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Proteomics and
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Human Kallikrein 8: Immunoassay Development and Identification in Tissue Extracts and Biological Fluids

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Background: **The serine protease human kallikrein 8 (hK8; neuropsin), a new member of the human kallikrein family, was predicted to be secreted; thus, it is expected to be present in biological fluids. The aim of this study was to develop a sensitive and specific immunoassay for hK8 (hK8-ELISA) and establish the distribution of hK8 in tissue extracts and biological fluids.**

Methods: **Recombinant hK8 was produced in a baculovirus expression system and purified with a three-step chromatographic procedure. Purified hK8 was injected into mice and rabbits for antibody generation. A highly specific and sensitive sandwich-type immunoassay (ELISA) was developed using the rabbit and mouse antisera to hK8. The hK8-ELISA was then used to study the distribution of hK8 in various biological fluids and tissue extracts.**

Results: **The dynamic range of the hK8-ELISA was 0.2 (detection limit) to 20 μg/L, and imprecision (CV) was <10% within this range. This hK8-ELISA was specific for hK8 and had no detectable cross-reactivity with other members of the human kallikrein family. With this assay, hK8 was detected in tissue extracts of esophagus (highest concentrations), skin, testis, tonsil, kidney, breast, and salivary gland and in the biological fluids breast milk (highest concentrations), amniotic fluid, seminal plasma, and serum. Furthermore, in some cancer cell lines, the concentration of hK8 was regulated by steroid hormones.**

Conclusions: **We report for the first time production of recombinant hK8 protein, generation of antibodies, and development of a highly sensitive and specific immunoassay for quantification of hK8 in tissue extracts and biological fluids. This assay can be used to explore the potential of hK8 as a marker of cancer or other conditions.**

The human tissue kallikrein gene family is now known to include 15 members *(1, 2)*. All genes in this family localize to chromosome 19q13.4 and share significant similarities at both the DNA and the amino acid level. The human kallikrein family includes human kallikrein 3 (hK3),[4] also known as prostate-specific antigen (PSA), an important biomarker for prostate cancer *(3)*. Recently, three other members of this family, hK6 (also known as neurosin), hK10 [also known as normal epithelial cell specific 1 (NES1)], and hK11 [also known as trypsin-like serine protease (TLSP)], have been shown to be potential biomarkers for ovarian and prostate cancer *(4–6)*. Recent reports implicate many other members of this family in cancers of the breast, ovary, prostate, and testis, as well as diverse diseases of the central nervous system, skin, and other systems [reviewed in Ref. *(7)*].

KLK8/neuropsin is a member of the human kallikrein family *(1, 2)*. [According to the official kallikrein gene nomenclature, *KLK8* is the gene and hK8 is the protein *(8)*.] Originally, *KLK8* was cloned from a human skin

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[4] Nonstandard abbreviations: hK, human kallikrein; *KLK*, kallikrein gene; PSA, prostate-specific antigen; NES1, normal epithelial cell specific 1; TLSP, trypsin-like serine protease; AcNPV, *Autographa californica* nuclear polyhedrosis virus; and SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

cDNA library as a homolog of mouse neuropsin *(9)*. The mouse homolog has highest expression in skin and brain, especially the hippocampus, and was assumed to be associated with neural plasticity, memory formation, and some forms of epilepsy *(10–13)*. *KLK8* mRNA is increased in the hippocampus of individuals with Alzheimer disease compared with controls, which suggests that *KLK8* may play a role in neurodegeneration *(14)*. *KLK8* transcripts in ovarian cancer tissues are expressed at higher concentrations than in controls *(15)*, and two splice variants of *KLK8* have been detected in ovarian cancer *(16)*. Because the *KLK8* gene is predicted to encode for a secreted serine protease, this enzyme may have value as a biomarker, similar to hK3 (PSA) *(3)*, hK6, hK10 (NES1), and hK11 (TLSP) *(4–7)*. To date, there have been no reports in the literature describing any relationship between hK8 protein expression and human disease. In the present study, recombinant hK8 was produced in a baculovirus expression system, purified by column chromatography, and injected into mice and rabbits for antibody generation. These antibodies were used to develop a highly sensitive and specific immunoassay for hK8 (hK8-ELISA). The assay was applied to the measurement of native hK8 in tissue extracts and biological fluids.

Materials and Methods

CLONING OF FULL-LENGTH *KLK8* INTO BACULOVIRUS

The 887-base fragment containing the full-length *KLK8* cDNA was cut with *Eco*RI restriction enzyme from the *KLK8*/pGEM-T Easy plasmid *(9)* and ligated into the *Eco*RI sites of the pVL1393 transfer vector (PharMingen) to create plasmid *KLK8*/pVL1393. This plasmid was transferred into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome by homologous recombination so that High Five™ insect cells were transfected with the transfer vector and AcNPV DNA. The baculovirus containing the full-length *KLK8* cDNA was amplified for hK8 protein production as described below.

PROTEIN PRODUCTION

High Five insect cells were cultured in polystyrene flasks (75-cm^2) with 25 mL of TNM-FH complete medium (PharMingen) at 27 °C until almost confluent. The medium in each flask was then changed with 25 mL of serum-free medium (Invitrogen). The cells were then infected with 100 μL of stock baculovirus solution containing full-length *KLK8* cDNA (1.3×10^8-pfu/mL) and cultured at 27 °C for 4 days. The extracellular medium in each flask was harvested and dialyzed against 10 mmol/L HEPES buffer (pH 7.4) at 4 °C for 3 days.

PROTEIN PURIFICATION

Recombinant hK8 in the dialyzed medium was purified by a three-step column chromatography procedure. The medium was first applied onto a cation-exchange column, HiTrap SP Sepharose HP (bed volume, 5 mL; Amersham Pharmacia Biotech) and equilibrated with 10 mmol/L HEPES buffer (pH 7.4). Proteins were eluted with a three-step gradient of 10 mmol/L HEPES buffer (pH 7.4) containing 50, 100, and 150 mmol/L NaCl (10 mL of each NaCl concentration). Fractions (1 mL) were collected separately in tubes. The hK8 content of fractions was determined by Western blotting with a rabbit antiserum as described elsewhere *(17)*. See below for details. Fractions containing hK8 were diluted 1:2 (1 mL of buffer for each 1-mL fraction) with 10 mmol/L HEPES buffer (pH 7.4) and applied to an affinity column, HiTrap Heparin HP (bed volume, 5 mL; Amersham Pharmacia Biotech), equilibrated with 10 mmol/L HEPES buffer (pH 7.4). Proteins were eluted with a two-step gradient of 10 mmol/L HEPES buffer (pH 7.4) containing 200 and 250 mmol/L NaCl (10 mL of each NaCl concentration) and collected as 1-mL fractions. Fractions containing hK8 were then applied onto another affinity column, HiTrap Benzamidine FF (bed volume, 1 mL; Amersham Pharmacia Biotech) and equilibrated with 10 mmol/L HEPES buffer (pH 7.4) containing 500 mmol/L NaCl. The flow-through from the benzamidine column was collected. At each purification step, all fractions were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie G-250 staining and Western blotting. Each fraction was evaluated with the NuPAGE Bis-Tris electrophoresis system using two 4–12% gradient polyacrylamide gels (Invitrogen). One gel was stained with a Coomassie G-250 staining solution, SimplyBlue SafeStain (Invitrogen). The proteins on the other gel were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). After blocking with 50 g/L skim milk in 0.1 mol/L Tris-HCl buffer (pH 7.5) containing 0.15 mol/L NaCl and 1 mL/L Tween 20 for 30 min at room temperature, the membranes were reacted with a rabbit antiserum to hK8 *(17)* and further reacted with goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Inc.) in 50 g/L skim milk in 0.1 mol/L Tris-HCl buffer (pH 7.5) containing 0.15 mol/L NaCl and 1 mL/L Tween 20. The secondary antibody was detected on x-ray film by a chemiluminescent substrate (Diagnostic Products Corporation). The band intensities on the film were analyzed by an image analysis software (Lab Works; Ultra-Violet Products Ltd.).

MASS SPECTROMETRY

The recombinant hK8 protein was identified and characterized by trypsin digestion and nanoelectrospray mass spectrometry, as described previously in detail for recombinant hK10 *(18)*.

PRODUCTION OF POLYCLONAL ANTIBODIES

The purified hK8 was used as an immunogen to immunize rabbits and mice. The protein solution containing 100 μg of hK8 (in 150 μL of solution for mice and 400 μL for rabbits) was mixed with the same volume of complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections. The mixed

solution was injected subcutaneously into female Balb/c mice and New Zealand White rabbits. Injections were repeated three times for mice and six times for rabbits at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of polyclonal antibodies to hK8, the following immunoassay was used. Sheep anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch) was immobilized on 96-well white ELISA plates. The mouse/rabbit serum was applied to the plates at different dilutions ranging from 1:500 to 1:50 000. After incubation and washing, biotinylated recombinant hK8 was added (5–10 ng/well). Finally, after incubation and washing, alkaline phosphatase-conjugated streptavidin was added, and the alkaline phosphatase activity was detected with time-resolved fluorescence as described in detail elsewhere *(19)*.

STANDARD IMMUNOASSAY PROCEDURE

White polystyrene microtiter plates were coated with sheep anti-mouse IgG, Fc fragment-specific antibody (Jackson ImmunoResearch). We applied 100 μL of coating antibody solution [50 mmol/L Tris-HCl buffer (pH 7.8) containing 5 mg/L antibody] to each well and incubated the plates overnight. The plates were washed four times with the washing buffer [10 mmol/L Tris-HCl buffer (pH 7.4) containing 150 mmol/L NaCl and 0.5 mL/L Tween 20]. Mouse antiserum to hK8 was diluted 2000-fold in a general diluent [50 mmol/L Tris-HCl buffer (pH 7.8) containing 60 g/L bovine serum albumin and 0.5 g/L sodium azide], and 100 μL of the diluted antiserum was applied to each well. After incubation for 2 h with shaking, the plates were washed six times with the washing buffer. hK8 calibrators or samples were applied in each well (50 μL/well) along with 50 μL of general diluent and incubated for 2 h with shaking. The plates were washed six times with washing buffer. Subsequently, 100 μL of rabbit antiserum to hK8 diluted 1000-fold in buffer A [50 mmol/L Tris-HCl buffer (pH 7.8) containing 0.5 mol/L KCl, 60 g/L bovine serum albumin, 50 mL/L goat serum, 25 mL/L mouse serum, 10 g/L bovine gamma globulin, and 5 mL/L Tween 20] was applied to each well and incubated for 1 h. Plates were washed as above. Finally, 100 μL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific, diluted 3000-fold in buffer A, was added to each well and incubated for 1 h, and plates were washed as above. We added 100 μL of diflunisal phosphate solution [0.1 mol/L Tris-HCl buffer (pH 9.1) containing 1 mmol/L diflunisal phosphate, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$] to each well and incubated the plates for 10 min. We then added 100 μL of developing solution (1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, 3 mmol/L EDTA) to each well and mixed the solution for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion). Calibration and data reduction were performed automatically, as described in detail elsewhere *(18, 19)*.

SENSITIVITY OF THE IMMUNOASSAY

Recombinant hK8 was used to generate the calibration curve. hK8 calibrators were prepared by diluting the purified recombinant hK8 in the general diluent. These calibrators were used to determine the detection limit of the assay.

PREPARATION OF HUMAN TISSUE EXTRACTS AND BIOLOGICAL FLUIDS

Adult human tissues were collected at autopsy from a 51-year-old woman and a 41-year-old man within 24 h after death from myocardial infarction. Fetal tissues were collected from two 20-week-old aborted fetuses (male and female). The following human tissues (adult and fetal) were used for screening: esophagus, tonsil, skin, testis, kidney, salivary gland, breast, fallopian tube, adrenal, bone, cerebellum, colon, endometrium, liver, lung, muscle, ovary, pancreas, pituitary, prostate, seminal vesicle, small intestine, spinal cord, spleen, stomach, thyroid, trachea, and ureter. Human tissue extracts were prepared as follows: Frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Extraction buffer [2 mL; 50 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl, 5 mmol/L EDTA, and 10 mL/L NP-40 surfactant] 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 centrifuged at 14 000*g* at 4 °C for 30 min. The supernatants containing the tissue extracts were collected and stored at −80 °C until use. The biological fluids (amniotic fluid, breast milk, cerebrospinal fluid, follicular fluid, serum, and seminal plasma) were leftovers of samples submitted for routine biochemical testing and stored at −80 °C until use. Our procedures have been approved by the Institutional Review Board of Mount Sinai Hospital.

SPECIFICITY OF THE IMMUNOASSAY

Amniotic fluid, esophagus extract, tonsil extract, and recombinant hK8 were used to determine the specificity of the hK8-ELISA. These samples were measured by the standard assay procedure described above. The mouse and rabbit anti-hK8 antisera were then successively replaced with sera from the same animals obtained before immunization (preimmune sera). The samples were then measured again, and the fluorescence counts were compared with the counts obtained by the standard assay. The cross-reactivities of other homologous proteins were also investigated using recombinant hK1, hK2, hK3, hK4, hK5, hK6, hK7, hK9, hK10, hK11, hK12, hK13, hK14, and hK15, all at a concentration of 1 mg/L (produced in-house). Recombinant hK8 (20 μg/L) and these reference samples were measured with the standard procedure described above; their fluorescence counts were then compared.

LINEARITY

To determine the linearity of the hK8-ELISA, recombinant hK8, milk, and amniotic fluid samples were serially

Table 1. Purification of recombinant hK8 produced by the baculovirus system.

Purification step	Protein,[a] mg	hK8,[b] mg	Recovery, %	Purification,[c] fold
Culture medium	359×10^3	1411[c]	100	1
SP Sepharose	600	334	23.7	142
Heparin	163	154	10.9	240
Benzamidine	108	108	7.7	254

[a] Measured with the bicinchoninic acid assay with albumin as standard (Pierce Chemical Co.).
[b] Calculated from Western blot band intensities on an x-ray film. See text for details.
[c] Based on 400 mL of culture medium.

diluted in general diluent, and the hK8 concentrations in these samples were measured with the standard assay.

RECOVERY

Recombinant hK8 was added to breast cytosols, seminal plasma, and normal sera (male and female) at different concentrations and measured with the developed hK8 immunoassay. Recoveries were calculated after subtraction of the endogenous concentrations.

FRACTIONATION OF BIOLOGICAL FLUIDS WITH GEL-FILTRATION HPLC

To determine the molecular mass of the protein detected in the biological fluids and tissue extracts, we fractionated amniotic fluid, ascites fluid, and breast milk by gel-filtration chromatography, as described elsewhere *(20)*. The fractions were then collected and analyzed for hK8 in the hK8-ELISA.

CANCER CELL LINES AND HORMONAL STIMULATION EXPERIMENTS

The breast cancer cell lines MDA-MB-231, BT-474, T-47D, MCF-7, and ZR-75; the ovarian cancer cell line HTB-75 (Caov-3); and the prostate cancer cell line LNCaP were purchased from the American Type Culture Collection. The BG-1 ovarian cancer cell line was kindly provided by Dr. Henri Rochefort (Montpellier University, Montpellier, France), and the PC-3 cell line, stably transfected with androgen receptor [PC-3 $(AR)_6$] was provided by Dr. Theodore Brown (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). Cells were cultured to near confluency in RPMI medium (Invitrogen) supplemented with glutamine (200 nmol/L) and fetal bovine serum (100 mL/L) in plastic flasks. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. Twenty-four hours before the experiments, the culture medium was changed into a medium containing 100 mL/L charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in absolute ethanol were added into the culture medium at a final concentration of 10^{-8} mol/L (final ethanol concentration, 10 mL/L). Steroids tested were aldosterone (mineralocorticoid), dexamethasone (glucocorticoid), norgestrel (androgenic progestin), dihydrotestosterone (androgen), and estradiol (estrogen). All steroids were purchased from Sigma Chemical Co. Cells stimulated with ethanol (10 mL/L final concentration) were included as controls. The cells were grown for 7 days, and the cell culture supernatants were collected for hK8 examination. The experiments were repeated at least twice.

Results

PRODUCTION AND PURIFICATION OF hK8 PROTEIN

The 31-kDa recombinant hK8 was expressed and secreted into the medium of baculovirus-infected High Five insect cells. The hK8 in the medium was detected by Western blot analysis 1 day after infection, and the strongest signal was detected at 4 days. Almost pure hK8 was detected at the SP Sepharose, heparin, and benzamidine purification steps in 100 mmol/L NaCl, 200 mmol/L NaCl, and the flow-through fractions, respectively. We obtained 108 μg of purified hK8 from 400 mL of culture medium with the three-step column chromatography purification procedure (Table 1). Purified hK8 was detected as a single band on a 4–12% SDS-PAGE gel stained with Coomassie G-250 solution (Fig. 1). This band was subjected to matrix-assisted time-of-flight and tandem mass spectrometric analysis as described elsewhere *(18)* and confirmed to be human hK8. For example, five sequenced peptides of the trypsin-digested recombinant hK8 exactly matched the corresponding regions of human hK8 (data not shown).

Table 2. hK8 concentration in biological fluids.

Fluid	Samples tested, n	hK8, mg/L			Positivity rate, %
		Range[a]	Mean (SD)	Median	
Breast milk	13	17–2665	599 (840)	174	100
Amniotic fluid	10	1.0–22	6.7 (6.5)	5.6	100
Follicular fluid	5	1.4–5.4	3.1 (1.5)	3.0	100
Seminal plasma	13	1.0–7.4	4.3 (3.6)	2.8	100
Serum (male)	10	2.0–6.9	3.9 (1.5)	3.6	100
Serum (female)	25	0.4–6.0	2.2 (2.3)	2.3	100
Cerebrospinal fluid	13	0.0–1.4	0.4 (0.4)	0.3	69

[a] Concentrations <0.2 mg/L are shown as 0.0.

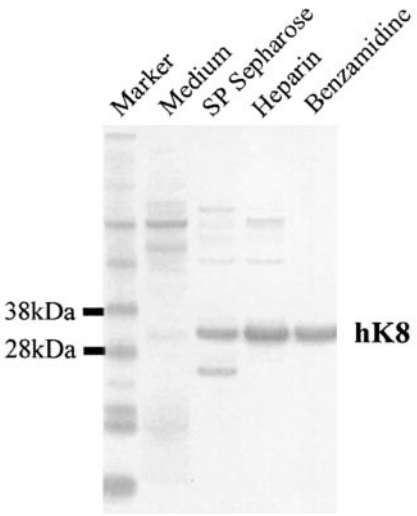

Fig. 1. SDS-PAGE of purified recombinant hK8 at each chromatographic step.

Each *lane* (except marker) was loaded with 3 μg of total protein. hK8 is essentially pure after the benzamidine step (see also Table 1).

STANDARD IMMUNOASSAY PROCEDURE

A typical calibration curve for the hK8 immunofluorometric ELISA format is shown in Fig. 2. The detection limit, defined as the concentration of analyte that could be distinguished from zero with 95% confidence, was 0.2 μg/L. The near-linear dynamic range extended to 20 μg/L. The within-run and day-to-day CVs for the developed hK8-ELISA were <10% within the measurement range, consistent with the precision of typical microtiter plate-based immunoassays.

SPECIFICITY

When amniotic fluid, esophagus extract, tonsil extract, and recombinant hK8 (20 μg/L) were measured with the developed assay, fluorescence counts >100 000 arbitrary units were obtained (Fig. 3). However, when either mouse or rabbit anti-hK8 polyclonal antibody was replaced with preimmune serum, fluorescence counts were reduced to background signals (<2000 arbitrary units). No immunoreactivity was detected when hK1, hK2, hK3, hK4, hK5, hK6, hK7, hK9, hK10, hK11, hK12, hK13, hK14, and hK15 solutions (all at 1000 μg/L) were measured with the developed assay for hK8. These data suggest that the assay is very specific for hK8 protein. Measured values for linearly diluted breast milk and amniotic fluid samples were within 10% of the expected values for the undiluted samples, suggesting an absence of matrix effects.

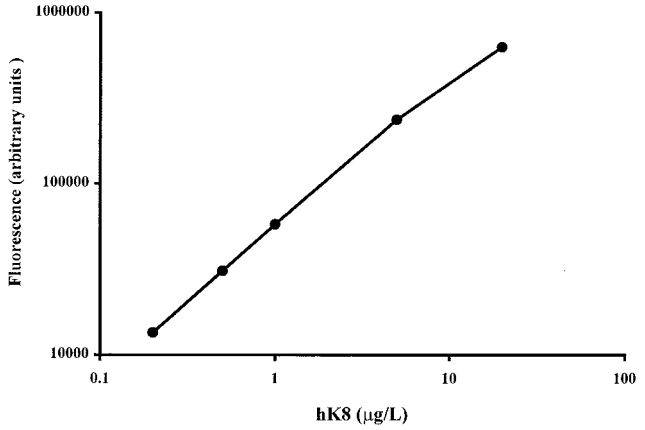

Fig. 2. Typical calibration curve for the hK8 immunoassay.

The background fluorescence (zero calibrator) was subtracted from all measurements. The dynamic range of this assay is 0.2–20 μg/L.

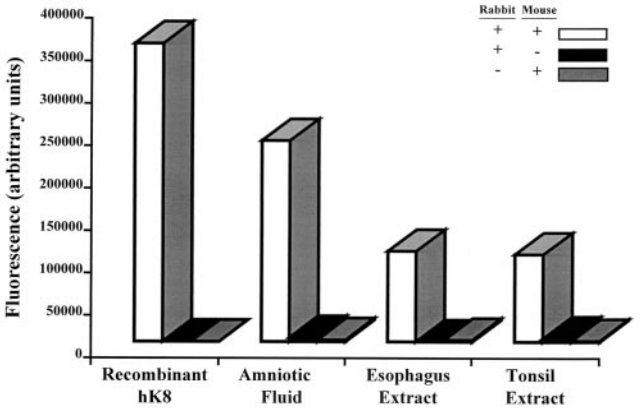

Fig. 3. Specificity of the hK8 immunoassay.

The assay was performed in the presence of both mouse and rabbit antibodies or in the absence of mouse or rabbit antibody (substituted by nonimmune serum). Samples tested were amniotic fluid and tissue extracts from esophagus and tonsil. For a discussion, see the text.

RECOVERY

Recovery of added recombinant hK8 was 95–100% in breast cytosols, 97–100% in seminal plasmas, 51–62% in male sera, and 36–78% in female sera, indicating almost complete recovery in tissue extracts and seminal plasma but less complete recovery in serum.

PRODUCTION OF hK8 BY CANCER CELL LINES AND HORMONAL REGULATION

hK8 is predicted to be a secreted protein. Breast, ovarian, and prostate cancer cell lines were cultured and stimulated with various steroids at a final concentration 10^{-8} mol/L, and the tissue culture supernatants were analyzed with the hK8-ELISA after 7 days of incubation. Among all cell lines, MDA-MB-231, BT-474, LNCaP, and ZR-75 did not produce detectable amounts of hK8 either before or after stimulation with the five different steroids at 10^{-8} mol/L. Detectable hK8 was found in supernatants from the cell lines PC-3 $(AR)_6$ (range, 38–156 μg/L), BG-1 (range, 48–65 μg/L), Caov-3 (range, 9–15 μg/L), MCF-7 (range, 1–10 μg/L), and T-47D (range, 1–5 μg/L). In terms of hormonal stimulation, the steroid(s) that produced a significant increase (at least 2-fold) in hK8 concentration above baseline (alcohol stimulation, 10 mL/L final concentration) were norgestrel and dihydrotestosterone for PC-3 $(AR)_6$ cells (3.5-fold increase over control), estradiol for T-47D cells (3.5-fold increase over control), and dexa-

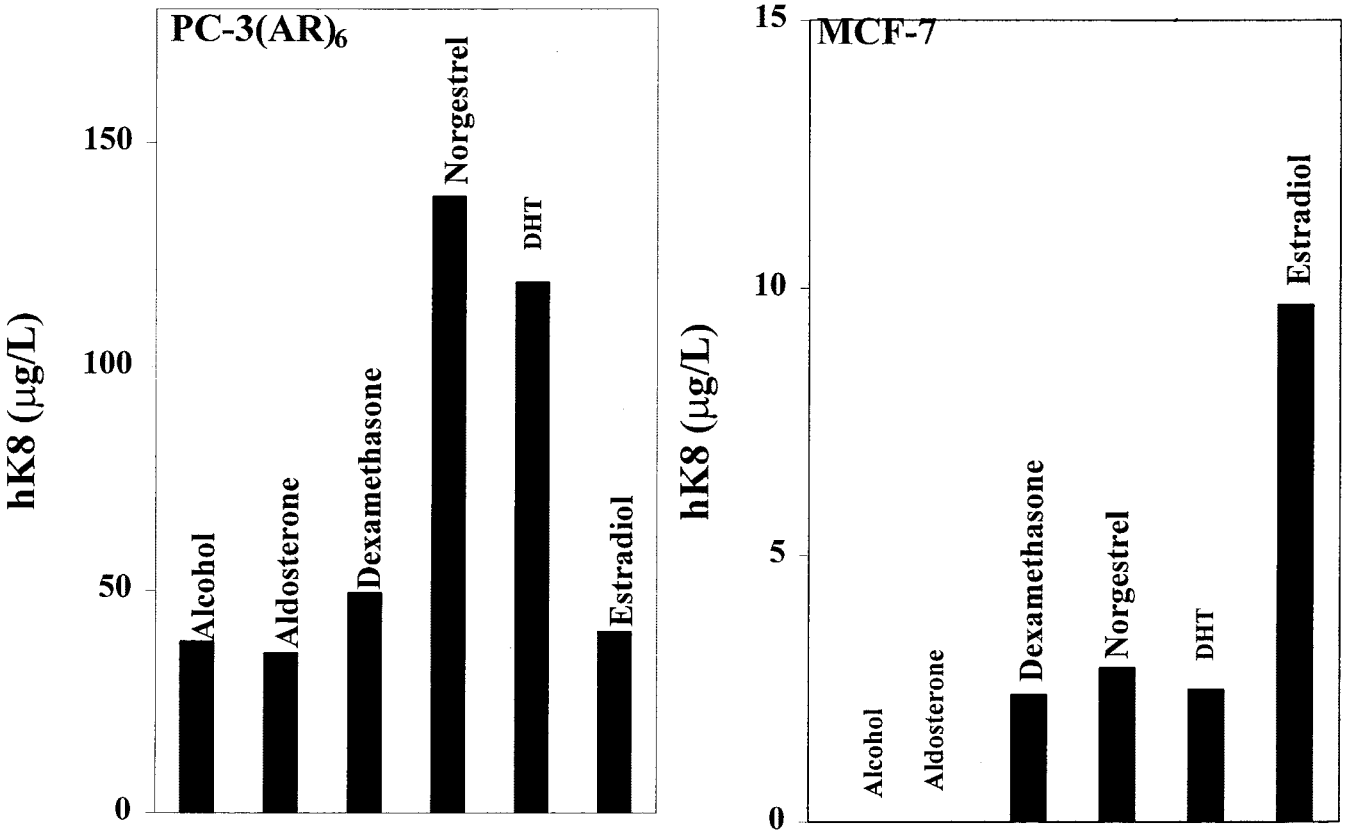

Fig. 4. Hormonal regulation of hK8 in the prostate carcinoma cell line PC-3 $(AR)_6$ and the breast carcinoma cell line MCF-7.

In the PC-3 $(AR)_6$ cell line (*left*), hK8 is up-regulated by norgestrel (an androgenic progestin) and dihydrotestosterone (*DHT*). In the MCF-7 cell line (*right*), hK8 is up-regulated by estradiol. For more discussion, see the text. All steroids were tested at a final concentration of 10^{-8} mol/L in the presence of 10 mL/L solvent (alcohol). Values represent means of three independent experiments; the SE was <10% in all cases.

methasone (4-fold), norgestrel (4-fold), dihydrotestosterone (3-fold), and estradiol (10-fold) for MCF-7 cells. For the cell lines BG-1 and Caov-3, there was not much change in hK8 concentration with any of the tested steroids. These data suggest that *KLK8* gene expression can be significantly up-regulated by a variety of steroids, including androgens, glucocorticoids, and estrogens, in different prostate and breast cancer cell lines but not in ovarian cancer cell lines. Examples of steroid hormone regulation of the *KLK8* gene in the cell lines PC-3 $(AR)_6$ and MCF-7 are shown in Fig. 4.

hK8 in human tissue extracts

We quantified hK8 in various adult and fetal male and female tissue extracts. The results are presented graphically in Fig. 5. The amount of hK8 in these extracts was corrected for the total protein content and is expressed as ng hK8/mg of total protein.

For adult tissues, highest amounts of hK8 were seen in esophagus, followed by skin, testis, tonsil, kidney, breast, salivary gland, axillary lymph node, and fallopian tube; all other tissues, including extracts from various parts of the brain, were negative (for a list of negative tissues, please see legend of Fig. 5). For fetal tissues, the highest amounts were seen in the ureter, followed by tonsil, esophagus, testis, prostate, kidney, seminal vesicle, thymus, skin, salivary gland, breast, and lung.

HPLC

To investigate whether hK8 in biological fluids and tissue extracts is circulating in various molecular forms, we fractionated one amniotic fluid sample, one milk extract, and one esophageal extract by gel-filtration chromatography, as described previously *(20)*, and analyzed all fractions with the hK8-ELISA (Fig. 6). In all cases, the detected hK8 eluted predominantly as a single peak with a molecular mass of ~30 kDa, consistent with the molecular mass of free (uncomplexed) hK8.

Discussion

The human kallikrein gene locus on chromosome 19q13.4 has now been well characterized *(1, 2)*. This gene family includes 15 members, all encoding for secreted serine proteases. Among all members, PSA is the premier biomarker for prostate cancer *(3)*.

The *KLK8* gene was first cloned from a human skin cDNA library as a homolog of mouse neuropsin *(9)*.

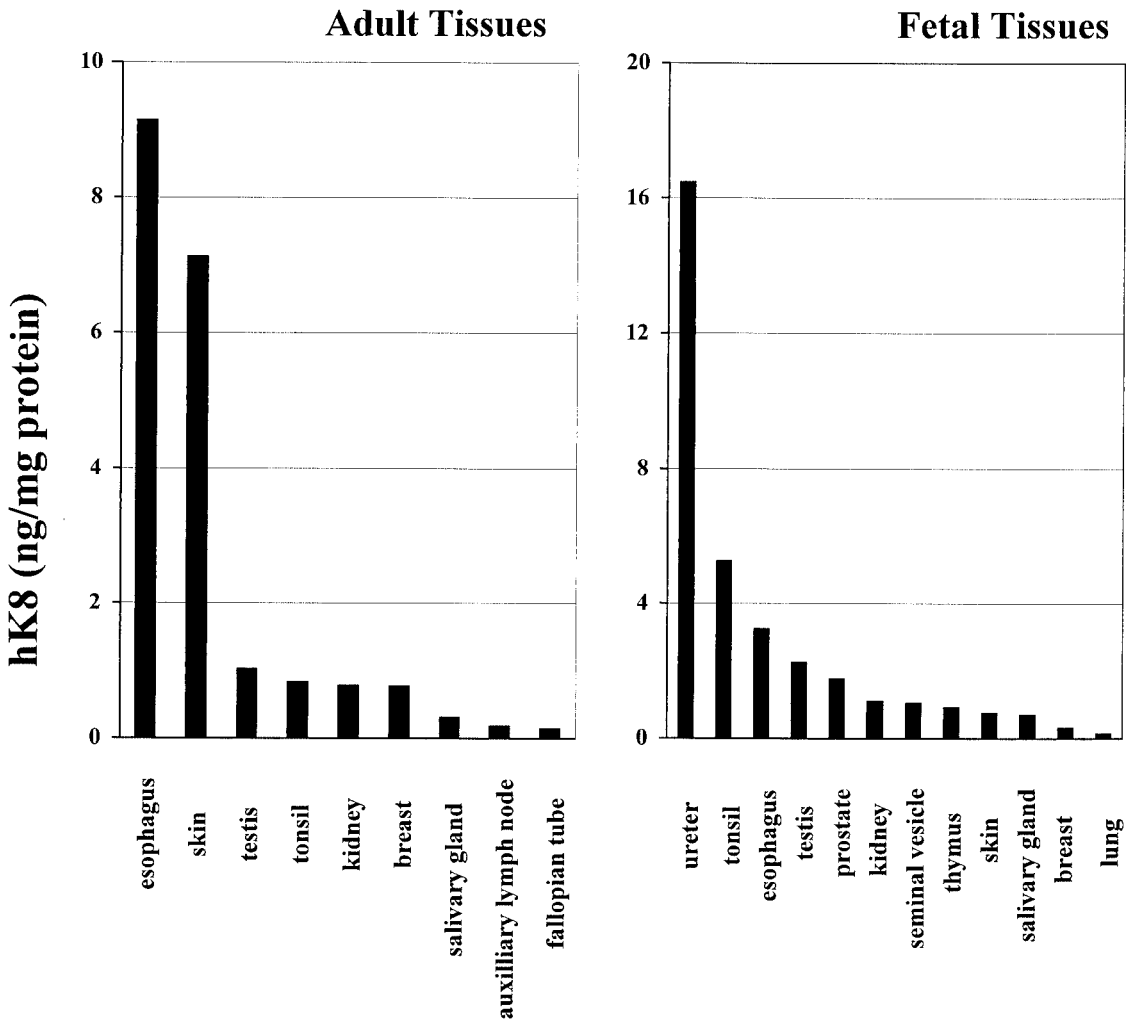

Fig. 5. Expression of hK8 in adult (*left*) and fetal (*right*) tissue extracts from males and females.

For a discussion, see the text. The following tissues were negative: *Adult Tissues*, adrenal, bone, cerebellum, colon, endometrium, frontal cortex, hippocampus, liver, lung, medulla, mesentery lymph node, midbrain, muscle, occipital cortex, ovary, pancreas, pituitary gland, pons, prostate, seminal vesicle, small intestine, spinal cord, spleen, stomach, temporal lobe, thyroid, trachea, ureter, and uterus; *Fetal Tissues*, adrenal, bone, bone marrow, cerebellum, colon, cortex, endometrium, fallopian tube, heart, liver, muscle, ovary, pancreas, pituitary gland, small intestine, spinal cord, spleen, stomach, thyroid, and trachea.

Human neuropsin (hK8) has 72% identity to mouse neuropsin. Recombinant mouse neuropsin was produced in a baculovirus expression system and was found to be secreted into the culture medium as a pro-protein containing the tetrapeptide Glu-Gly-Ser-Lys at the amino terminus *(21)*. The homologous amino terminus in human neuropsin would contain the tetrapeptide Glu-Gln-Asp-Lys. If this propeptide is removed by another protease, hK8 is activated *(21)*.

Expression of the mouse homolog is highest in skin and brain, especially in the hippocampus, and is assumed to be associated with neural plasticity, memory formation, and some forms of epilepsy *(10–13)*. However, characterization of tissue expression and other activities of human neuropsin have not been studied to date, primarily because of a lack of suitable reagents and methods for measuring the human enzyme. For these reasons, we have undertaken this study to develop recombinant human hK8 protein, specific antibodies directed to hK8, and highly sensitive and specific immunologic method for hK8 quantification in tissue extracts and biological fluids. These methods allowed us to preliminarily examine the expression of the human protein, as well as its potential value as a cancer biomarker. Previously, several other kallikreins, including *KLK4*, *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK9*, *KLK10*, *KLK11*, *KLK14*, and *KLK15*, have been associated with various forms of malignancy and especially cancers of the ovary, breast, prostate, and testis [reviewed in Refs. *(1, 2)*].

We have succeeded in producing and purifying relatively large amounts of human hK8 in a baculovirus expression system. This recombinant protein was used as an immunogen in mice and rabbits to produce highly specific polyclonal antibodies against hK8. We used these

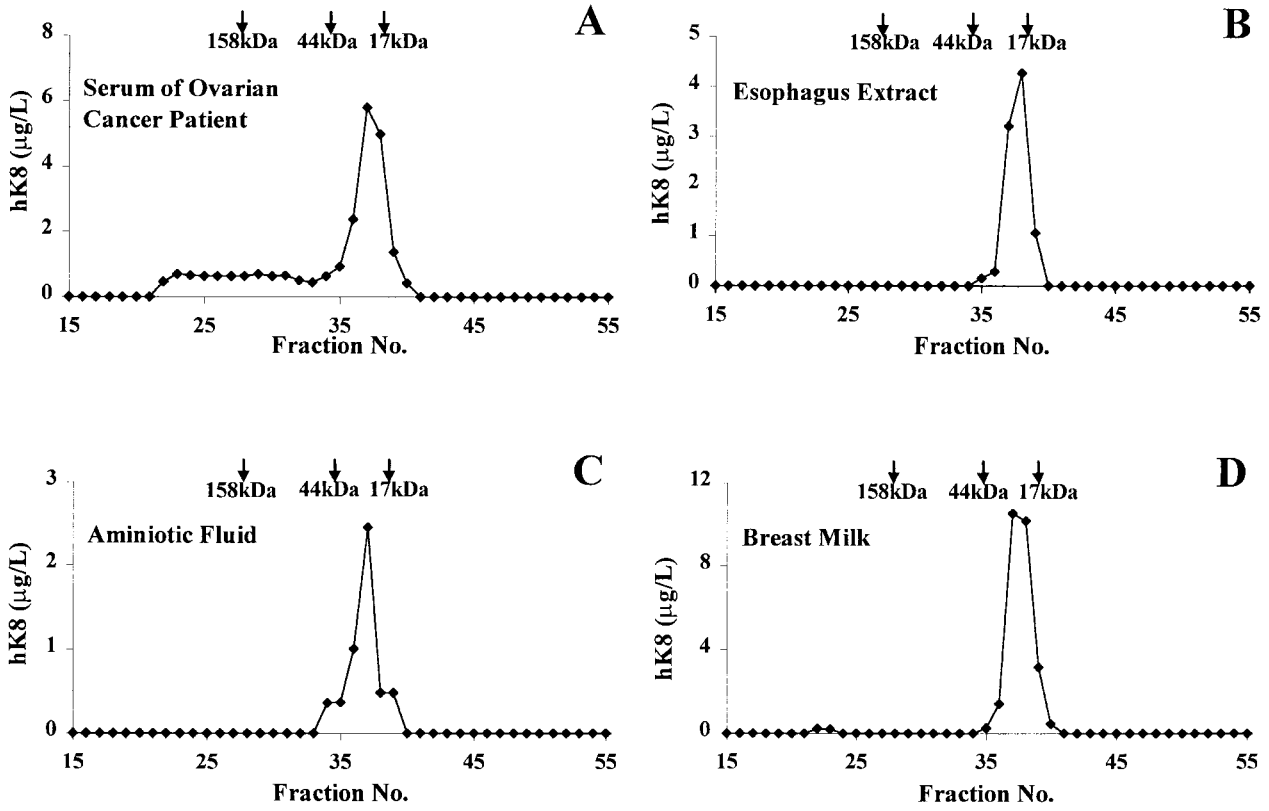

Fig. 6. HPLC separation on a gel-filtration column of a serum from an ovarian cancer patient (*A*), an esophageal extract (*B*), amniotic fluid (*C*), and breast milk (*D*).

The *peak* represents the free, 30-kDa form of hK8. The molecular masses of three markers and their elution times are shown by *arrows*. For a discussion, see the text.

antibodies to develop a noncompetitive immunofluorometric procedure (hK8-ELISA) suitable for quantifying hK8 with high sensitivity and specificity. None of the other known human kallikreins cross-reacts to a significant degree with the developed immunoassay. With this method, we were able to demonstrate presence of hK8 in many biological fluids, especially human breast milk. These data confirm that, in vivo, hK8 is a secreted protein. We also established the tissue expression of hK8 by analyzing extracts from diverse adult and fetal tissues. In general, we found concomitant expression of hK8 in both adult and fetal tissues, including esophagus, skin, testis, tonsil, kidney, breast, and salivary gland. A few other tissues in the fetal period produced hK8 as well. One important difference between the expression of mouse and human hK8 is related to the central nervous system. In the mouse, *KLK8* is expressed at high concentrations in the central nervous system and seems to be associated with neural plasticity, memory formation, and some forms of epilepsy *(10–13)*. Although *KLK8* expression at the mRNA level has been reported in human brain *(14)*, we did not detect any hK8 protein in the areas of human brain examined, including the cerebellum, frontal cortex, hippocampus, medulla, midbrain, occipital cortex, pons, spinal cord, and temporal lobe. Furthermore, the concentrations in cerebrospinal fluid were extremely low. These data suggest that hK8 may not be significantly expressed in human brain. This is in contrast to some other kallikreins, most prominently hK6 (protease M/neurosin), which is highly expressed in human brain and is present at very high concentrations in cerebrospinal fluid *(22, 23)*.

It has been reported that most, if not all, kallikrein genes are regulated by steroid hormones *(1, 2)*. In studies using breast and prostatic carcinoma cell lines, it has been shown that certain kallikreins are up-regulated only by androgens and androgenic progestins, whereas others are predominantly up-regulated by estrogens. However, the promoters of these genes have only been functionally characterized in very few cases *(24, 25)*. Hormonal regulation of the *KLK8* gene has not been reported previously. In the present study, we used breast, ovarian, and prostatic carcinoma cell lines to show that this gene is significantly up-regulated by androgens, androgenic progestins, and estrogens, depending on the cell line. Particularly, very high concentrations of hK8 were seen with the prostatic carcinoma cell line PC-3 that had been stably transfected with the androgen receptor. In the MCF-7 breast carcinoma cell line, which contains high concentrations of estrogen receptor, *KLK8* is up-regulated by estradiol. Interestingly, in the ovarian cancer cell lines in which basal expression of hK8 was observed (BG-1 and Caov-3), steroid hormone stimulation did not produce

any significant changes in expression. This finding underlines the possible involvement of tissue-specific co-regulators of hK8 expression. These data suggest that *KLK8* transcription is controlled by steroid hormones in some but not all tissues. Functional promoter analysis of this gene is warranted. These data are also in accordance with our tissue expression studies showing that in the breast (a steroid hormone-regulated tissue), there is significant expression of hK8; the protein is then secreted and found at high concentrations in breast milk.

Our serum recovery experiments indicated that recovery of hK8 is incomplete (36–78%). This is a common phenomenon among many kallikreins, including PSA. Many kallikreins interact with various circulating proteinase inhibitors, and especially when bound to α_2-macroglobulin, the complex is not detectable by the immunoassays used *(26–30)*. We examined the molecular forms of hK8 in various biological fluids (Fig. 6). Our assay detects a single peak of ~30 kDa, which corresponds to the free hK8 protein. It is thus possible that hK8 circulates in tissues and biological fluids, including serum, in its free form. Alternatively, it is conceivable that the fraction of hK8 that is bound to proteinase inhibitors (e.g., α_2-macroglobulin) is not recognized by the hK8-ELISA. More studies targeting hK8/inhibitor complex formation will be necessary to clarify these issues.

In conclusion, we report here for the first time the production of human recombinant hK8 and development of highly specific antibodies and a highly sensitive immunofluorometric procedure for hK8 quantification. These tools will be invaluable in future studies investigating the potential utility of this new kallikrein in diagnostics.

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