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Human Kallikrein 13: Production and Purification of Recombinant Protein and Monoclonal and Polyclonal Antibodies, and Development of a Sensitive and Specific Immunofluorometric Assay

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Background: **The aims of this study were to develop immunologic reagents and a sensitive and specific immunoassay for human kallikrein 13 (hK13) and to examine the presence of hK13 in human tissues and biological fluids.**

Methods: **Recombinant hK13 protein was produced and purified with use of a *Pichia pastoris* yeast expression system. The protein was used as an immunogen to generate mouse monoclonal and rabbit polyclonal anti-hK13 antibodies. A sandwich-type immunoassay was developed with these antibodies. The assay was used to measure hK13 in various biological fluids and tissue extracts. Immunohistochemical analysis was also performed on nondiseased and cancerous prostatic sections.**

Results: **The hK13 immunoassay had a detection limit of 0.05 μg/L and showed no cross-reactivity with homologous kallikreins. The assay was linear at 0–20 μg/L, and within-and between-run CVs were <10% (n = 12). hK13 was detected in tissues, including esophagus, tonsil, trachea, lung, cervix, and prostate. hK13 was also found in seminal plasma, amniotic fluid, follicular fluid, ascites of ovarian cancer patients, breast milk, and cytosolic extracts of ovarian cancer tissues. hK13 was immunohistochemically localized in epithelial cells of both nondiseased and cancerous prostate. hK13 appears to be overexpressed in 50% of ovarian cancer tissues compared with healthy ovarian tissues. Recovery of active enzyme added to milk or amniotic fluid was 70–98%, but was <20% when added to serum, suggesting rapid sequestration by protease inhibitors. In fluids and tissue extracts, hK13 was found in its free (~30 kDa) form.**

Conclusions: **This immunofluorometric assay for hK13 may be used to examine the value of hK13 as a disease biomarker and to further explore the physiologic and pathobiologic role of this enzyme in human disease.**

Serine proteases are proteolytic enzymes and play important roles in physiologic processes, including digestion, coagulation, fibrinolysis, apoptosis, cell migration, tissue remodeling, and inflammation. The kallikreins are a subfamily of the serine protease enzyme family *(1, 2)*. The human kallikrein gene family was, until recently, thought to include only three members: *KLK1*, which encodes for pancreatic/renal kallikrein (hK1);[4] *KLK2*, which encodes for human glandular kallikrein 2 (hK2); and *KLK3*, which encodes for prostate-specific antigen (PSA; hK3) *(3)*. The best known of the three classic human kallikreins is PSA, an important biomarker for prostate cancer diagnosis and monitoring *(4)*. Recently, new serine proteases with high degrees of homology to the three classic kallikreins were cloned. These newly identified serine proteases have now

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[4] Nonstandard abbreviations: hK, human kallikrein; PSA, prostate-specific antigen; MS/MS, tandem mass spectrometry; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; and AMC, 7-amido-4-methylcoumarin.

been included in the expanded human kallikrein gene family. The entire human kallikrein gene locus on chromosome 19q13.4 now includes 15 genes, designated *KLK1–KLK15*; their respective proteins are known as hK1–hK15 *(5)*. The genomic organization and other structural and functional characteristics of these genes and their encoded proteins have recently been reviewed *(6)*.

KLK13, previously known as *KLK-L4*, is one of the newly identified kallikrein genes. *KLK13* was cloned with use of the positional candidate approach. The gene resides on chromosome 19q13.4, spans ~10 Kb of genomic DNA sequence, and has five coding exons and four introns *(7)*. The protein has 47% and 45% sequence identity with PSA and hK2, respectively *(7)*. At the mRNA level, *KLK13* expression is highest in the mammary gland, prostate, testis, and salivary glands *(7)*. The function of the protein is still unknown. *KLK13*, like all other members of the human kallikrein family, is predicted to encode a secreted serine protease that is likely present in biological fluids. However, no suitable method for measuring hK13 protein with high sensitivity and specificity has been described. Given the prominent role of PSA as a cancer biomarker and the recent demonstration that other members of this gene family are also potential cancer biomarkers *(8–12)*, we hypothesized that hK13 may also have utility as a disease biomarker. To further investigate the diagnostic and other clinical applications of this protein, it will be necessary to develop highly sensitive and specific procedures for its quantification in biological fluids and tissues. Because no known natural source of hK13 exists, we expressed this protein in yeast, purified the recombinant protein to homogeneity, generated monoclonal and polyclonal antibodies, and developed an immunofluorometric method for hK13 quantification. Using this assay, we studied the tissue expression of hK13 and preliminarily report that hK13 is overexpressed in ovarian cancer tissues.

Materials and Methods

CLONING OF KLK13 cDNA INTO THE YEAST EXPRESSION SYSTEM

Recombinant hK13 was produced in the *Pichia pastoris* yeast expression system (Invitrogen). Two primers were designed to amplify the *KLK13* cDNA sequence: the forward primer was 5′-TCC AAG GAA TTC AAC ACC AAT GGG ACC-3′, and the reverse primer was 5′-CCA TTG TCT AGA TTG GGA CAT TCA GGT-3′. Human salivary cDNA was used as a template. PCR was carried out in a 20-μL reaction mixture, containing 1 μL of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 100 ng of primers, and 2.5 U of *Pfu* DNA polymerase (Stratagene), in an Eppendorf master cycler. The PCR conditions were 94 °C for 5 min, followed by 94 °C for 30s, 62 °C for 30s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product was then cloned into the yeast expression vector pPICZαA by standard procedures *(13)*. The sequence of the construct was confirmed with an automated DNA sequencer.

PRODUCTION OF hK13 IN YEAST

The pPICZαA vector containing the *KLK13* cDNA sequence was introduced into the yeast strains X-33, KM71, and GS115. A stable clone was selected from the X-33 strain according to the manufacturer's recommendations (Invitrogen). hK13 was produced by growing the stable yeast clone in a medium containing 10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate (pH 6.0), 13.4 g/L yeast nitrogen base, and 40 mg/L biotin in a 30 °C shaking incubator (250 rpm). hK13 production was induced with 5 mL/L methanol over 6 days. The cells were subsequently pelleted by centrifugation, and the supernatant was collected. A hK13 rabbit polyclonal peptide antibody (produced in house) was used to monitor hK13 production by Western blot analysis.

CHARACTERIZATION OF hK13 BY MASS SPECTROMETRY

Polyacrylamide gels were stained with Coomassie G-250 for visualization, and selected bands were subsequently excised and destained with 300 mL/L acetonitrile in 100 mmol/L ammonium bicarbonate. Each band was then reduced (10 mmol/L dithiothreitol in 50 mmol/L ammonium bicarbonate, pH 8.3) and alkylated (50 mmol/L 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).

All nanoelectrospray mass spectrometry (MS) experiments were conducted on a Q-star (PE/Sciex) hybrid quadrupole/time-of-flight instrument, for high resolution and online tandem MS (MS/MS) experiments *(14)*. Conventional mass spectra were obtained by operating the quadrupole in a radiofrequency-only mode while a pusher electrode was pulsed at a frequency of ~7 kHz to transfer all ions to the time-of-flight analyzer. MS/MS experiments on trypsin-digested peptides identified in survey scans were conducted with use of a nanoelectrospray source. Precursor ions were selected by the first quadrupole while a pusher electrode was pulsed (frequency ~7 kHz) to transfer fragment ions formed in the radiofrequency-only quadrupole cell to the time-of-flight analyzer. Mass spectral resolution was typically 9000–10 000. Scan durations of 1 and 2 s were set for conventional and MS/MS mass spectral acquisition, respectively. Collisional activation was performed using nitrogen collision gas with typically a 30-V offset between the DC voltage of the entrance quadrupole and the radiofrequency-only quadrupole cell. Data were acquired and processed by LC Tune and Biomultiview programs from PE/Sciex.

PURIFICATION OF hK13 WITH CATION-EXCHANGE AND REVERSED-PHASE CHROMATOGRAPHY

The recombinant hK13 was purified from yeast culture supernatants by cation-exchange chromatography using CM-Sepharose fast flow (Pharmacia Biotech) and reversed-phase liquid chromatography using a C_4 column (0.45 × 5 cm; Vydac). The presence of hK13 in various fractions was identified by Western blotting with an anti-hK13 peptide antibody. In summary, CM-Sepharose beads previously activated with 1 mol/L KCl were equilibrated in 10 mmol/L MES buffer (pH 6.5). The yeast culture supernatant was first absorbed on CM-Sepharose beads by incubation at 4 °C overnight under agitation. The beads were then washed with 10 mmol/L MES buffer (pH 6.5), and hK13 was eluted with 300 mmol/L KCl in 10 mmol/L MES buffer (pH 6.5). Trifluoroacetic acid as ion-pairing agent was added into this eluate (final concentration, 10 mL/L), which was then loaded on a C_4 column equilibrated with 1 mL/L trifluoroacetic acid in water. A linear gradient (1% per min) of acetonitrile from 15% to 50% in 1 mL/L trifluoroacetic acid was then performed. The fraction containing hK13 was evaporated on a SpeedVac (Savant). The purified material was then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue to assess its purity and its molecular mass. The protein concentration of the purified hK13 was determined by the bicinchoninic acid method, which uses bovine serum albumin (BSA) as calibrator (Pierce Chemical Co.).

ASSESSMENT OF hK13 PROTEIN GLYCOSYLATION

hK13 protein glycosylation was assessed by treatment with the deglycosylation enzyme PNGaseF and the Gel Code glycoprotein staining reagent set according to the manufacturer's recommendations (Pierce).

ASSESSMENT OF hK13 ENZYMATIC ACTIVITY

The enzymatic activity of recombinant hK13 protein was assessed with use of the fluorogenic substrates FSR-7-amido-4-methylcoumarin (AMC) and VPR-AMC. We used 10 mmol/L Tris (pH 7.5), 0.1 mol/L NaCl, 5 g/L BSA as a buffer and mixed 5 μL of 10 mmol/L substrate solution, 1 μL of enzyme (1–600 ng), and 94 μL of buffer. We then incubated the samples at 37 °C for 30 min and added 700 μL of 125 mL/L acetic acid to stop the reaction. We measured the fluorescence at an excitation wavelength of 300 nm and emission of 460 nm. We used trypsin as the positive control. The fluorogenic substrates were purchased from Bachem.

N-TERMINAL SEQUENCING OF hK13 RECOMBINANT PROTEIN

N-Terminal sequencing of purified recombinant hK13, blotted on polyvinyldifluoride membranes and stained with Coomassie blue, was performed with the Edman degradation method on an automated amino acid sequencer.

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST hK13

The purified recombinant hK13 protein was used as an immunogen to immunize rabbits and mice. hK13 (100 μg) was injected subcutaneously into BALB/c female mice and New Zealand White female rabbits. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of anti-hK13 polyclonal antibodies in mice and rabbits (see below), we used the following immunoassay. Sheep anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch) was immobilized on 96-well white ELISA plates. The mouse/rabbit serum was then applied to the plates at different dilutions ranging from 1:500 to 1:50 000 [100 μL/well; diluted in 60 g/L BSA, 50 mmol/L Tris (pH 7.80), 0.5 g/L sodium azide]. After incubation (1 h) and washing, biotinylated recombinant hK13 was then added to each well (5–10 ng/well). Finally, after incubation (1 h) and washing, alkaline phosphatase-conjugated streptavidin was added, the wells were incubated (15 min) and washed, and the alkaline phosphatase activity was detected with time-resolved fluorescence (for details, see below).

PRODUCTION AND CHARACTERIZATION OF 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 3-weeks 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 removed. To generate monoclonal anti-hK13 antibodies, the splenocytes were fused with the Sp2/0 myeloma cells with use of 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 strepta-

vidin 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 *(15)*. The positive clones were expanded sequentially in 24-well and 6-well plates in complete medium (reducing the fetal calf serum to 150 mL/L and changing the HAT to HT). Supernatants were further tested by performing IgG isotyping, and clones 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 twofold in 20 mmol/L sodium phosphate buffer (pH 7.0) and injected into a HiTrap 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.

IMMUNOFLUOROMETRIC ASSAY FOR hK13

Standard assay procedure. For the polyclonal assay (mouse polyclonal/rabbit polyclonal), white polystyrene microtiter plates were coated with sheep anti-mouse IgG, Fc fragment-specific antibody (100 μL of coating antibody solution containing 500 ng of antibody diluted in 50 mmol/L Tris buffer, pH 7.80 in each well) by incubation overnight. 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 7.40). Mouse anti-hK13 polyclonal antiserum was diluted 500-fold in a general diluent [60 g/L BSA, 50 mmol/L Tris (pH 7.80), 0.5 g/L sodium azide], and 100 μL was applied to each well. After a 1-h incubation, the plates were washed six times with washing buffer.

hK13 calibrators or samples were then pipetted into each well (50 μL/well along with 50 μL of the general diluent) and incubated for 1 h with shaking; the plates were then washed with washing buffer six times. Subsequently, 100 μL of rabbit anti-hK13 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 30 min; plates were then washed as described above. Finally, 100 μL/well alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc fragment-specific), diluted 2000-fold in buffer A, was added to each well and incubated for 30 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). The calibration and data reduction were performed automatically, as described elsewhere *(15)*. For the monoclonal antibody assay (two monoclonal antibodies), white polystyrene microtiter plates were coated directly with one monoclonal antibody specific for hK13 (code 2-17; 500 ng/well). The plates were then washed six times with the washing buffer. Subsequently, hK13 calibrators were added along with a second biotinylated monoclonal antibody (code 11C1; 50 ng/well). Detection was achieved with alkaline phosphatase-conjugated streptavidin. Diflunisal phosphate and developing solution were added as stated above. For the monoclonal/polyclonal immunoassay configuration, mouse monoclonal 11C1 was used for directly coating microtiter plates and the rabbit polyclonal antibody was used for detection, with the procedures described above. More details about such procedures have been described elsewhere for similar immunoassays for hK6 *(8)* and hK10 *(9)*.

Determination of the sensitivity of the hK13 immunoassay. Recombinant hK13 was used to generate the calibration curve. hK13 calibrators were prepared by diluting the purified recombinant hK13 in the general diluent. These calibrators were then used to define the detection limit of the assay.

Determination of the specificity of the hK13 immunoassay. Biological fluids and recombinant hK13 were used to determine the specificity of the developed immunoassay. These samples were first measured by the standard assay procedure described above. The mouse and rabbit anti-hK13 antisera were then successively replaced with sera from the same animals, obtained before immunization (preimmune sera). The samples were measured again, and the fluorescence counts were compared with the counts obtained by the standard assay. The cross-reactivities of other homologous proteins were investigated using purified recombinant hK1–hK15 (available in house), all diluted in the general diluent.

HUMAN TISSUE CYTOSOLIC EXTRACTS AND BIOLOGICAL FLUIDS

Human tissue cytosolic extracts were prepared as follows: Various frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Extraction buffer [1 mL, containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L NP-40 surfactant, 1 mmol/L phenylmethylsulfonyl fluoride, 1 g/L aprotinin, and 1 g/L leupeptin] was added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and vortex-mixing every 10 min. Mixtures were then centrifuged at 14 000*g* at 4 °C for 30 min. The

supernatants (cytosolic extracts) were then collected. The biological fluids were leftovers of samples submitted for routine biochemical testing. All tissue cytosolic extracts and biological fluids were stored at −80 °C until use.

RECOVERY

Recombinant hK13 was added to the general diluent (control), to male and female normal sera, and to various biological fluids at different concentrations; the samples with added hK13 were measured with the monoclonal/monoclonal, monoclonal/polyclonal, and polyclonal/polyclonal hK13 immunoassays. Recoveries were then calculated after subtraction of the endogenous concentrations.

FRACTIONATION OF BIOLOGICAL FLUIDS WITH SIZE-EXCLUSION HPLC

To determine the molecular mass of the protein detected in the biological fluids and tissue extracts, various samples were fractionated with gel-filtration chromatography, as described elsewhere *(8, 9)*. The fractions were collected and analyzed for hK13 by the developed immunoassay.

IMMUNOHISTOCHEMISTRY

Rabbit polyclonal antibody was raised against full-length hK13, as described above. Immunohistochemical staining for hK13 was performed according to a standard immunoperoxidase method. Briefly, paraffin-embedded tissue sections (4 μm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with 30 mL/L aqueous hydrogen peroxide for 15 min. Sections were then treated with 4 g/L pepsin at pH 2.0 for 5 min at 42 °C and blocked with 20 mL/L protein blocker (Signet Laboratories) for 10 min. The primary antibody was then added at a 1:1000 dilution for 1 h at room temperature. After the plates were washed, biotinylated anti-rabbit antibody (Signet), diluted fourfold in antibody dilution buffer (Dako), was added. After incubation and washing, streptavidin-tagged horseradish peroxidase was added for 30 min at room temperature. After washing, detection was achieved with diaminobenzidine for 5–10 min. The slides were then counterstained with hematoxylin and then mounted with coverslips.

Results

PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT hK13 PROTEIN

hK13 is predicted to be a secreted serine protease. Hydrophobicity and structural homology analysis suggested that the active form of hK13 starts from amino acid 26 (segment 1–20 represents the signal peptide and segment 21–25 is the activation peptide) *(7)*. The cDNA encoding for this active form of hK13 was cloned into a *P. pastoris* yeast expression system. Expression in yeast produced a higher molecular mass protein (~ 50 kDa) and much smaller amounts of a 28-kDa protein. Both proteins were visible on Western blots (data not shown). The bands representing the 50- and 28-kDa proteins were excised from Coomassie-stained gels, and selected trypsin digested fragments were sequenced by MS/MS. The partial sequence VSGWGTTTSPQVNYPK was identified from the peptide fragments and precisely matched the fragment expected for amino acids 159–174 of hK13 *(7)*. Four other fragments also matched the sequence (data not shown).

We postulated that the higher molecular mass recombinant hK13 protein is highly glycosylated. To explore this possibility, we separated this protein by SDS-PAGE and stained the gels with Coomassie blue before and after digestion with the deglycosylating enzyme PNGaseF (Fig. 1A). On treatment with PNGaseF, the molecular weight of the recombinant hK13 protein was reduced to 25–28 kDa, consistent with the molecular mass of the nonglycosylated hK13 protein *(7)*. Staining for glycoproteins with acidic fuchsin sulfite was abolished by treatment with PNGaseF (Fig. 1B).

The N-terminal sequence of the purified recombinant hK13 was VLNTNG, confirming that the protein represented the active form of the enzyme because activation occurs after cleavage of a K ↓ V bond between amino acids 25 and 26 of the pre-proenzyme *(6, 7)*.

hK13 cleaved the two trypsin substrates, FSR-AMC and VPR-AMC, in a concentration-dependent manner. For example, under the experimental conditions outlined, hK13 activity produced an increase in the fluorescence of the substrate FSR-AMC, from 1.1 to 1.9 arbitrary units with 10 ng of hK13, to 6.9 arbitrary units with 100 ng of hK13, and to 14.5 arbitrary units with 160 ng of hK13 in 30 min at 25 °C. For the substrate VPR-AMC, the enzyme increased the fluorescence from 0.5 arbitrary units to 1.9 arbitrary units with 10 ng of enzyme, to 13.9 arbitrary units with 100 ng of enzyme, and to 35.5 arbitrary units with 160 ng of enzyme.

PRODUCTION OF ANTIBODIES AND DEVELOPMENT OF THE IMMUNOFLUOROMETRIC ASSAY

Recombinant hK13 protein was used as an immunogen in mice and rabbits to generate polyclonal and monoclonal antibodies. These antibodies were then used for immunohistochemical localization of hK13 (see below) and for the development of three different versions of an ELISA-type immunofluorometric assay. Among the three assay configurations (polyclonal mouse coating antibody/polyclonal rabbit detection antibody, monoclonal mouse coating antibody/polyclonal rabbit detection antibody, and monoclonal mouse coating antibody/biotinylated monoclonal mouse detection antibody), the most sensitive was based on a monoclonal/polyclonal configuration. In selected cases, all three research configurations were used, as indicated in the text. Below, we will describe the characteristics of the developed ELISA-type immunofluorometric procedure.

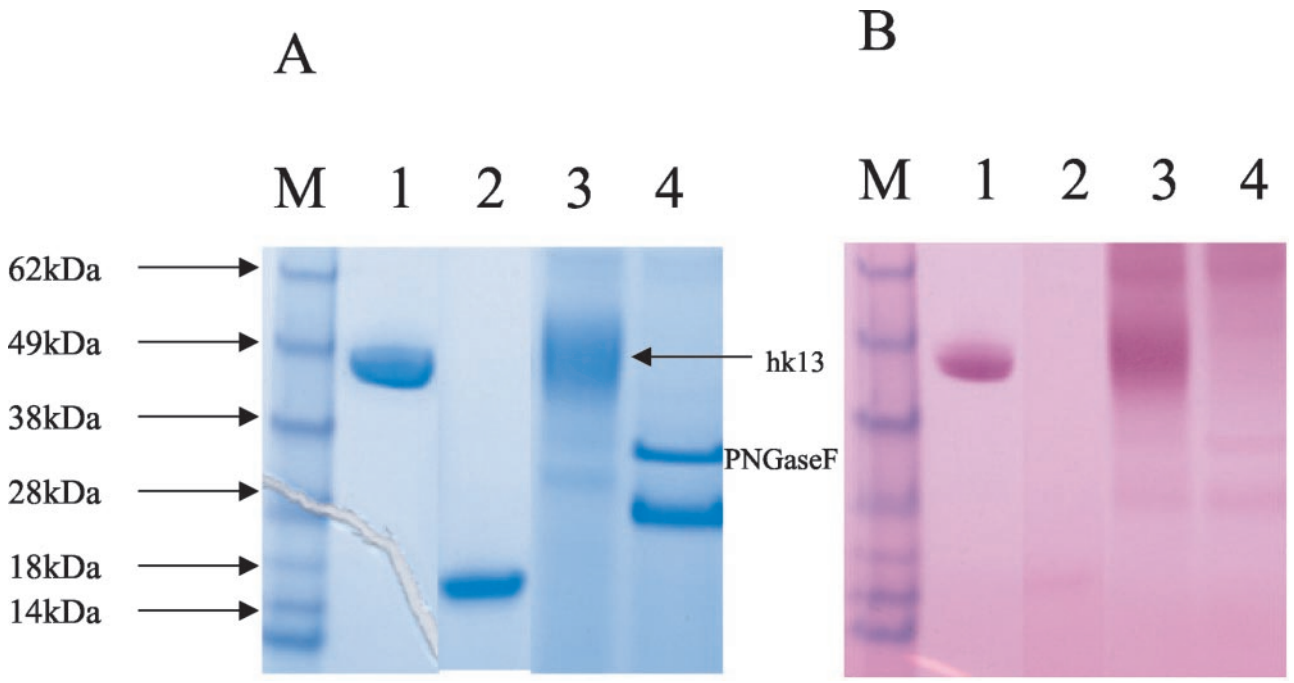

Fig. 1. SDS-PAGE of purified recombinant hK13 before and after treatment with PNGaseF.

(*A*), *lane 1*, positive control (horseradish peroxidase); *lane 2*, negative control (soybean trypsin inhibitor); *lane 3*, recombinant hK13 (identified by *arrow*); *lane 4*, recombinant hK13 after treatment with PNGaseF. The higher molecular mass band represents PNGaseF, and the lower molecular mass band (~ 28 kDa) represents deglycosylated hK13. (*B*), gel was stained with acidic fuchsin sulfite, a glycoprotein stain (Pierce). Note the staining of the glycoproteins, horseradish peroxidase (positive control), and hK13 in *lanes 1* and *3*. *Lane M*, molecular mass markers.

SENSITIVITY, DETECTION LIMIT, SPECIFICITY, LINEARITY, AND PRECISION OF THE hK13 IMMUNOFLUOROMETRIC ASSAY

Sensitivity. A typical calibration curve for the hK13 immunofluorometric assay is shown in Fig. 2. The detection limit, defined as the concentration of hK13 that can be distinguished from zero with 95% confidence, was 0.05 μg/L. These data were obtained with the mouse monoclonal (11CI)/rabbit polyclonal immunoassay configuration, which was more sensitive than the other two assay configurations (monoclonal/monoclonal and polyclonal/polyclonal immunoassays).

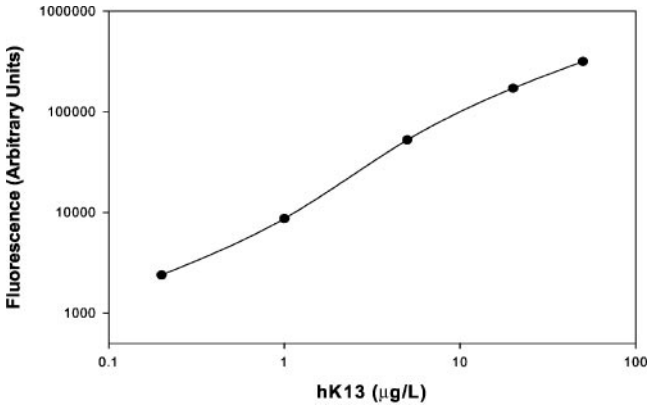

Fig. 2. Typical calibration curve for the hK13 immunoassay.

The background fluorescence (zero calibrator) has been subtracted from all measurements. The dynamic range of the assay is 0.05–20 μg/L.

Specificity. For all assay configurations, we confirmed that the assay specifically measures hK13. When we replaced mouse and rabbit antisera with preimmune mouse and rabbit sera, the fluorescence signals of hK13-positive samples were reduced to nearly zero (data not shown). Recombinant hK1, hK2, hK3, hK4, hK5, hK6, hK7, hK8, hK9, hK10, hK11, hK12, hK14, and hK15 proteins did not produce measurable readings, even at concentrations 1000-fold higher than that of hK13.

Linearity and precision. Samples of milk, seminal plasma, and amniotic fluid were linear on dilution. Within- and between-run imprecision was assessed with various hK13 calibrators and clinical samples. In all cases, the CVs were 3–8% (n = 12).

hK13 IN SELECTED HUMAN TISSUE EXTRACTS AND BIOLOGICAL FLUIDS

The highest tissue extract concentrations of hK13 were in esophagus and tonsil, followed by salivary gland, prostate, kidney, skin, trachea, ureter, testis, breast, lung, and thyroid (Fig. 3). A similar pattern was also seen in fetal tissues (Fig. 3).

In biological fluids, the highest concentrations were seen in seminal plasma, followed by amniotic fluid, breast milk, and follicular fluid (Table 1). Cerebrospinal fluid contained only traces of hK13. hK13 was below the detection limit of the assay in all male and female serum samples from healthy individuals.

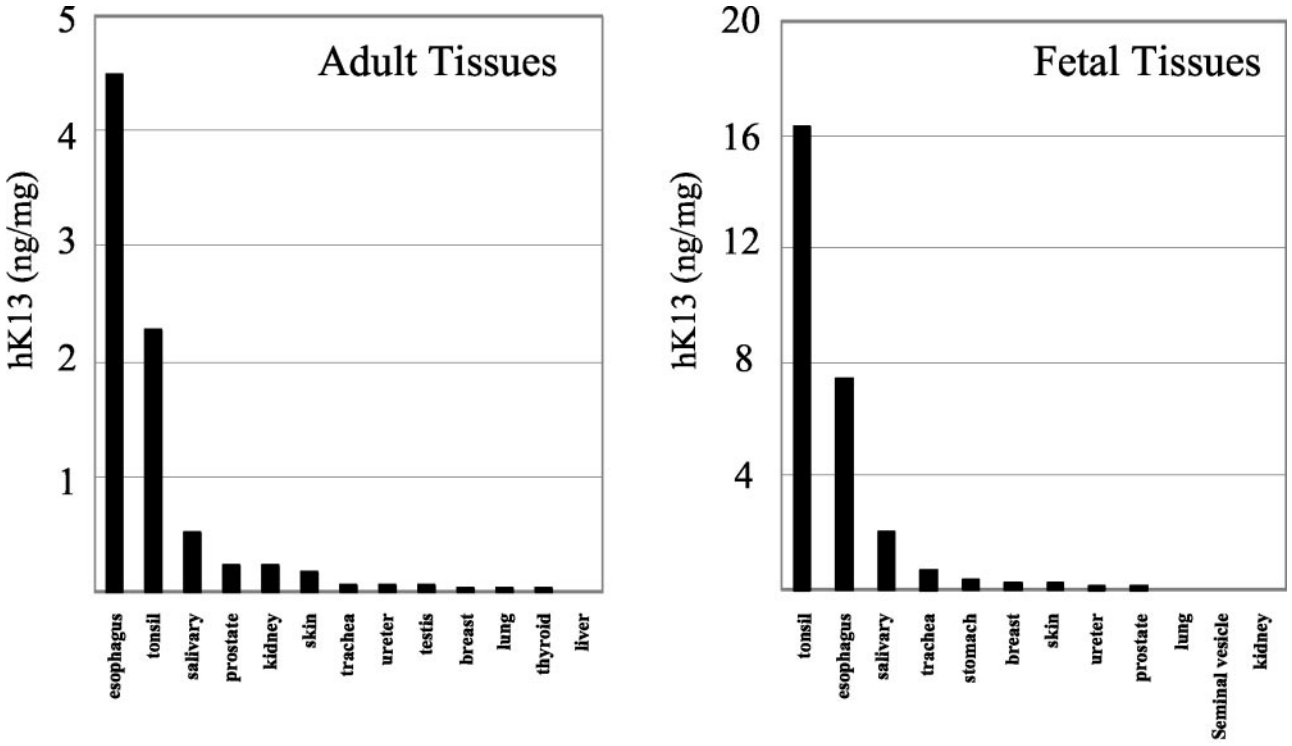

Fig. 3. Tissue expression of hK13 protein.

Human tissues were pulverized, and cytosolic extracts were prepared, as described in *Materials and Methods*. The hK13 concentration was then measured with the ELISA. Results were normalized for total protein content and are expressed as ng of hK13/mg of total protein.

OVARIAN CANCER CYTOSOLS AND ASCITES FLUIDS

Among 20 ovarian carcinoma extracts, 10 were positive for hK13, with concentrations ranging from 0.2 to 15 μg/L. None of the 10 extracts from either healthy ovarian tissue or benign ovarian disease were highly positive (Fig. 4). Among 44 ascites fluid samples from women with advanced ovarian carcinoma, all were positive for hK13, with values ranging from 0.1 to 20 μg/L. The mean (SD) and median values were 2.3 (4.2) and 0.8 μg/L, respectively.

RECOVERY OF hK13 FROM BIOLOGICAL FLUIDS

Recoveries of recombinant hK13 added to amniotic fluid and milk were 70–98% (range) but were 5–10% with male and female sera. This low recovery was verified with all three versions of the developed immunoassay. Recoveries were similar (5–10%) for serum (from men and women) when we used hK13 from amniotic fluid and seminal plasma (data not shown).

Table 1. Analysis of hK13 in biological fluids.

Biological fluid	n[a]	hK13, μg/L Range	Mean (SD)	Median	Positivity rate, %
Seminal plasma	10	86–406	274 (108)	284	100
Amniotic fluid	20	16–88	45 (20)	41	100
Breast milk	10	3.8–30	8.6 (7.9)	5.9	100
Follicular fluid	6	0.08–5.6	1.5 (1.0)	0.81	100
Cerebrospinal fluid	10	0–0.24	0.15 (0.14)	0.09	90
Male/female sera	(21/21)	0	0	0	0

[a] Number of samples.

FRACTIONATION OF BIOLOGICAL FLUIDS WITH SIZE-EXCLUSION HPLC

In gel-filtration column chromatography, immunoassayable hK13 of tissue extracts consistently eluted in a peak corresponding to a molecular mass of ~30 kDa, consistent with the molecular mass of free (noncomplexed) hK13. This single peak was present in an esophageal extract, seminal plasma, amniotic fluid, and follicular fluid. By contrast, in ascites fluid from ovarian cancer patients, we also detected a small peak corresponding to a molecular mass of ~100 kDa (Fig. 5).

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We localized hK13 immunohistochemically in both nonmalignant and malignant prostatic tissues (Fig. 6). Staining was mostly cytoplasmic in epithelial cells, whereas stroma was negative. Some nuclear staining of epithelial cells was also evident.

Discussion

The *KLK13* gene was cloned using the positional candidate approach and found to be down-regulated in breast cancer tissues and breast cancer cell lines *(7)*. On the basis

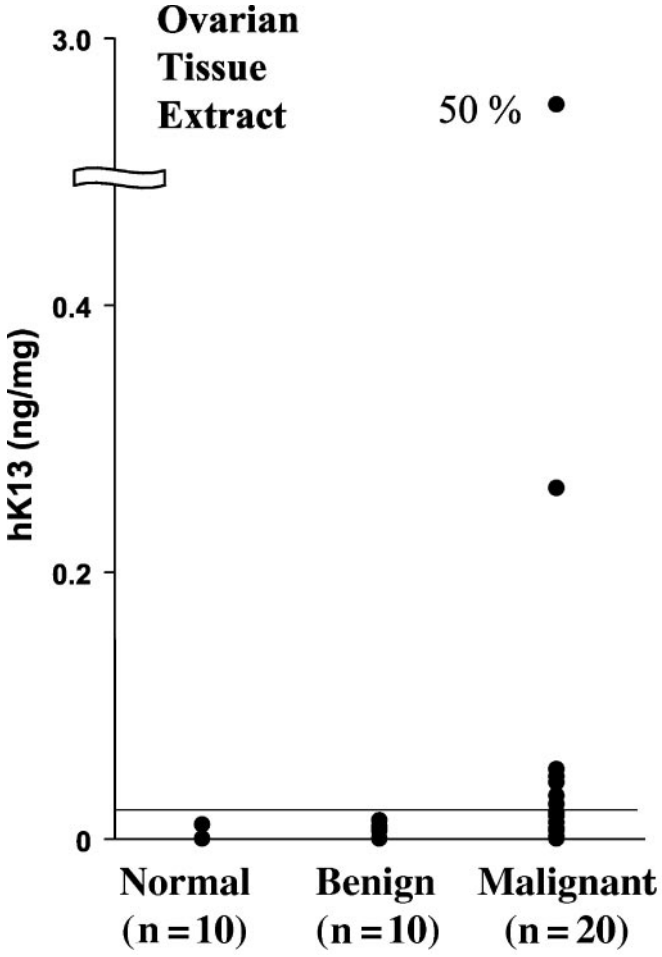

Fig. 4. Quantification of hK13 protein in ovarian tissue extracts.

We analyzed extracts from 10 healthy ovaries, 10 ovaries with benign disease, and 20 with ovarian cancer. Results are expressed as ng of hK13/mg of total protein. In 50% of the ovarian cancer tissue extracts, hK13 protein is overexpressed compared with extracts from healthy and benign tissue.

of its chromosomal location and structural similarities with other kallikreins, *KLK13* was classified as a novel member of the kallikrein gene family *(5–7)*. Because there is no known natural source of hK13, we expressed it in relatively large amounts in a *P. pastoris* expression system. Compared with the predicted molecular mass of hK13 *(7)*, the protein in yeast is present in two forms, nonglycosylated (~28 kDa) and heavily glycosylated (50 kDa), the latter being the predominant species. This protein represents the active form of the enzyme and has trypsin-like cleavage specificity, as demonstrated by synthetic substrates.

Our most sensitive assay was based on a mouse monoclonal coating/rabbit polyclonal detection antibody configuration. This assay is highly sensitive and specific, detecting hK13 at concentrations ≥0.05μg/L. Size-exclusion HPLC of biological fluids indicated that this assay detects only one immunoreactive peak of the expected molecular mass (~30 kDa). These data suggest that our immunoassay detects the free fraction of hK13, although

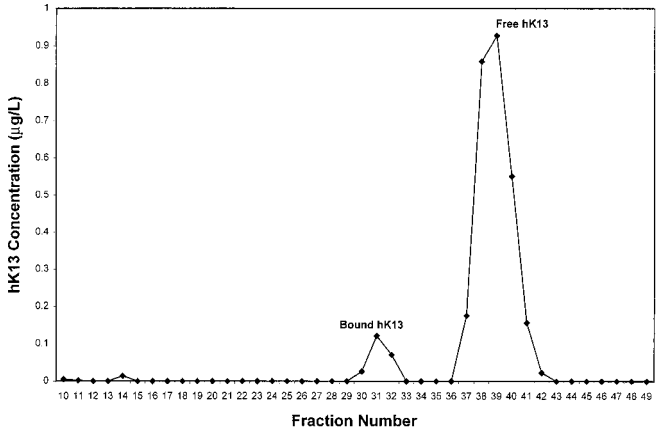

Fig. 5. Fractionation of ascites fluid from an ovarian cancer patient by size-exclusion HPLC.

The fractions were analyzed for hK13 by the developed ELISA. There are two immunoreactive peaks, one at *fractions 38* and *39*, corresponding to a molecular mass of ~30 kDa (free hK13). Another, smaller peak elutes at *fractions 30–32* and corresponds to a molecular mass of ~100 kDa. We postulate that this peak represents hK13 bound to a proteinase inhibitor.

in ascites fluid from an ovarian cancer patient, there is another peak, likely representing hK13 bound to a proteinase inhibitor or another interacting protein. These possibilities are currently under investigation.

We identified hK13 in seminal plasma, amniotic fluid, follicular fluid, and breast milk, suggesting that this enzyme is secreted by cells of male and female reproductive organs. The enzyme was immunohistochemically localized in prostatic tissues (Fig. 6). Unlike other kallikreins (e.g., hK6 and hK10) *(8, 9)*, hK13 is not present at appreciable amounts in cerebrospinal fluid. Similarly, the concentration of hK13 in serum of healthy men and women appears to be very low, below the detection limit of our assay (<0.05 μg/L). The availability of this highly specific and sensitive immunoassay will facilitate further studies to examine whether the hK13 concentration in serum is altered in disease states, including cancer. It is already known that at least four kallikreins (hK2, hK3, hK6, and hK10) are serologic cancer biomarkers *(4, 10–12)*.

Our immunoassay revealed that the recovery of recombinant hK13 from serum was incomplete, with values ranging from 5% to 10%. We did not observe differences among the monoclonal/monoclonal, monoclonal/polyclonal, and polyclonal/polyclonal immunoassay configurations. We postulated that serum contains proteinase inhibitors or other interacting proteins that bind to free hK13. Our recombinant protein is enzymatically active and may be sequestered by excess proteinase inhibitors such as α_2-macroglobulin. It is already well known that two other kallikreins, hK3 (PSA) and hK2, form complexes with many proteinase inhibitors in serum *(16–25)*. This proposal merits further investigation.

It has already been demonstrated that multiple kallikreins are overexpressed in ovarian carcinoma [re-

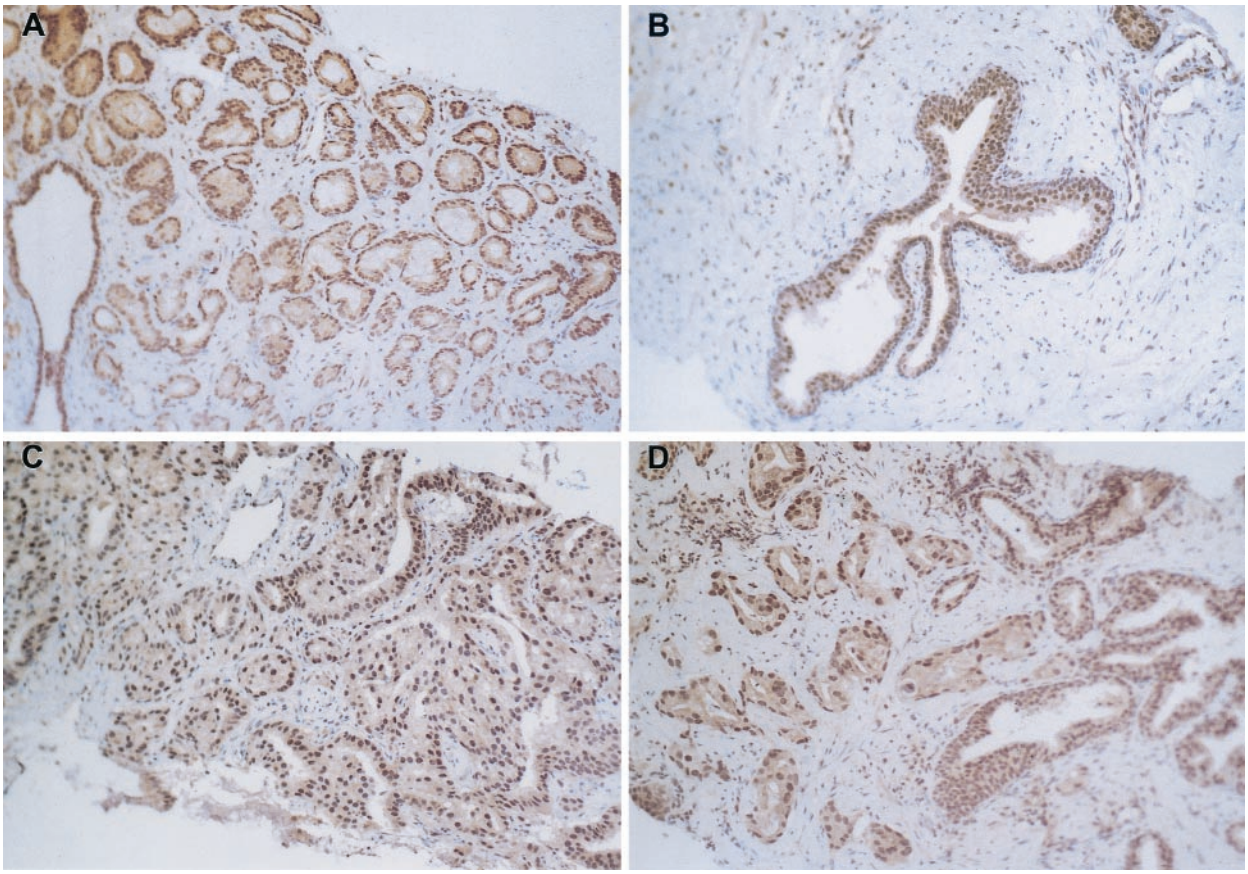

Fig. 6. Immunohistochemical localization of hK13 protein in paraffin-embedded prostatic tissues (needle biopsies) using the polyclonal hK13 rabbit antibody.

There is moderate cytoplasmic positivity with minimal nuclear staining and negative stroma. (*A*), prostate cancer, case 1; (*B*), prostate hyperplasia, case 1; (*C*), prostate cancer, case 2; (*D*), prostate cancer and hyperplasia, case 3. (Original magnification for *panels A–D*, ×400).

viewed in Ref. *(26)*]. There is experimental evidence that these kallikreins may form a cascade enzymatic pathway similar to the pathways of coagulation and fibrinolysis. In this report, we present the first evidence that hK13 is also overexpressed in a subset of patients with ovarian carcinoma (Fig. 4). We also report relatively high concentrations of hK13 in ascites fluid of women with ovarian cancer. These data suggest that hK13 should be added to the growing number of kallikreins that are overexpressed in ovarian carcinoma.

In conclusion, we describe for the first time production of recombinant hK13 proteins, production of antibodies, and development of highly sensitive and specific immunologic assays for hK13 quantification. We further demonstrate the presence of hK13 in human tissues and biological fluids. In the future, it may be worthwhile to study hK13 expression in disease states and examine whether hK13 concentration in serum can serve as a disease biomarker similar to other kallikreins (hK2, hK3, hK6, and hK10).

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