

Characterization of Human Kallikreins 6 and 10 in Ascites Fluid from Ovarian Cancer Patients

Liu-Ying Luo^{a,b} Antoninus Soosaipillai^a Linda Grass^a Eleftherios P. Diamandis^{a,b}

^aDepartment of Pathology and Laboratory Medicine, Mount Sinai Hospital, and ^bDepartment of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

Key Words

Human kallikrein 6 · Human kallikrein 10 · Ovarian cancer biomarkers · Ascites fluid · Tissue kallikreins

Abstract

Objectives: Human kallikreins 6 (hK6) and 10 (hK10) are secreted serine proteases. We previously found that hK6 and hK10 are highly overexpressed in epithelial ovarian tumors and demonstrated that serum levels of hK6 and hK10 are valuable biomarkers for ovarian cancer diagnosis and prognosis. Our aim is to purify and characterize these two kallikreins from ascites fluid of ovarian cancer patients. **Methods:** Protein concentrations of hK6 and hK10 in ovarian cancer ascites fluids were measured with ELISA-type immunoassays. hK6 and hK10 were purified from the ascites fluids with immunoaffinity columns, followed by reverse-phase high performance liquid chromatography. Purified hK6 and hK10 were then subjected to N-terminal sequencing. Enzymatic analyses were performed with synthetic fluorogenic peptides. **Results:** hK6 and hK10 were present in ovarian cancer ascites fluid at concentrations ranging from 0.2–571 and 0.7–220 µg/l, respectively. The majority of hK6 and hK10 in the ascites fluids were present in the free (uncomplexed) form. Both hK6 and hK10 purified from the ascites fluid were zymogens with a molecular mass of 30 kDa. Purified hK6 exhibited trypsin-like enzymatic activity, whereas no enzymat-

ic activity was observed for purified hK10. The enzymatic activity of hK6 could be suppressed by a neutralizing monoclonal antibody. **Conclusions:** The majority of hK6 secreted by the ovarian tumor cells into the ascites fluid are present in the uncomplexed, zymogen form, possessing weak trypsin-like enzymatic activity. All hK10 present in ovarian cancer ascites fluids are in the uncomplexed, zymogen form and have no detectable enzymatic activity.

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Introduction

Human tissue kallikreins are a group of 15 secreted serine proteases, hK1–hK15, encoded by genes (KLK1–KLK15) that are tandemly clustered in a 300-kb region on chromosome 19q13.4 [1]. hK6 and hK10 are 2 members of this protease family. hK6, also known as zyme/neurosin/protease M, is a protein consisting of 244 amino acids [2–4]. hK6 is expressed in many tissues, such as the brain, ovary, breast and skin [5, 6]. It is also found in many biological fluids, such as cerebrospinal fluid, milk and ascites fluid [7]. The physiological functions of hK6 remain unclear. Recent investigations with recombinant hK6 produced in yeast and insect cells have shown that hK6 has trypsin-like enzymatic activity. hK6 hydrolyzes peptide bonds preferentially after an arginine residue [8, 9].

In *in vitro* experiments, hK6 can cleave amyloid precursor protein, myelin basic protein, fibrinogen and extracellular matrix components, including fibronectin, laminin and collagen [2, 8, 10]. X-ray crystal structure analysis revealed that its features are more similar to trypsin than to other regulatory enzymes [10, 11]. These results suggest that hK6 may have broad substrate specificity and may function as a degradative protease. Therefore, hK6 may cleave different substrates in different cell types and microenvironments and may play different roles in different tissues. hK6 is implicated with many diseases. It has been reported that hK6 is differentially expressed in Alzheimer's disease, multiple sclerosis, uterine, gastric breast and ovarian cancer [4, 12–16]. hK10, also known as normal epithelial cell specific 1, was first identified as a 276-amino-acid protease that is downregulated in malignant breast epithelial cells [17]. Similar to hK6, hK10 is expressed in a variety of tissues, with high levels in the tonsil, skin, ovary and breast [18]. It is also detected in many biological fluids, such as milk and seminal plasma [19]. hK10 is predicted to have trypsin-like enzymatic activity [17]. However, this prediction has not been experimentally confirmed so far. The physiological functions of hK10 are not known. hK10 has been found to be downregulated in both breast and testicular cancer [17, 20, 21], suggesting that it may function as a tumor suppressor. This hypothesis was further supported by the finding that overexpression of hK10 in breast cancer cell lines could suppress tumor formation in nude mice [22]. The expression of KLK10 may be regulated by promoter hypermethylation [23] and KLK10 has a number of splice variant forms [24].

In our previous studies, we found that hK6 and hK10 were overexpressed in epithelial ovarian cancer. High levels of hK6 and hK10 in ovarian tumor cytosolic extracts are associated with more aggressive tumor phenotypes and poor prognosis [14, 25–28]. Overexpression of hK6 and hK10 in ovarian tumors also results in elevation of hK6 and hK10 serum levels in ovarian cancer patients. It has been demonstrated that hK6 and hK10 are valuable diagnostic biomarkers for ovarian cancer [29, 30]; however, the biological mechanisms underlying the overexpression of hK6 and hK10 in ovarian cancer are not clear. To gain insights into the possible roles of hK6 and hK10 in ovarian cancer progression, we purified and characterized hK6 and hK10 secreted by ovarian tumors. Ascites fluid develops when ovarian tumor cells exfoliate and seed in the peritoneal cavity. This fluid is rich in proteins secreted by the ovarian tumor cells and provides an excellent source for native hK6 and hK10.

Materials and Methods

Ascites fluid samples were obtained from 25 ovarian cancer patients during therapeutic drainage. Informed consent was obtained from all patients and our protocol was approved by the Mount Sinai Hospital Ethics Committee.

Immunoassays for hK6 and hK10

The concentrations of hK6 and hK10 in the ascites fluids were measured with 'sandwich-type' immunoassays as previously described [7, 19]. Briefly, the procedures are outlined below. For the hK6 immunoassay, one hK6-specific monoclonal antibody (clone 27-4) was immobilized in 96-well microtiter plates by incubating 500 ng/well in coating buffer (50 mM Tris, 0.05% sodium azide, pH 7.8) overnight. After three washings with washing buffer (5 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.8), 50 μ l of ascites fluids or standards was pipetted into each well and mixed with 50 μ l of assay buffer (6% BSA, 50 mM Tris, 10% goat IgG, 2% mouse IgG, 1% bovine IgG, 0.5 M KCl, 0.05% sodium azide, pH 7.8). After 1 h incubation, the plate was washed six times. Subsequently, another biotinylated hK6-specific monoclonal antibody (code E24) was added (50 ng/100 μ l/well), diluted in the assay buffer. After the plate was incubated and washed as above, alkaline phosphatase-conjugated streptavidin was added (5 ng/100 μ l/well) diluted in the BSA buffer (6% BSA, 50 mM Tris, 0.05% sodium azide, pH 7.8). Finally, the alkaline phosphatase substrate diflunisal phosphate was added and incubated for 10 min, followed by the addition of a developing solution containing Tb^{3+} , EDTA and NaOH, as described elsewhere [7, 19]. Fluorescence was then measured as described [7]. For the hK10 immunoassay, hK10-specific monoclonal antibodies (clone B14 and biotinylated clone 5D3) were used for coating and detection. Other steps were similar to the hK6 immunoassay.

Immunoaffinity Purification of hK6 and hK10 from Ascites Fluid

Immunoaffinity beads were prepared by coupling hK6- and hK10-specific monoclonal antibodies (clones E24 and B14, respectively) to agarose beads using the Affi-Gel Hz Immunoaffinity Kit (Bio-Rad Labs, Hercules, Calif., USA), following the manufacturer's recommendations. To purify hK6/hK10, ascites fluid samples with high levels of hK6/hK10 were pooled and mixed with E24/B14 immunoaffinity beads at 4°C overnight. The agarose beads were then packed in Econo chromatography columns (Bio-Rad) and sequentially washed with 10 bed volumes of 20 mM Tris, 150 mM NaCl, pH 7.5, and 20 mM Tris, 500 mM NaCl, pH 7.5. hK6/hK10 were eluted with 5 bed volumes of 0.2 M glycine-HCl, pH 2.5. Fractions of 1 ml were collected and immediately neutralized with 0.5 M NaHCO_3 . The fractions containing hK6/hK10 were determined with ELISA immunoassays as described above.

Western Blot Analysis

Eluted fractions from the hK6/hK10 immunoaffinity columns were first separated with SDS-PAGE and transferred to Hybond C membranes (Amersham Biosciences, Piscataway, N.J., USA). The membranes were then blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.6) for 2 h at room temperature. Subsequently, membranes were incubated with rabbit anti-hK6/hK10 polyclonal antibodies (diluted 200-fold in 1% nonfat dry milk TBS-T) for 1 h at room temperature.

After the membranes were washed three times for 15 min in TBS-T, they were incubated in alkaline phosphatase-conjugated goat antirabbit IgG (final concentration of 0.5 mg/l in 1% nonfat dry milk in TBS-T; Jackson ImmunoResearch, West Grove, Pa., USA) for 30 min. Finally, after the membranes were washed again as above, a dioxetane-based substrate was added (Pierce Chemical Co., Rockford, Ill., USA) and chemiluminescence emission was captured on X-ray film.

Reverse-Phase High-Performance Liquid Chromatography

After immunoaffinity purification, hK6 and hK10 were further purified by reverse-phase high-performance liquid chromatography (HPLC). The eluted fractions containing hK6/hK10 from the immunoaffinity purification were supplemented with trifluoroacetic acid (TFA; final concentration 1%) and then loaded on a C4 column equilibrated with 0.1% TFA in water. A linear gradient of 10–90% (1%/min) acetonitrile with 0.1% TFA was then performed. The peaks containing hK6/hK10 were collected and acetonitrile was evaporated by nitrogen gas.

N-Terminal Sequencing

Purified hK6/hK10 were separated on SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were stained with Coomassie blue G-250 and the bands were excised. N-terminal sequencing was performed with an ABI 492 sequencer.

Enzymatic Analysis of hK6

The enzymatic activity of purified hK6 was measured with synthetic fluorogenic peptides coupled to 7-amino-4-methylcoumarin (AMC), purchased from Bachem Bioscience, King of Prussia, Pa., USA. They included Val-Pro-Arg-AMC, Phe-Ser-Arg-AMC, Pro-Phe-Arg-AMC, Val-Leu-Lys-AMC, Glu-Lys-Lys-AMC and Ala-Ala-Pro-Phe-AMC. The reaction was set up in a microtiter plate at a volume of 100 μ l as follows: 120 nM purified hK6, 0.4 mM substrates, 100 mM Na_2HPO_4 , 0.01% Tween-20, pH 8.0. The microtiter plate was then incubated in the Wallac Victor Fluorometer (Peter-Elmer, Wellesley, Mass., USA) at 37°C. Fluorescence was measured with wavelengths set at 355 nm for excitation and 460 nm for emission.

Effect of Anti-hK6 Monoclonal Antibody E24 on hK6

Enzymatic Activity

To examine the effect of hK6 monoclonal antibody E24 on hK6 enzymatic activity, 500 nM E24 was included in a reaction mixture containing 120 nM purified hK6, 100 mM Na_2HPO_4 , 0.01% Tween-20, pH 8.0. After 1 h incubation at room temperature, Val-Pro-Arg-AMC was added at a final concentration of 0.4 mM. The plate was then incubated at 37°C and fluorescence was measured as described above.

Results

Concentrations of hK6 and hK10 in the Ascites Fluid of Ovarian Cancer Patients

Ascites fluid was collected from 25 ovarian cancer patients during therapeutic drainage, and hK6 and hK10

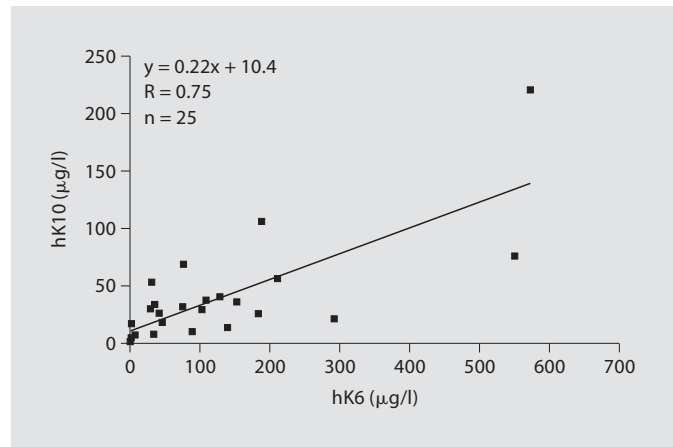


Fig. 1. Distribution and correlation of the protein concentrations of hK6 and hK10 in 25 ascites fluids from ovarian cancer patients. For more detail, see text. R = Pearson correlation coefficient.

concentrations were measured with ELISA immunoassays. hK6 concentrations ranged from 0.2 to 571 μ g/l, with a mean of 125 μ g/l and a median of 78 μ g/l. hK10 levels ranged from 0.7 to 220 μ g/l, with a mean of 38 μ g/l and a median of 29 μ g/l. hK6 and hK10 concentrations in ascites fluid were positively correlated ($R = 0.75$) (fig. 1).

Immunoaffinity Purification of hK6 and hK10 from Ascites Fluid

Ascites fluid samples with high levels of hK6 or hK10 were pooled. hK6 and hK10 were then purified with columns containing immobilized hK6- or hK10-specific monoclonal antibodies (clones E24 and B14, respectively). Proteins bound to the columns were subsequently eluted at low pH. Proteases present in biological fluids are often bound to protease inhibitors. To determine whether hK6 and hK10 also form complexes with protease inhibitors in ascites fluid, we performed Western blot analysis with rabbit anti-hK6/hK10 polyclonal antibodies. As shown in figure 2, in the eluate from the hK6 immunoaffinity column (E24), there is only one band of about 30 kDa. No higher molecular weight complex was detected. This suggests that in ascites fluid, hK6 is present mainly in its free, uncomplexed form. This result is in agreement with our earlier finding that only a very small percentage (less than 2%) of hK6 binds to protease inhibitors [31]. Similarly, in the eluate from the hK10 immunoaffinity column, we could only detect one band of

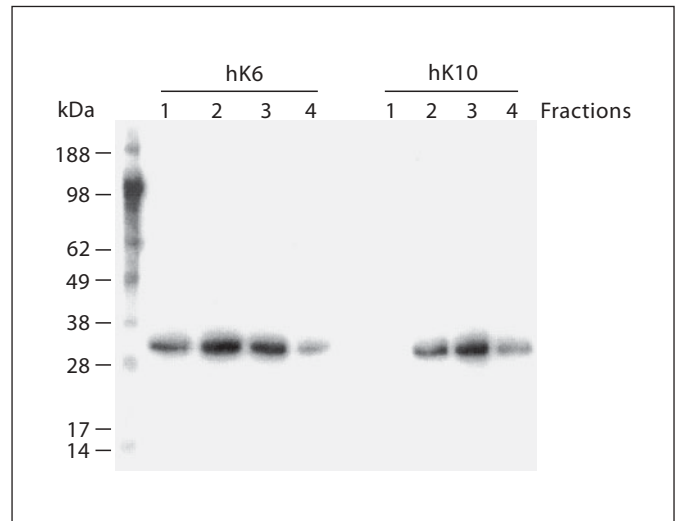


Fig. 2. Western blot analysis of fractions eluted from hK6 and hK10 immunoaffinity columns. Both kallikreins have a molecular mass of approximately 30 kDa.

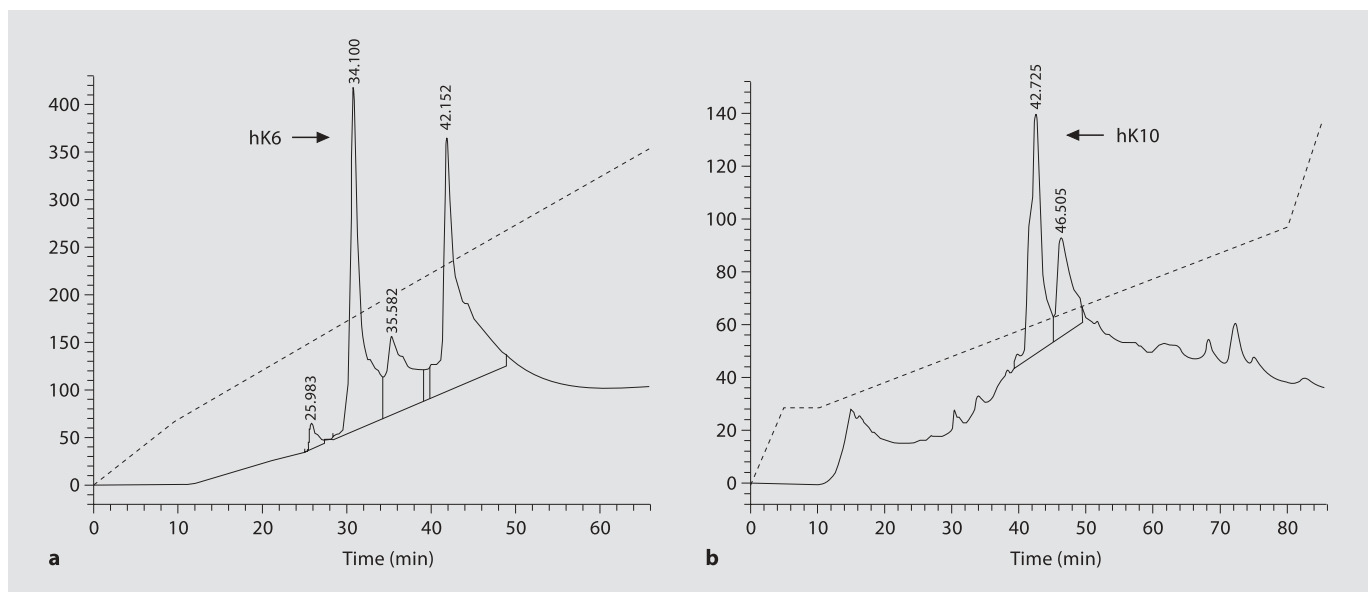


Fig. 3. Purification of hK6 and hK10 from the ascites fluid with reverse-phase HPLC after immunoaffinity purification. Typical chromatograms for hK6 (**a**) and hK10 (**b**). hK6 and hK10 are eluted at around 34–42% acetonitrile. The dotted lines represent the acetonitrile gradient.

about 30 kDa. No higher molecular weight complex was found, indicating that hK10 present in the ascites fluid is also uncomplexed.

Reverse-Phase HPLC

To purify hK6 and hK10 to homogeneity, eluates from the hK6 and hK10 immunoaffinity columns were further subjected to reverse-phase HPLC. Figure 3 shows two typical chromatograms. The peaks corresponding to hK6

and hK10 (identified by ELISA assays) were collected, concentrated and their purity was examined with SDS-PAGE. Both hK6 and hK10 in the ascites fluid are proteins with an approximate molecular mass of 30 kDa (fig. 4).

N-Terminal Sequence

Purified hK6 and hK10 were subjected to N-terminal sequencing. Through protein sequence homology com-

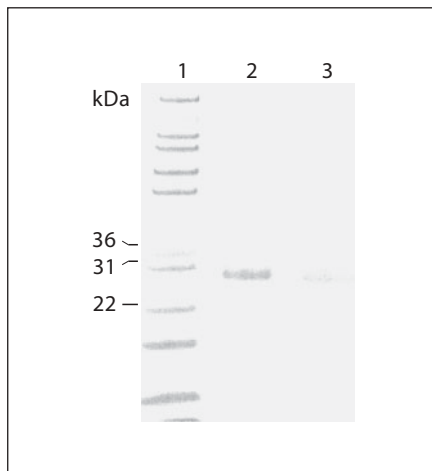


Fig. 4. Purified hK6 and hK10 from ascites fluid as shown by Coomassie blue-stained SDS-PAGE. Lane 1 = Molecular mass marker; lane 2 = purified hK6; lane 3 = purified hK10.

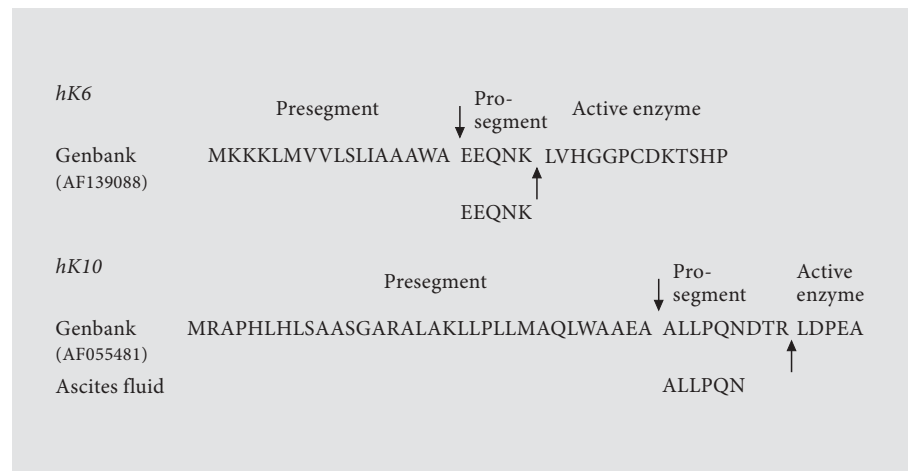


Fig. 5. Alignment of the N-terminal sequences of hK6 and hK10. Downward and upward arrows indicate pro- and active enzyme cleavage sites, respectively. hK6 and hK10 purified from the ascites fluid are zymogens, with the prosegment in their N-terminal sequence.

Table 1. Enzymatic kinetic analysis of hK6 purified from ascites fluid with synthetic peptides as substrates

Substrate	K_m mM	K_{cat} min^{-1}	$K_{cat}/K_m \cdot$ $\text{mM}^{-1} \text{min}^{-1}$
Val-Pro-Arg-AMC	0.36 ± 0.09	0.08	0.22
Phe-Ser-Arg-AMC	0.30 ± 0.08	0.05	0.17
Pro-Phe-Arg-AMC	0.23 ± 0.06	0.005	0.020
Val-Leu-Lys-AMC	no reaction		
Glu-Lys-Lys-AMC	no reaction		
Ala-Ala-Pro-Phe-AMC	no reaction		

parison, pro-hK6 has been predicted to start from amino acid 17 (Glu; E), and pro-hK10 starts from amino acid 34 (Ala; A) (1). The obtained N-terminal sequences matched the predicted sequences of pro-hK6 and pro-hK10 (fig. 5). Our results provide direct experimental evidence for this prediction and confirm that a majority of hK6 and hK10 in ascites fluid are present in their zymogen form.

Enzymatic Activities of Purified hK6 and hK10

The enzymatic activities of hK6 and hK10 purified with ascites fluid were examined with synthetic fluorogenic AMC peptides. Previous work from our laboratory has shown that recombinant hK6 (produced in mamma-

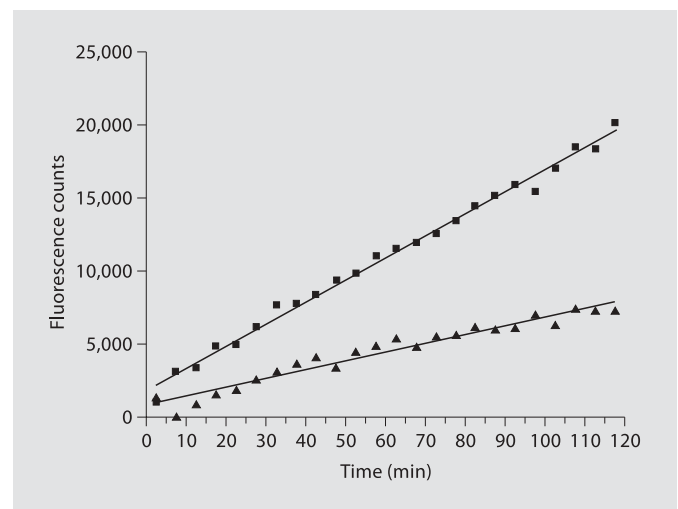


Fig. 6. Typical enzyme kinetic curves showing the inhibition of native hK6 enzymatic activity by its specific monoclonal antibody clone E24. ■ = Purified hK6 alone; ▲ = purified hK6 + E24. For more detail, see text.

lian cells) can cleave preferentially after arginine and that it has no chymotryptic activity [8]. With native hK6 purified from ascites fluid, similar results were obtained (table 1). Further experiments showed that the enzymatic activity of native hK6 could be suppressed by about 60% after binding to monoclonal antibody E24 (fig. 6). Although N-terminal sequence results indicate that the pu-

rified hK6 from ascites fluid is a zymogen, the enzymatic activity we observed could be due to a small amount of active hK6 which could not be identified by N-terminal sequencing, or to minimal but detectable activity of the proform itself. Similar experiments were performed with purified hK10. However, no activity was detected with any of the synthetic peptides tested (data not shown).

Discussion

We purified native hK6 and hK10 from ovarian cancer ascites fluid and characterized their structure and enzymatic activities. We found that hK6 and hK10 were present in the ascites fluid predominantly in their free, zymogen form. hK6, but not hK10, was enzymatically active, and this activity could be inhibited by a monoclonal antibody raised against recombinant hK6 produced in mammalian cells.

Of importance is our finding that free, active hK6 is present in the ascites fluid. These data provide evidence that hK6 may be involved in ovarian cancer progression. Previous investigations have shown that hK6 can cleave extracellular matrix proteins, suggesting that it may promote tumor metastasis [8, 28]. Others have reported that recombinant hK6 can cleave plasminogen to release angiostatin [9], an inhibitor of angiogenesis. Besides hK6, other members of the human tissue kallikrein family have been implicated in angiogenesis. Like hK6, hK3 (also known as prostate-specific antigen), can cleave plasminogen to generate angiostatin [32]. Furthermore, hK3 can inhibit endothelial cell proliferation and migration by blocking the response of endothelial cells to angiogenic stimulants, including fibroblast growth factor 2 and vascular endothelial growth factor [33]. Additionally, hK9 has been identified as an interacting partner of a protein that is involved in blood vessel formation in premalignant skin lesions [34]. These findings suggest that the contribution of hK6 to ovarian cancer progression may be complex and could include extracellular matrix degradation, tumor cell migration and regulation of angiogenesis.

The finding that active hK6 is present in ovarian cancer ascites fluid provides a rationale for the development of novel therapeutic strategies. Previously, we found that hK6 is overexpressed in ovarian tumor tissues and that high levels of hK6 in tissue and serum are associated with more aggressive disease phenotypes and poor prognosis [14, 29]. However, proteases are secreted into the extracellular milieu as inactive zymogens and then activated

by autoactivation or by other proteases. Therefore, elevation in protease protein level does not necessarily imply an increase in its proteolytic activity. Activity-based assays in ascites fluid may correlate better with disease phenotypes. We have shown that hK6 enzymatic activity can be suppressed by an hK6-specific monoclonal antibody, a candidate therapeutic agent [35].

We were not able to detect any enzymatic activity of hK10 purified from ascites fluid. Possible reasons may include the following: (1) no active hK10 is present in the ascites fluid, only the inactive zymogen is present; (2) hK10 may cleave very specific substrates that have not been tested in this investigation. Although hK10 is predicted to have trypsin-like enzymatic activity, this has not been confirmed experimentally. Dhar et al. [20] were also unable to detect hK10 enzymatic activity. Comparison of the amino acid sequence of hK10 with other tissue kallikreins reveals some distinguishing features. hK10 is the only 'nonclassical' kallikrein that has an almost complete 'kallikrein loop', a stretch of 11 amino acids that is playing a major role in substrate specificity [1]. In addition, the sequence forming the oxyanion hole (located around the catalytic serine) in the other kallikreins is the same, being GDSGGP (S is the catalytic serine residue). However, in hK10, the first glycine residue is replaced by serine [1]. These sequence differences may confer unique substrate specificity for hK10.

Serum and tissue hK6 and hK10 concentrations are positively correlated with each other in ovarian cancer patients. This result supports the previously developed hypothesis that hK6, hK10 and other kallikreins may be members of an enzymatic cascade pathway [36, 37]. Many other tissue kallikreins, including hK4, hK5, hK8, hK11, hK13, hK14 and hK15, have also been found to be differentially expressed in ovarian cancer [38–44]. Therefore, a multikallikrein cascade enzymatic pathway may be operating in ovarian cancer. If it exists, this pathway may represent a good candidate therapeutic target.

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