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The human kallikrein protein 5 (hK5) is enzymatically active, glycosylated and forms complexes with two protease inhibitors in ovarian cancer fluids

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Abstract

The kallikrein family is a group of 15 serine protease genes clustered on chromosome 19q13.4. Binding of kallikreins to protease inhibitors is an important mechanism for regulating their enzymatic activity and may have potential clinical applications. Human kallikrein gene 5 (*KLK5*) is a member of this family and encodes for a secreted serine protease (hK5). This kallikrein was shown to be differentially expressed at the mRNA and protein levels in diverse malignancies. Our objective was to study the enzymatic activity and the interaction of recombinant hK5 protein with protease inhibitors. Recombinant hK5 protein was produced in yeast and mammalian expression systems and purified by chromatography. HPLC fractionation, followed by ELISA-type assays, immunoblotting and radiolabeling experiments were performed to detect the possible interactions between hK5 and proteinase inhibitors in serum. Enzymatic deglycosylation was performed to examine the glycosylation pattern of the protein. The enzymatic activity of hK5 was tested using trypsin and chymotrypsin-specific synthetic fluorogenic substrates. In serum and ascites fluid, in addition to the free (~ 40 kDa) form, hK5 forms complexes with α_1-antitrypsin and α_2-macroglobulin. These complexes were detected by hybrid ELISA-type assays using hK5-specific coating antibodies and inhibitor detection antibodies. The ability of hK5 to bind to these inhibitors was further verified in vitro. Spiking of serum samples with ^{125}I-labeled hK5 results in the distribution of the protein in two higher molecular mass (bound) forms, in addition to the unbound form. The hK5 mature enzyme is active and shows trypsin, but not chymotrypsin-like, activity. The pro-form of hK5 is not active. Recombinant hK5 shows a higher than predicted molecular mass due to glycosylation. hK5 is partially complexed with α_1-antitrypsin and α_2-macroglobulin in serum and ascites fluid of ovarian cancer patients. The recombinant protein is glycosylated and its mature form shows trypsin-like activity.

Keywords: Kallikrein; Human kallikrein 5; *KLK5*; *KLK-L2*; Human stratum corneum tryptic enzyme; Serine protease; Serpin; Immunofluorometric assay; Serine protease inhibitor

1. Introduction

The human kallikrein gene family is a group of 15 serine proteases that cluster close to the telomere of chromosome 19 [1,2]. Kallikreins exhibit diverse physiological functions in different tissues, and more recently, it has been shown that they may be implicated in the initiation and progression of cancer [3–5]. We have recently cloned the human kallikrein gene 5 (*KLK5*, according to the human gene nomenclature committee [6]), previously known as the kallikrein-like gene-2 (*KLK-L2*) [7] or human stratum corneum tryptic enzyme (HSCTE) [8]. This gene is a new member of the human kallikrein family which maps to chromosome 19q13.4, close to other kallikreins [9,10]. The gene is mainly expressed in the skin, testis, breast and brain [7,8].

The hK5 protein is predicted to be synthesized as an inactive pre-pro-enzyme, consisting of a signal (pre) peptide of 29 amino acids, followed by a 37-amino acid activation (pro) peptide and an active enzyme of 237 amino acids with

Abbreviations: *KLK*, human kallikrein (gene); hK, human kallikrein (protein); PSA, prostate-specific antigen; CSF, cerebrospinal fluid; BSA, bovine serum albumin; PEG, polyethylene glycol; HSCTE, human stratum corneum tryptic enzyme; CHO, Chinese hamster ovary; PNGaseF, peptide *N*-glycosylase F; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AMC, aminomethyl coumarin; DMSO, dimethyl sulfoxide

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a predicted molecular mass of 25 kDa [1,7]. Cleavage of the activation peptide is predicted to occur after an arginine residue (Arg ↓ Ile), thus a trypsin-like enzyme is needed for activation of the protein [1]. hK5 has the conserved catalytic triad of serine proteases [7] and was found to have proteolytic activity [8].

We have recently shown that *KLK5* is differentially expressed, at the mRNA level, in different endocrine-related malignancies including ovarian [11], breast [12], prostate [13] and testicular cancer [14], and has the potential of being a cancer biomarker. More recently, we developed a sensitive immunoassay for measuring hK5 protein and found that serum levels of hK5 are elevated in ovarian and breast cancer patients [15]. In addition, the hK5 protein was reported to have a role in skin desquamation [15].

There are different mechanisms for controlling serine protease activity to avoid any unwanted protein degradation and to allow spatial and temporal regulation of the proteolytic activity. One mechanism is by producing the enzyme in the form of an inactive "proenzyme" which will be activated when necessary. Once activated, serine proteases are controlled by ubiquitous endogenous inhibitors. Laskowski and Qasim [16] divide all known inhibitors into two categories; inhibitors devoid of significant class specificity and class-specific inhibitors. The former includes proteins of the α_2-macroglobulin family, which bind proteases through a molecular trap mechanism and inhibit them by steric hindrance [17]. With respect to specific serine protease inhibitors, at least 23 structurally distinct families are currently known, including the Kunitz, STI (soybean trypsin inhibitor)-Kunitz, Kazal, and hirudin families as well as the Serpins (*ser*ine *p*roteinase *in*hibitors) [16]. Many of the specific inhibitors are capable of inhibiting the same serine protease, and the same inhibitor may inhibit several serine proteases [16]. Some molecular complexes of kallikreins with protease inhibitors have clinical applicability since they can improve the sensitivity and specificity of total kallikreins as cancer biomarkers [18].

In the current study, we analyze the molecular forms of hK5 in different biological fluids. Using recombinant hK5 from yeast and mammalian cells, we provide evidence that hK5 has trypsin-like substrate specificity. We also report for the first time, the identification of two protease inhibitors which can form complexes with hK5 in serum and ascites fluid from ovarian cancer patients.

2. Materials and methods

2.1. Production of recombinant hK5 in mammalian cells

Recombinant hK5 was produced in Chinese hamster ovary (CHO-CCL-61) cells using the pcDNA3.1 directional TOPO expression kit (Invitrogen, Carlsbad, CA) as follows: According to our GenBank accession number AF135028, two *KLK5*-specific primers (L2TF: 5′ CACCATGGCTACAGCAAGACCCC 3′ and L2TR: 5′ GGAGTTGGCCTGGATGGTTTCCT 3′) were used to amplify the pro-form of *KLK5* from human breast cDNA . The PCR reaction was carried out in a 50-μl mixture, containing 1 μg of cDNA, 5 μl *Pfu* DNA polymerase reaction buffer (Stratagene, La Jolla, CA), 10 μM deoxynucleoside triphosphates, 30 μM primers, and 0.5 μl of *Pfu Turbo* DNA polymerase (Stratagene), using an Eppendorf gradient cycler. The PCR conditions were 95 °C for 1 min, followed by 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min for 35 cycles, and a final extension at 72 °C for 10 min. The PCR product was then cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen) according to the manufacturer's instructions. Sequence of the construct was confirmed with an automated DNA sequencer.

The vector containing *KLK5* cDNA was transformed into TOP10 *Escherichia coli* cells (Invitrogen). Positive clones were selected, propagated and the *KLK5* plasmid was purified using standard techniques. The construct was then linearized and transfected into CHO cells (ATCC, Rockville, MD) using the PolyFect transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's protocol; β-galactosidase cDNA was co-transfected as a reporter gene. Cells were harvested after 48 h and tested for reporter gene expression. Positive clones were grown for 2 weeks in a selection medium (F12 medium with fetal calf serum and Genticin antibiotic), which allows growth of only stable transfectants. Surviving clones were picked after 2 weeks, expanded, and limiting dilutions were performed. Single cells were picked and expanded to ~ 50% confluency. Cells were then grown in CHO serum-free medium (Invitrogen) with antibiotic for 10 days. Supernatant was collected and tested for hK5 expression using Western blots with anti-hK5 rabbit polyclonal peptide antibody (produced in-house).

Human hK5 was also produced in a *Pichia pastoris* yeast expression system (Invitrogen). The details of this procedure have been described elsewhere [19].

2.2. Characterization of hK5 by mass spectrometry and N-terminal sequencing

Polyacrylamide gels were stained with Coomassie G-250 and selected bands were excised and destained with 300 ml/l acetonitrile in 100 mM ammonium bicarbonate. Each band was then reduced (10 mmol/l dithiothreitol in 50 mM ammonium bicarbonate, pH 8.3) and alkylated (50 mM iodoacetamide in 50 mmol/l ammonium bicarbonate, pH 8.3) before overnight trypsin digestion. Peptide fragments were then extracted with 50 ml/l acetic acid, evaporated dry on a Savant concentrator, and reconstituted in 10 μl of a solution of methanol–water–acetic acid (500:495:5 by volume). Mass spectrometric analysis was then performed as previously described [20]. N-terminal sequencing was performed with the automated Edman degradation method.

2.3. Purification of hK5 with cation-exchange chromatography

Recombinant hK5 was purified from cell culture supernatants by cation-exchange chromatography using a HiTrap FF CM-Sepharose column on an FPLC system (Amersham Biosciences, Piscataway, NJ). The presence of hK5 in various fractions was identified with Western blotting. The pooled fractions were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue to assess the purity and molecular mass of hK5. The protein concentration of the purified hK5 was determined by the bicinchoninic acid method, which uses bovine serum albumin (BSA) as calibrator (Pierce Chemical Co., Rockford, IL).

2.4. Immunofluorometric assay for hK5

We utilized an in-house developed monoclonal–polyclonal ELISA-type sandwich assay with a high degree of sensitivity and specificity. Details were reported elsewhere [19]. In summary, the assay was as follows: White polystyrene microtiter plates were coated with monoclonal anti-hK5 antibody by overnight incubation of 100 μl of coating antibody solution (containing 500 ng of antibody diluted in 50 mmol/l Tris buffer, pH 7.8) in each well. The plates were then washed six times with the washing buffer (9 g/l NaCl and 0.5 g/l Tween 20 in 10 mmol/l Tris buffer, pH 7.4). hK5 calibrators or samples were then pipetted into each well (50 μl/well) (diluted 1:1 in a 60 g/l solution of BSA, referred to as 'general diluent'), and incubated for 2 h with shaking, followed by washing for six times. Subsequently, 100 μl of rabbit anti-hK5 antiserum diluted 1000-fold in buffer A (containing the components of the general diluent plus 25 ml/l normal mouse serum, 100 ml/l normal goat serum, and 10 g/l bovine IgG) was applied to each well and incubated for 1 h; plates were then washed as described earlier. Finally, 100 μl/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific (Jackson ImmunoResearch), diluted 3000-fold in buffer A was added to each well and incubated for 45 min, and plates were washed as above.

Diflunisal phosphate (100 μl of a 1 mmol/l solution) in substrate buffer (0.1 mol/l Tris, pH 9.1, 0.1 mol/l NaCl, and 1 mmol/l $MgCl_2$) was added to each well and incubated for 10 min. Developing solution (100 μl, containing 1 mol/l Tris base, 0.4 mol/l NaOH, 2 mmol/l $TbCl_3$, and 3 mmol/l EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically, as described elsewhere [21].

2.5. hK5 hybrid assays

Hybrid immunoassays were performed by utilizing the anti-hK5 monoclonal antibody as the capture antibody and replacing the rabbit polyclonal anti-hK5 detection antibody with a rabbit antibody against each of six common serine protease inhibitors (α_2-macroglobulin, α_1-antitrypsin, α_1-antichymotrypsin, inter-α-trypsin inhibitor, α_2-antiplasmin and antithrombin III) at optimal concentrations (1:2000-fold dilution in all of them except 1:4000 for α_2-macroglobulin antibody). An additional blocking step (1 h incubation in a 60 g/l BSA solution) was added to the above-described protocol to further reduce background.

2.6. Fractionation of biological fluids with size-exclusion HPLC

To determine the molecular mass of the protein detected in the biological fluids, and to identify different

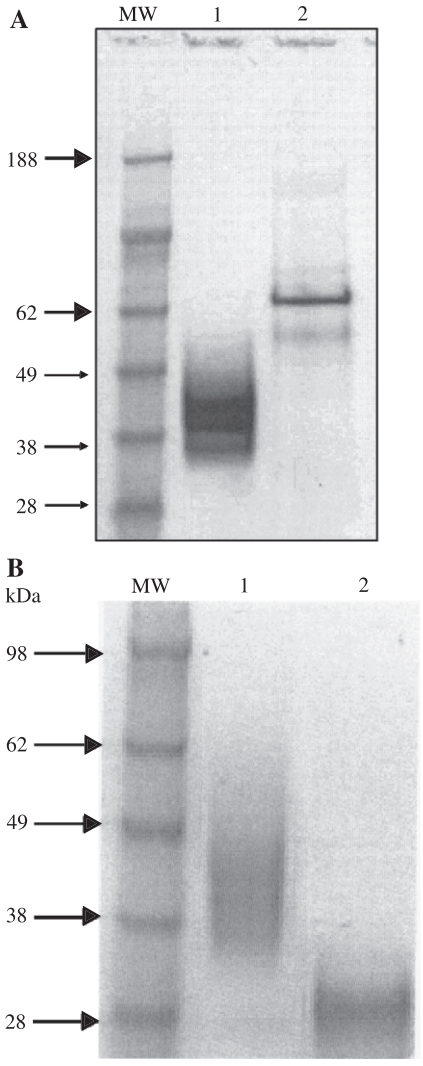

Fig. 1. Coomassie-stained SDS-PAGE gel with recombinant hK5 purified from yeast (lane 1) and CHO (lane 2) cells. Three bands are seen for hK5 from yeast and two from CHO cells. Bands were higher than the expected molecular mass (~ 30 kDa) due to glycosylation. After deglycosylation, one band is seen at a molecular mass of approximately 28 kDa (Panel B, lane 2). MW, molecular mass markers.

biological forms of hK5, serum, ascites fluid from advanced ovarian cancer patients and milk from lactating women were fractionated with gel filtration chromatography, as described elsewhere [22]. The fractions were collected and analyzed for hK5 using the developed immunoassay.

2.7. Radioactive labeling of hK5

Purified, recombinant hK5 protein was radiolabeled with ^{125}I by using Iodobeads® and the protocol provided by the manufacturer (Pierce). In summary, one bead was washed with 500 μl reaction buffer (0.1 M sodium phosphate buffer, pH 6.5), added to $Na^{125}I$ solution (5 μl of $Na^{125}I$; specific activity = 0.1 mCi/μl) in 500 μl reaction buffer and incubated for 5 min. Fifty micrograms of protein was diluted in 500 μl reaction buffer, added to the above solution and incubated for 8 min. Beads were then removed and the solution was transferred to a clean tube. The solution was then loaded into a PD-10 desalting column (Amersham) pre-equilibrated with reaction buffer. Twenty fractions (1 ml each) were then eluded with reaction buffer. Two peaks of radioactivity were detected: tubes 3–5 (radiolabeled hK5) and tubes 8–12 (free iodine). Tubes containing the protein were further purified by size-exclusion HPLC.

2.8. Glycosylation analysis

hK5 was incubated with peptide *N*-glycosylase F (PNGaseF) (New England Biolabs, Beverly, MA). Fifteen micrograms of purified hK5 was first denatured in 2 μl of denaturing buffer (5% SDS, 10% β-mercaptoethanol) at 100 °C for 5 min and immediately put on ice for 5 min. One-tenth volume of both G7 buffer [0.5 M sodium phosphate (pH 7.5)] and 10% NP-40 surfactant were then added, followed by 1 μl of PNGaseF. The reaction was incubated at 37 °C for 2 h. Two identical polyacrylamide gels containing 10 μg/lane of hK5, deglycosylated hK5, as well as horseradish peroxidase (a glycoprotein of ~ 40 kDa; positive control) and soybean trypsin inhibitor (an unglycosylated protein of ~ 21.5 kDa; negative control) were subjected to SDS-PAGE. One gel was stained with SimplyBlue SafeStain (Invitrogen) and the other with GelCode® Glycoprotein staining kit (Pierce). This gel was treated with periodic acid, which oxidizes the glycols present in glycoproteins to aldehydes, followed by immersion in the GelCode® Glycoprotein Stain, containing acidic fuchsin sulfite; the reaction produces a magenta color.

2.9. Enzymatic activity of recombinant hK5

The enzymatic activities of both the mammalian (proform) and yeast (mature) forms of hK5 were evaluated using two trypsin-like substrates (VPR-AMC and FSR-AMC) and a chymotrypsin-like substrate (AAPF-AMC) (Bachem, Bubendorf, Switzerland). Substrates were prepared from a lyophilized powder in dimethyl sulfoxide (DMSO) to a stock solution of 10 mM. Upon cleavage of these peptide substrates by an enzyme, the fluorochrome aminomethyl coumarin (AMC) is released. AMC fluoresces with an excitation wavelength of 370 nm, and an emission wavelength of 460 nm.

The peptide substrates, at a final concentration of 0.5 mM, were incubated with various concentrations of mature hK5 (28, 55, 85 and 111 μg/ml final concentrations) or prohK5 (12.5 and 25 μg/ml final concentrations) in 50 mM Tris–HCl, pH 8.0 and incubated for 15–35 min at 37 °C. A negative control without enzyme was also performed in parallel. The change in fluorescence was monitored during incubation using either the Roche COBAS FARA instrument (Roche, Basel, Switzerland) or the Perkin Elmer Life

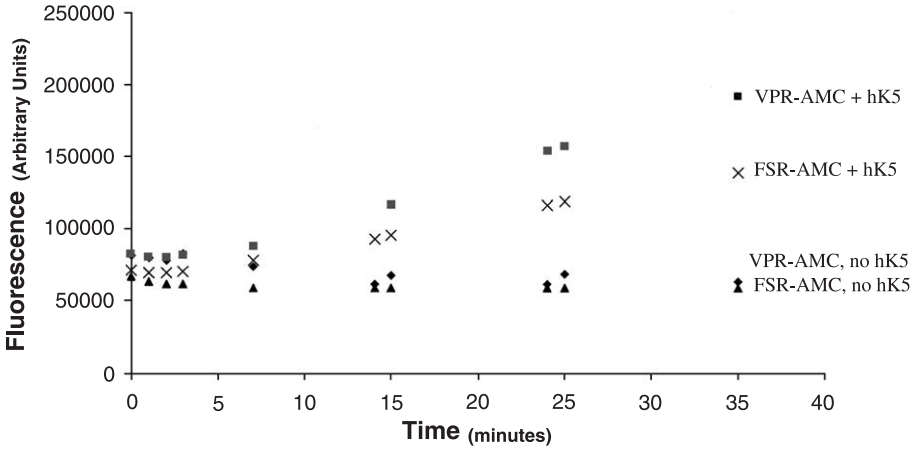

Fig. 2. Kinetics of the enzymatic activity of mature hK5 with two trypsin-like substrates (VPR-AMC and FSR-AMC) at pH 8.0 and 37 °C. Higher activity was observed with VPR-AMC compared to FSR-AMC. Substrates without hK5 were used as negative controls. No activity was observed with the chymotrypsin-like substrate AAPF-AMC (data not shown).

Sciences Wallac Victor^2 1420 Multilabel Counter and plotted as the change in fluorescence over time.

3. Results

3.1. Production and purification of recombinant hK5 proteins

Recombinant hK5 was produced in a *P. pastoris* yeast expression system as described elsewhere [19] and in CHO mammalian cells. Expression in yeast produced three molecular mass proteins (~ 35, 40 and 44 kDa) (Fig. 1). In mammalian cells, a main band of ~ 64 kDa and a minor band of ~ 55 kDa were obtained. The identity of all bands was confirmed by mass spectrometry.

3.2. Glycosylation analysis

hK5 proteins were subjected to in vitro deglycosylation by PNGaseF and separated by SDS-PAGE on two identical polyacrylamide gels. One gel was stained with Coomassie blue and the other with a glycoprotein stain (acidic fuchsin sulfite). Horseradish peroxidase, a glycoprotein, and soybean trypsin inhibitor, a non-glycosylated protein, were also included as positive and negative controls, respectively. After deglycosylation, the three bands from the yeast expression system co-migrate as a smaller molecular mass band of ~ 30 kDa (the expected mass of the non-glycosylated enzyme) (Fig. 1, lane 3). In vitro deglycosylation abolished staining of glycosylated hK5 with acidic fuchsin sulfite (data not shown).

3.3. Enzymatic activity

The mature, recombinant hK5 produced in yeast was tested against different trypsin-like and chymotrypsin-like substrates. As expected by the presence of aspartate in the binding pocket of the enzyme [7,8], hK5 was found to have trypsin-like, but not chymotrypsin-like, substrate specificity. Fig. 2 shows the activity of hK5 on two trypsin-like substrates (VPR-AMC and FSR-AMC). This substrate specificity was further confirmed by dose-response analysis (data not shown). Maximum activity was observed at 37 °C. No hK5 activity was detected when either the pro-form or the mature form of hK5 was incubated with the chymotrypsin-like substrate AAPF-AMC (data not shown). The pro-form of recombinant hK5 produced in mammalian cells showed only very minimal activity with the above-mentioned trypsin-like substrates (< 5% of the mature enzyme), indicating that it is enzymatically inactive.

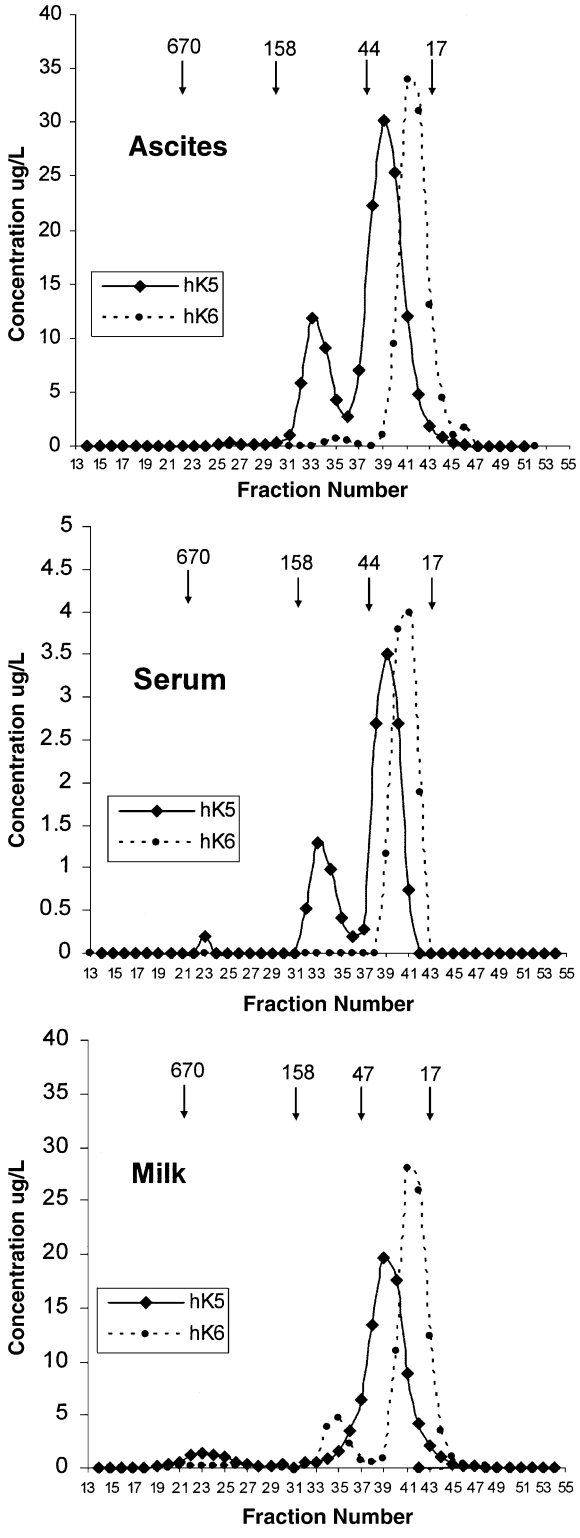

Fig. 3. Fractionation of an ascites fluid from an ovarian cancer patient, hK5 spiked serum and milk from a lactating woman with size-exclusion HPLC. The presence of hK5 in different fractions was measured with the hK5-specific ELISA immunoassay. In all fluids, there is an immunoreactive peak around fractions 38–40, corresponding to the free form of the protein (~ 40 kDa). In spiked serum and ascites fluid, a second peak is seen around fraction 33, corresponding to a molecular mass of ~ 100 kDa. Another peak was observed in milk at fraction 22. Molecular mass standards were also separated on the same column, and their corresponding elution profile is shown in kilodaltons at the top of the chromatogram. Human kallikrein 6 (hK6; MW of ~ 30 kDa) was included as a control. For discussion, see text.

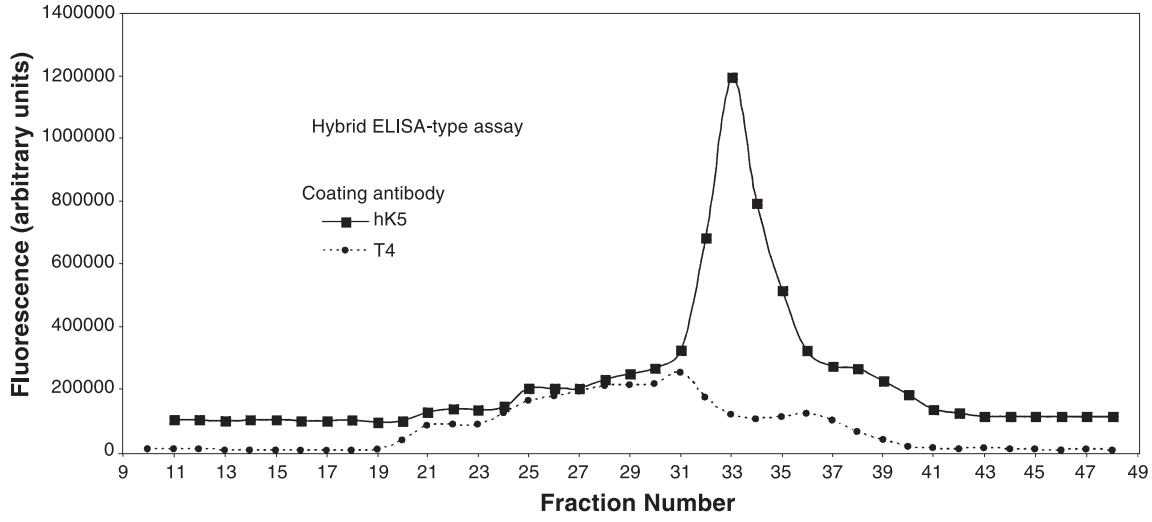

Fig. 4. Results of the hK5/α_1-antitrypsin hybrid ELISA-type assay for HPLC fractionated ascites fluid from ovarian cancer. A single immunoreactive peak is seen around fraction 32 (~ 100 kDa), corresponding to hK5 bound to α_1-antitrypsin (α_1-AT). Background signal was assessed by coating with a monoclonal anti-thyroxine antibody (T_4). For discussion, see text.

3.4. hK5 recovery from biological fluids

We tested the recovery of added recombinant hK5 in a 60 g/l BSA solution (as a control) in milk or lactating women, ascites fluid from ovarian cancer patients and serum. Recoveries ranged from 80% to 90% in BSA and milk, compared to 60–75% in ascites fluid and 50–60% in male and female serum. These data prompted us to speculate that hK5 may be interacting with serum protease inhibitors, similarly to other kallikreins, including hK3 (prostate-specific antigen, or PSA) and hK2 [23–26] (please see below).

3.5. Fractionation of biological fluids with size-exclusion HPLC

To determine the molecular mass of the hK5 protein detected in different biological fluids, samples were fractionated on a gel filtration column. The presence of hK5 in various fractions was then assessed with the hK5-specific ELISA-type immunoassay [19]. When the hK5 concentration in fractions was plotted against the fraction number, a peak around fractions 38–40 (corresponding to a molecular mass of ~ 40 kDa) was detected. Representative data

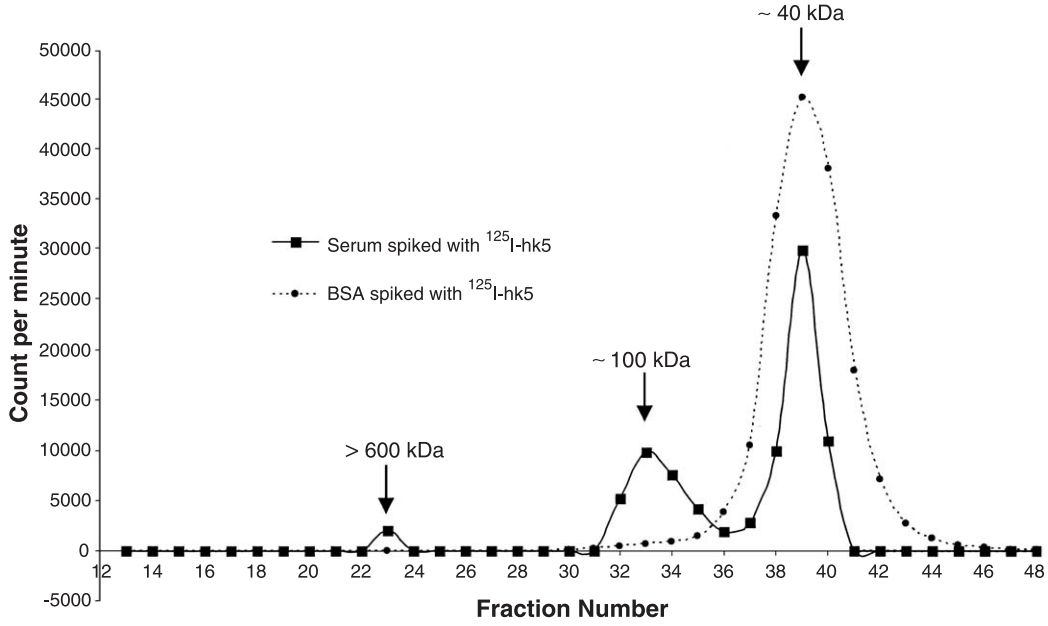

Fig. 5. Fractionation of serum and a 60 g/l BSA solution spiked with ^{125}I-labeled hK5 protein, with size-exclusion HPLC. The presence of hK5 in different fractions was assessed by counting radioactivity. In addition to the free form of hK5 in BSA (~ 40 kDa), two additional peaks at ~ 100 and >600,000 kDa were found in serum. For discussion, see text.

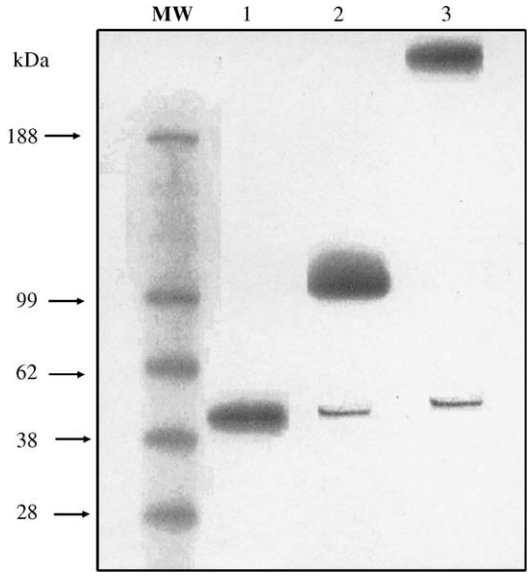

Fig. 6. Autoradiography of an SDS-PAGE gel containing ^{125}I-labeled hK5 mixed with BSA alone (lane 1), BSA and α_1-antitrypsin (lane 2) and BSA and α_2-macroglobulin (lane 3). MW, molecular mass marker with masses shown in kilodaltons. For discussion, see text.

obtained from ascites fluid of ovarian cancer patients, serum and milk are shown in Fig. 3. The higher than predicted molecular mass of free hK5 (~ 40 kDa instead of ~ 30 kDa) was verified by measuring another kallikrein, hK6 (molecular mass ~ 30 kDa), in the same samples (Fig. 3). We hypothesized that native hK5 is either glycosylated or it may interact with the gel filtration column, leading to a slightly delayed retention.

In ascites fluid from ovarian cancer patients, in addition to the peak around 40 kDa (fraction 39), another, smaller peak, corresponding to a molecular mass of ~ 100 kDa (fraction 33) was also detectable (Fig. 3). We verified that this peak represents hK5 bound to α_1-antytrypsin (see below). Normal serum contains trace amounts of hK5 which cannot be detected after HPLC fractionation. When serum is spiked with recombinant hK5, another peak is detected, in addition to the free form (Fig. 3, middle panel). These peaks are similar to those seen in ascites fluid. In milk, two peaks were detectable; the free form (40 kDa) and traces of a higher molecular mass (>600 kDa) form.

3.6. Interaction of hK5 with protease inhibitors

Recovery experiments and HPLC fractionation suggested that hK5 may be bound to protease inhibitors in serum, ascites fluid and milk. We verified the interaction of hK5 with α_1-antitrypsin and α_2-macroglobulin, as follows:

(1) We performed hybrid ELISA-type assays using an anti-hK5 monoclonal antibody for capture and each one of six polyclonal rabbit antibodies against protease inhibitors for detection, as described in Materials and methods. HPLC fractions of ascites fluid and spiked serum were used for analysis. A peak was observed at fraction 33 (corresponding to a molecular mass of ~ 100 kDa) using polyclonal rabbit anti-α_1-antitrypsin detection antibody (Fig. 4). The signal was substantially higher than the background signal of a negative control [replacing anti-hK5 with anti-thyroxine (T_4) antibody as the capture antibody]. These data were con-

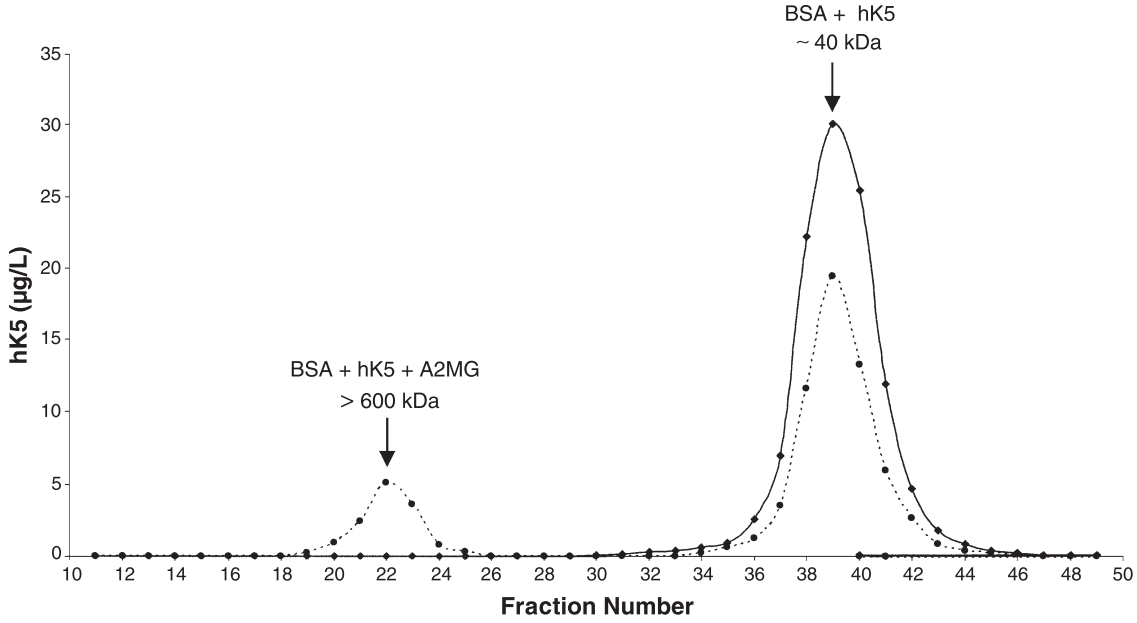

Fig. 7. Results of the hK5 ELISA assay of HPLC fractions of BSA spiked with hK5 only (solid line) and BSA spiked with both hK5 and α_2-macroglobulin (α2MG) (dotted line). The first sample shows a single immunoreactive peak around fraction 39, corresponding to the free form of the protein. In the second sample, the height of this peak decreased and a second peak is seen around fraction 22.

firmed for both ascites fluid and spiked serum, indicating that α_1-antitrypsin is one of the protease inhibitors that complexes with hK5 in these fluids. The α_2-macroglobulin hybrid assay showed a slight (3-fold) increase over background signal and we could not conclusively verify the interaction of hK5 with α_2-macroglobulin with this experiment. Hybrid assays with four other inhibitors (α_1-antitrypsin, inter-α-trypsin inhibitor, α_2-antiplasmin and antithrombin III) showed no significant signal increases (data not shown).

(2) We spiked either a 60 g/l BSA solution (control) or a normal serum with ^{125}I-radiolabeled recombinant hK5, incubated overnight and then fractionated both samples with HPLC gel filtration chromatography. The results are shown in Fig. 5. While the radioactivity in the BSA solution elutes mainly around fraction 39, corresponding to a molecular mass of ~40–45 kDa, radioactivity was also detected around fraction 33 (corresponding to a molecular mass of ~100 kDa) and traces around fraction 23 (corresponding to a molecular mass >600,000) in the serum.

(3) ^{125}I-radiolabeled hK5 was incubated with either 60 g/l BSA alone (control), BSA plus α_1-antitrypsin and BSA plus α_2-macroglobulin, incubated overnight, then run on a gel. The gel was dried and exposed to an X-ray film. In the BSA sample, we detected the expected band of ~40 kDa. When α_1-antitrypsin and α_2-macroglobulin were added to hK5, a gel shift was detected, indicating an interaction between hK5 and these inhibitors (Fig. 6). These shifted bands were seen at the expected masses of 100 kDa for α_1-antitrypsin, and >600 kDa for α_2-macroglobulin, respectively.

(4) The ability of hK5 to form a complex with α_2-macroglobulin was further verified in vitro. The same amount of hK5 was added to either a 60 g/l BSA solution or to BSA plus α_2-macroglobulin. Samples were size-fractionated and measured by the hK5 immunoassay. Addition of α_2-macroglobulin resulted in decrease of the ELISA-detectable hK5 (before HPLC size fractionation) by about one-third (data not shown). Fig. 7 shows that after HPLC fractionation, the concentration of the free form of hK5 (around fraction 39) was reduced, while a new, smaller peak appeared around fraction 22 (molecular mass $\geq$ 600,000 kDa).

4. Discussion

The interactions between kallikreins and protease inhibitors are well known. For example, human kallikrein 1 interacts with kallistatin [27]. Human kallikrein 3 (hK3, PSA) forms complexes with many protease inhibitors such as α_1-antichymotrypsin, α_2-macroglobulin and α_1-antitrypsin [23,24,28] and hK2 binds to α_2-antiplasmin, antithrombin III, plasminogen activator inhibitor-1, and α_2-macroglobulin [25,26]. The identification of all possible protease inhibitors that bind to different kallikreins represents an important step toward our understanding of kallikrein homeostasis and their role in health and disease [29]. In addition, kallikrein/protease inhibitor complexes may find novel clinical applications [26].

In the current study, we identified two protease inhibitors, α_1-antitrypsin and α_2-macroglobulin that bind to hK5 in biological fluids. Identification of α_1-antitrypsin as an hK5 inhibitor is not surprising since hK5 is predicted to have trypsin-like activity [7,8] and inhibitors are usually weak substrates of serine proteases. The molecular mass of the hK5/α_1-antitrypsin complex is ~100 kDa, which is close to the predicted molecular mass. The α_2-macroglobulin protein is a tetramer assembled from pairwise disulfide-bridged 180-kDa subunits and is susceptible to cleavage by most proteases [30]. The hK5/α_2-macroglobulin complex was not readily detectable by our hybrid ELISA-type or the hK5-specific assay. This observation is in accord with previously published reports on the nature of the interaction between α_2-macroglobulin and other kallikreins [31,32]. When α_2-macroglobulin interacts with a protease, the protease is entrapped within the α_2-macroglobulin molecule. In the case of hK5, we assume that an epitope of hK5 may be partially exposed. Our experiments suggest that α_1-antitrypsin is the main hK5 inhibitor in ascites fluid and serum. It should be noted, however, that previous reports indicate that proteases in complex with α_2-macroglobulin are rapidly removed from the circulation [33] and this may also apply to hK5.

We found recombinant hK5 to be glycosylated; this is expected since hK5 has four potential glycosylation sites at positions 69, 173, 208 and 252 of the amino acid chain. The trypsin activity of the protein, predicted by multiple sequence alignment [7,34], was verified by enzymatic analysis with defined substrates. In the trypsin-like group (to which most of the kallikreins belong), the protease cleaves peptide bonds following basic amino acids such as arginine or lysine, since it has an aspartate (or glutamate) in the substrate-binding pocket which can form a strong electrostatic bond with these residues. Our recombinant mammalian hK5 did not show enzymatic activity, as expected, since it was in the pro-form.

The activity of proteolytic enzymes in cancer is highly regulated and the interaction between proteases and their inhibitors may have an effect on cancer development and/or progression. The possible coordinated expression of kallikreins and their inhibitors in malignancy has been previously suggested. Shigemasa et al. [35] reported a statistically significant elevation of hK7 and its inhibitor, antileukoprotease, in ovarian cancer. In addition, specific inhibitors have been identified for most proteolytic enzymes and it is documented that inhibitors can prevent extracellular degradation and thus tumor cell invasion. For example, plasminogen activator-inhibitor 1 is suggested to protect against the proteolytic effect of plasminogen activator [36].

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