elmer, Mass., USA). The excitation and emission wavelengths were set at 485 and 535 nm, respectively, and fluorescence was measured for 20 min. Enzyme-free reactions were used as negative controls, and background fluorescence was subtracted from each value. All experiments were performed in triplicate. As mentioned elsewhere [18], pro-hK6 is autoactivated during the first 5 min of incubation.

Zymography

Zymography was performed according to the method of Liotta and Stetler-Stevenson [30]. Nupage 1% gelatin gels were purchased from Invitrogen (Ont., Canada), along with the renaturation, developing and running buffer. Proteins were run in the gel according to the instructions of the manufacturer. After electrophoresis, the gel was incubated in the renaturation buffer for 30 min at room temperature followed by incubation in developing buffer overnight. The gel was stained with Coomassie blue for 1 h and destained with water. The enzyme spots were visible as white bands on a blue background.

Cleavage of ECM Proteins by hK6 and Western Blot Analysis

Biotinylation of ECM Proteins. Fibronectin, laminin, vitronectin and collagen were selected for biotinylation as they are considered important members of the ECM. Biotinylation of ECM proteins was performed as previously described [31]. The proteins were dialyzed in 100 m*M* sodium phosphate buffer (pH 7.4) overnight to remove salts. NHS-LC-biotin (Pierce Chemical Co., Rockford, Ill., USA) at a 100-fold molar ratio was added and left to react for 1 h at room temperature. The biotinylated proteins were then dialyzed for 72 h in 5 litres of sodium phosphate buffer (pH 7.4) at 4°C with 3 buffer changes per day to remove unbound biotin. These biotinylated proteins were used as substrates of hK6.

Incubation of Biotinylated ECM Proteins with hK6. The biotinylated proteins (50 μg) were incubated in a 250-μl reaction mixture with 100 m*M* phosphate buffer (pH 7.6), 0.1 *M* NaCl and 0.01% Tween-20 with 1–2 μg of purified hK6. This mixture was incubated in a 37°C incubator. At 0, 12, 24 and 48 h, 20 μl of the reaction mixture were removed for analysis. The same reaction mixture but

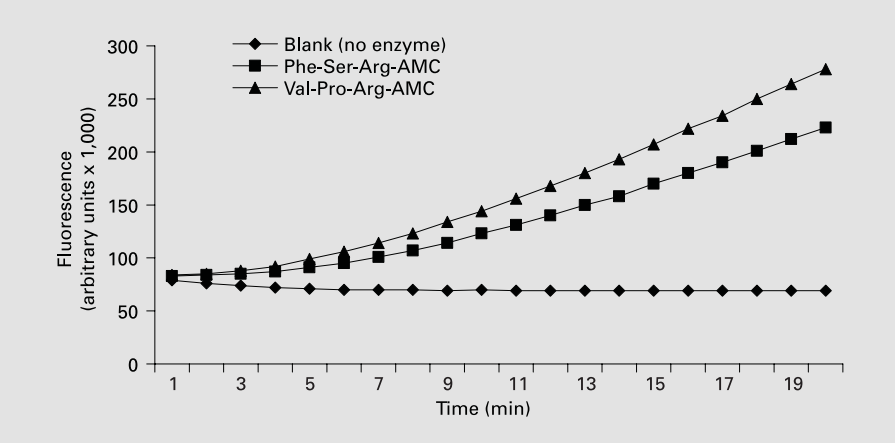

Fig. 1. Catalytic activity of purified hK6 with VPR-AMC and FSR-AMC substrates.

without any enzyme was run for 48 h in parallel as a negative control. Proteins were separated on 4–12% SDS-PAGE gels (Nupage, Invitrogen) under reducing conditions and were transferred to nitrocellulose membranes. Membranes were blocked by 5% skim milk overnight followed by incubation with a streptavidin-alkaline phosphatase conjugate (1:40,000) for 1 h. The membranes were washed in washing buffer (50 m*M* Tris, 50 m*M* NaCl, pH 7.6, with 0.1% Tween-20) and exposed to a chemiluminescence substrate for 1 min. They were then developed by exposure to an X-ray film.

ELISA of Tumour Cell Line Supernatants for Secreted hK6

We identified 5 cell lines: HTB75 (ovarian cancer), HTB161 (ovarian cancer), HTB19 (breast cancer), MDA468 (breast cancer) and PC3 $(AR)_6$ (prostate cancer) that secrete significant amounts of hK6 by ELISA analysis of tissue culture supernatants.

Inhibition of hK6 Catalytic Activity by a Monoclonal Antibody

hK6 monoclonal antibodies were produced by standard hybridoma technology using recombinant hK6 protein as immunogen [18]. One antibody clone, E24, was found to inhibit the enzymatic activity of hK6 by more than 99%. This monoclonal antibody was used to block the catalytic activity of hK6. In 100 µl of reaction mixture, 1 µg of hK6 and E24 antibody (2, 5, 10 and 20 µg) were used. The mixture was incubated for 2 h at 4°C. The enzymatic reaction was then started by the addition of 2 µl of 80 m*M* VPR-AMC substrate. The fluorescence was monitored for 20 min, at 1-min intervals. All assays were performed in duplicate.

Cell Migration in a Boyden Chamber

A 96-well reusable Boyden chamber with an 8-µm polycarbonate-framed filter was used to study the role of hK6 in the migration of tumour cells [32]. HTB19, a tumourigenic breast cancer cell line (also known as BT-20) that secretes significant amounts of hK6, was used in the migration assay. It was cultured in serum-free media and grown to confluency in 250-ml culture flasks. The filter was soaked overnight with ECM proteins, including fibronectin, laminin, vitronectin, collagen II and collagen III at 10 µg/cm^2. In parallel, the hK6 monoclonal antibody was used to block the activity of secreted hK6 from the cells in the culture media. The optimum concentration of the antibody was established from inhibition assays with the enzyme. In control wells, the same amount of dilution buffer was added. Fifty microlitres of culture media with 10% FBS were used in the lower chamber as a chemoattractant. The chamber was incubated in a CO_2 incubator at 37°C for 48 h. Removal of cells from the upper surface and staining of the migrated cells on the bottom surface of the filter was performed according to the method of Tarui et al. [33]. Finally, pictures of the migrated cells were taken with an inverted microscope (Olympus, Japan) at ×200 magnification with random selection of at least four fields.

Labelling of HTB19 Cells with Calcein and Migration Assay in a Boyden Chamber

The cultured HTB19 cells were labelled with calcein according to the method of Frevert et al. [34]. The labelled cells were then placed in the upper well of a Boyden chamber, keeping the other parameters constant. The migrated cells that reached the lower chamber were measured by calcein fluorescence with a fluorometer (Wallac, Perkin-Elmer).

Results

hK6 was produced and purified following the method described elsewhere [18]. hK6 produced by this method is greater than 98% pure, as judged by SDS-PAGE. The enzymatic activity of this protein was confirmed using synthetic fluorogenic substrates (fig. 1). As previously shown, this enzyme autoactivates itself and cleaves preferentially after an arginine residue [18]. Gelatin zymography for active hK6 indicated effective cleavage at the expected molecular weight of hK6 (fig. 2).

We tested the ability of hK6 to cleave ECM proteins such as fibronectin, laminin, vitronectin, collagen II and collagen III (fig. 3). Prolonged incubation time increased the fragmentation pattern of these proteins (fig. 3). Sometimes the proteins in control conditions showed some cleavages; as most of the ECM is composed of several polypeptide chains, those are separated due to the re-

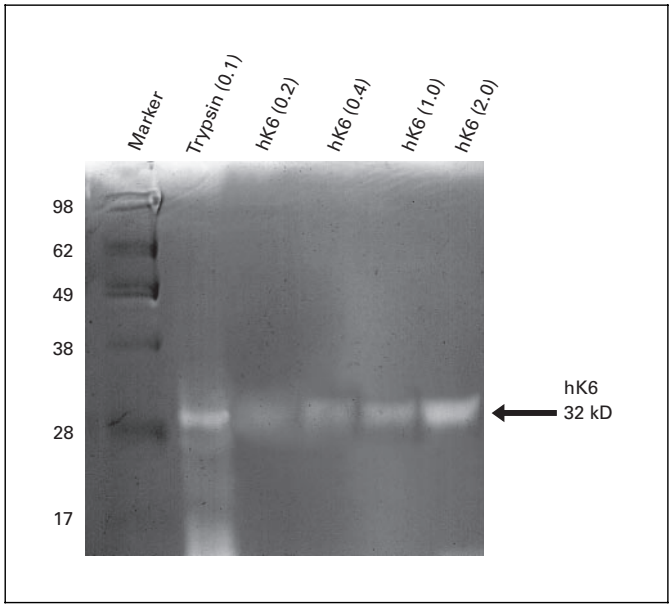

Fig. 2. Gelatin zymography of purified hK6 and trypsin (control). Numbers in parentheses represent amounts of enzyme per lane, in micrograms. Numbers on the left of the figure represent molecular weights in kD.

duced conditions of the gel. None of the bands visible in the blot were contributed by self-cleavage of hK6 as it was not biotinylated. There were several bands always visualized in the blot in the case of '0 h' of incubation of all the substrates as the enzyme had the opportunity to cleave the substrate within the ample time of mixing the enzyme into the reaction mixture and freezing it at –80°C.

In table 1, we present the concentration of secreted hK6 in tissue culture supernatants from various cancer cell lines. The same cell lines were tested by ELISA for production of other kallikreins such as hK5, hK7, hK8, hK10, hK11, hK13 and hK14 (data not shown). We chose the cell line HTB19 for migration and invasion assays since this cell line produced relatively large amounts of hK6 and much lower amounts of all other kallikreins.

In figure 4, we present the inhibitory activity of monoclonal antibody E24 on the enzymatic activity of hK6. The enzymatic activity was completely abolished at antibody concentrations of 20 μg/ml or higher.

In Boyden chamber assays, we found that cells treated with the neutralizing hK6 monoclonal antibody migrated less than control (non-treated) cells. Using calcein fluores-

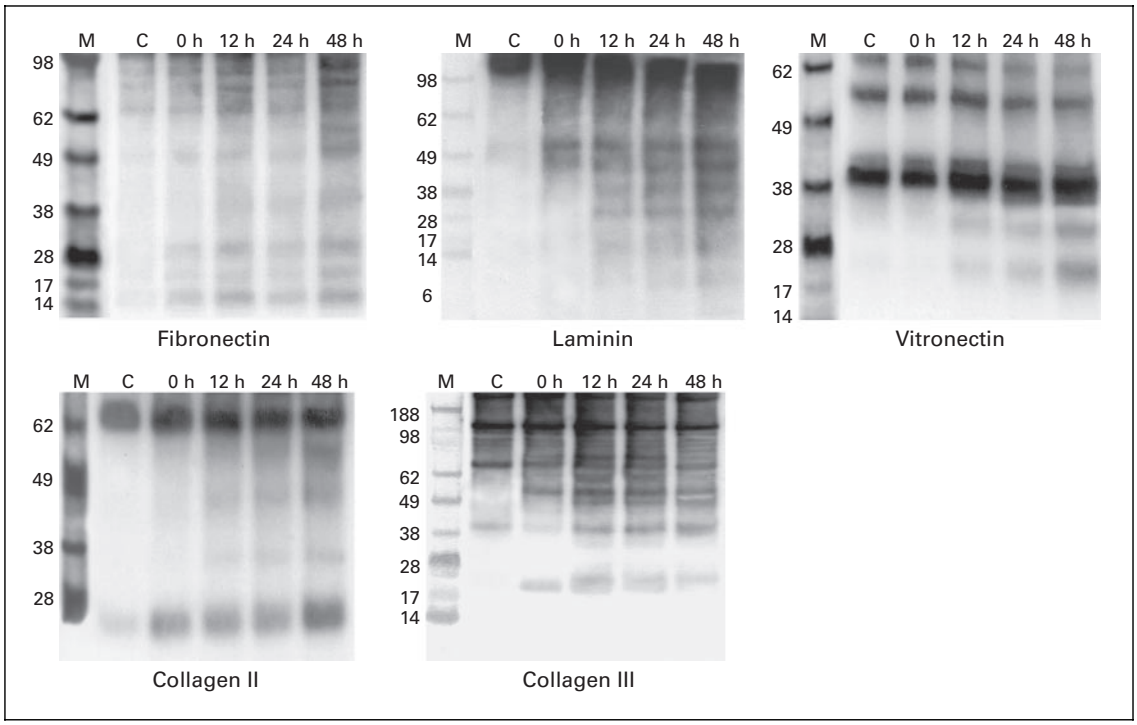

Fig. 3. Cleavage of ECM components by purified hK6. M = Molecular weight markers (in kD); C = control digestion without enzyme. Digestion was performed for variable times (0–48 h). Note digestion of proteins with generation of new bands over time. For discussion, see text. All proteins were biotinylated and detected by Western blot with streptavidin-alkaline phosphatase and chemiluminescence.

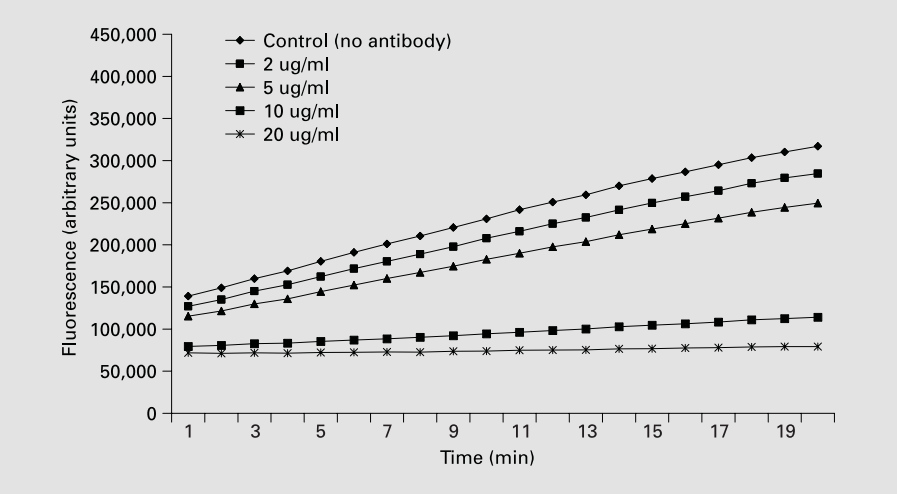

Fig. 4. Inhibition of catalytic activity of hK6 by monoclonal antibody E24. Purified hK6 and different concentrations of the monoclonal antibody were mixed and incubated for 2 h. Then, the reaction was started by addition of substrate (VPR-AMC).

cence measurements, we were able to quantify cell migration. In general, cell migration was reduced by approximately 40% when cells were treated with the neutralizing hK6 antibody ($p < 0.05$ in all cases).

Discussion

ECM 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 ECM proteins and represents 60% of the total protein in the human body. There are different types of collagen, according to its molecular weight and its structural and functional conformation, which associate with different components of the ECM. 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 ECM protein that could interact with maspin (mammary serine protease inhibitor), a tumour suppressor protein in human breast epithelial cells. A direct interaction between maspin and ECM collagen may contribute to cell adhesion, which plays a role in the prevention of tumour cell migration and angiogenesis [36]. The degradation of collagen III by hK6 could be a crucial event leading to tumour cell metastasis. For progression, invasion or metastasis, tumour cells must dissolve the collagen network [29]. Zymography is considered a standard technique to examine the activity of matrix-degrading enzymes produced by tumour cells [37, 38]. In our experiments, the zymogram revealed the ability of hK6 to degrade gelatin. As gelatin is derived from collagens, these data indicate the capability of hK6 to degrade collagen networks. Our Western blot data with collagen II and III are in agreement with the zymographic results. For both collagen types, many bands appeared after digestion with hK6, in comparison with control (fig. 3). The density and number of bands were generally proportional to the length of the incubation. Among the non-collagen components of the ECM, fibronectin, laminin and vitronectin are the most important. Collagen in association with these proteins forms the scaffold of ECM that regulates cell migration, differentiation, proliferation, metastasis and invasion [23, 39, 40]. Fibronectin is responsible for interaction with integrin molecules during migration of tumour cells [29, 37, 41, 42]. Degradation of this protein influences cell migration, attachment and signal transduction. So, degradation of fibronectin may have

Table 1. Concentration of hK6 in tissue culture supernatants of various cancer cell lines

Cell line	Origin	hK6, µg/l media with FCS	Serum-free media
MDA468	breast cancer	300	75
PC3 $(AR)_6$	prostate cancer[1]	800	230
HTB75	ovarian cancer	30	2
HTB161	ovarian cancer	60	5
HTB19 (BT-20)	breast cancer	75	50

FCS = Fetal calf serum.
[1] This cell line was stably transfected with androgen receptor.

multiple roles in metastasis. Our Western blots with fibronectin revealed many fragments after 48 h of incubation, indicating the ability of hK6 to hydrolyze this important protein. Laminin is another member of the ECM and basement membrane that forms a network with collagen IV [24]. Integrins can bind to laminin and fibronectin during migration, regulating focal adhesion kinase, one of the main kinases associated with cell migration [39–41]. Our data show that laminin can be degraded by hK6. Degradation of fibronectin and laminin by hK6 was also shown by Bernett et al. [19].

Vitronectin is a 75-kD extracellular glycoprotein deposited extravascularly in many tissues [43, 44]. In our experiments, it was shown that vitronectin generated many fragments after digestion with hK6. Previously, Magklara et al. [18] showed that hK6 cleaves collagen I and collagen IV and several other physiologically important proteins such as fibrinogen and casein. These data complement the results presented herein.

The Boyden chamber is now considered a powerful in vitro tool for testing cell migration [32]. We have chosen to work with the HTB19 tumour cell line for several reasons: first, this cell line secretes mostly hK6 in serum-free media (50 μg/l) but not other kallikreins, and secondly, these cells were reported to be metastatic. We cultured the cells in media which were protein- and serum-free, to eliminate the effects of other proteins on migration. Incubation was extended up to 48 h, which is sufficient for secretion of hK6, since this kallikrein was reported to appear in culture media within 24 h [5]. Tumour cells cleave ECM proteins through the action of their secreted proteases, and the metastatic or invasive cells move to the other side, through the dissolved matrix, and ultimately through the pore of the polycarbonate membrane due to chemoattraction. Addition of neutralizing hK6 antibody blocks the activity of hK6 and results in decreased migration and invasiveness. These preliminary data support a role of hK6 in metastasis and invasion and suggest that hK6 may be a therapeutic target. The fluorescence end point assay is technically superior and more quantitative than the visual end point assay [34]. Frevert et al. [34] showed that calcein fluorescence maintained a linear function with the number of cells and did not affect the migration of cells in any way. In the Boyden chamber, a major portion of metastatic cells always reaches the medium of the lower chamber rather than staying anchored on the bottom of the filter. So, measurement of the cells only from the bottom of the filter may not be enough to reveal the actual picture of the migration [34, 45]. In our experiment, cells labelled with calcein were added in the Boyden chamber. The bottom side of the filter was stained, and migrated cells that reached the wells of the lower chamber were counted by measurement of fluorescence.

hK6 is secreted as a pro-enzyme but is autoactivated [18]. Once activated, it acts similarly to rat myelencephalon-specific protease, which is characterized as a degradative protease with much greater catalytic efficiency for Arg versus Lys in the P1 position [18, 19]. In comparison to other proteases, its overall structural conformation, folding and catalytic motif seem to be similar to trypsin. The X-ray crystallographic data of hK6 suggest that the variable surface loop region that is responsible for activity is short and generally oriented away from the substrate binding site. This is a characteristic feature of a degradative type of protease, exemplified by the digestive enzyme trypsin [46]. Our data regarding the degradation of high-molecular-weight ECM proteins and our experiments with synthetic substrates are consistent with this hypothesis. The suggested role of hK6 for matrix protein degradation is important, in light of present knowledge that ovarian and other tumours overexpress hK6. If such a role of hK6 is confirmed in vivo, this enzyme may represent a novel target for anti-metastatic therapy.

Acknowledgement

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