

Detection of Human Kallikrein 4 in Healthy and Cancerous Prostatic Tissues by Immunofluorometry and Immunohistochemistry

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Background: Human kallikrein 4 (gene, *KLK4*; protein, hK4), a recently discovered member of the kallikrein gene family, shares many characteristics with prostate-specific antigen, the best available marker for prostate cancer. Because the protein has not been detected in any human tissue, we attempted to develop immunologic methods for hK4 analysis and use them to detect hK4 in healthy and cancerous tissue extracts and biological fluids.

Methods: We extracted total RNA from 20 pairs of matched (healthy–cancer) prostate tissue samples. *KLK4* cDNA was amplified by reverse transcription-PCR (RT-PCR) and cloned in a pPICZαA expression vector. We then transformed the construct product into *Pichia pastoris* yeast strains and induced secreted recombinant protein production by addition of methanol. We purified the recombinant protein by nickel ion-affinity chromatography and used it as an immunogen in rabbits and mice to generate polyclonal anti-hK4 antibodies. These antibodies were used to develop a sandwich-type immunoassay suitable for hK4 quantification in biological fluids and tissue extracts.

Results: The immunoassay had a detection limit of 0.1 μg/L. We detected hK4 in 10 of 21 matched (healthy–cancer) prostate tissues, and hK4 was frequently higher in healthy tissues. In one matched-sample pair, the hK4 content was relatively high in both the healthy [4.62

μg/g of total protein (TP)] and the cancerous (1.22 μg/g of TP) prostate tissue. Among tissue extracts, we found the highest concentrations of hK4 in healthy (0.0–4.62 μg/g of TP) and cancerous (0.0–1.72 μg/g of TP) prostatic extracts and in placental extracts (0.0–0.05 μg/g of TP). We also detected traces of hK4 protein immunoreactivity in amniotic fluid (<0.1–0.6 μg/L), human breast milk (<0.1–0.75 μg/L), and seminal plasma (0.2–0.9 μg/L). Immunohistochemical studies showed cytoplasmic staining for hK4 protein in both malignant and benign epithelial cells of the prostate. However, we did not detect hK4 in cerebrospinal fluid, healthy and cancerous ovarian tissue extracts, and many other human tissue extracts.

Conclusions: hK4 protein is present in some prostatic tissue extracts but at relatively low concentrations, although *KLK4* mRNA is readily detectable by RT-PCR. We propose that the protein either is not synthesized efficiently or is degraded very quickly.

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The human kallikrein gene family, which encodes a subgroup of serine proteases, is now known to consist of 15 members (1). The best known are the classic kallikreins: tissue/pancreatic/renal kallikrein (hK1),⁵ prostate-specific antigen (PSA; hK3), and human glandular kallikrein 2 (hK2). All kallikrein genes are localized to chromosome 19q13.4, have the same exon-intron organization, and share many similarities in their predicted protein sequences, including the signal-peptide motif and the location of the His-Asp-Ser catalytic triad (2). All

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⁵ Nonstandard abbreviations: hK, protein product of kallikrein gene; PSA, prostate-specific antigen; *KLK*, kallikrein gene; RT-PCR, reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline containing Tween 20; BSA, bovine serum albumin; MS, mass spectrometry; ALP, alkaline phosphatase; and TP, total protein.

members are predicted to have either trypsin or chymotrypsin-like proteinase activity.

Human kallikrein gene 4 (*KLK4*; protein, hK4) is one such new member of this family of serine protease genes. This gene was discovered independently by two groups who used a subtractive hybridization approach (3) or a positional candidate-gene approach (4, 5). On the basis of the amino acid sequences, the closest relatives of *KLK4* within the human kallikrein gene family are those that encode PSA (38% identity), the human stratum corneum chymotryptic enzyme (45% identity), and *KLK6* (zyme/neurosin/protease M; 38% identity) (6); the last and *KLK10* are implicated in ovarian cancer (7, 8). Initially, Northern blotting studies had determined that the expression of this gene was restricted to the male prostate gland (3); however, subsequent experiments based on reverse transcription-PCR (RT-PCR) have shown that *KLK4* mRNA is also expressed in testicular, mammary, adrenal, brain, uterine, and thyroid tissues, albeit in lower amounts than in the prostate (5, 9). *KLK4* expression is up-regulated by androgens in the prostate cancer-derived cell line LNCaP (3), whereas in the breast carcinoma cell line BT-474, it is up-regulated by both androgens and progestins (5). A putative androgen-response element has been located in the promoter of this gene (9), but it is not yet known whether it is functional. The proposed functions of *KLK4* include roles in tooth maturation (10) and/or carcinogenesis of hormonally regulated tissues (1, 2). We have demonstrated the up-regulation of *KLK4* mRNA in ovarian cancer compared with that in healthy ovarian tissues; indeed, *KLK4* is an independent indicator of poor prognosis in grade I and II ovarian tumors (11, 12). However, the function of this hormonally regulated protein in ovarian cancer has not yet been determined.

This lack of knowledge of hK4 has occurred because no quantitative studies at the protein level have as yet been performed. Because the open reading frame of *KLK4* predicts that its first 26 N-terminal amino acids are the signal peptide, we expected that the 224-amino acid mature enzymatic form would be a secreted protein (3, 5, 9). Therefore, we hypothesized that hK4 is present in biological fluids, especially in secretions from steroid hormone-regulated tissues. Consequently, hK4 may be a disease biomarker in prostate, breast, and ovarian cancers. In this study, we developed a highly specific and sensitive sandwich-type, enzyme-linked immunofluorometric assay capable of quantifying hK4. Using this assay, we examined the concentrations of hK4 protein in tissues and biological fluids obtained from healthy and ill patients.

Materials and Methods

TOTAL RNA EXTRACTION

We pulverized 50–100 mg of 20 prostate tissue pairs (healthy–cancerous) in liquid N₂ and extracted total RNA by the TRIzol[®] method (Invitrogen). We also extracted total protein from the same tissues and one more tissue

pair, as described below for protein extraction. We assessed total RNA concentration spectrophotometrically, and 2 µg was used for the synthesis of first-strand cDNA by the SuperScript[™] preamplification system, according to the directions supplied by the manufacturer (Invitrogen). To test the success of cDNA synthesis, we amplified 1 µL by PCR with primers specific for β-actin. Equal amounts of PCR product were visualized on a 2% agarose gel stained with ethidium bromide.

ASSESSMENT OF *KLK4* mRNA EXPRESSION

One microliter of first-strand cDNA was amplified with HotStar[™] Taq DNA polymerase in the PCRs, as prescribed by the manufacturer (Qiagen Inc), with the following PCR primers: 5'-GAATTCCTTCCGCAGGATGT-3' and 5'-TGACCCGCTGTACCACCCCA-3', which are specific for *KLK4*. The primers spanned at least two exons to avoid contamination with genomic DNA. PCRs were performed in an Eppendorf thermocycler (enzyme activation at 95 °C for 15 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 1 min, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min). After the PCRs were completed, 15 µL of the 278-bp *KLK4* product was visualized by staining with ethidium bromide on 2% agarose gels. We photographed the gels under ultraviolet light with the Speedlight Gel Documentation System (Lighttools Research) and ×20 magnification. Images were scored for the presence (positive) or absence (negative) of a *KLK4* PCR-generated band and its relative intensities within the tissue pairs.

RECOMBINANT hK4 PROTEIN PRODUCTION AND PURIFICATION

Expression construct. We generated *KLK4* cDNA, which encodes amino acids 31–254 (numbering based on GenBank accession no. NP_004908), in a PCR with 1 µL of human prostate cDNA as the template, 100 ng of the forward primer 5'-AGCTGCGAATTCATCATAAACGGC-3', 100 ng of the reverse primer 5'-TCCGCGGCCGCA-GAGTAACTGGCTGC-3', 200 µM dNTPs, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), and 2.5 U of pfu DNA polymerase (Stratagene). The total reaction mixture volume was 25 µL. The PCR was performed in an Eppendorf thermocycler (initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min). Using standard techniques (13), we cloned the PCR product into the pPICZαA expression vector of the EasySelect[™] *Pichia pastoris* yeast expression system (Invitrogen). The expression construct contained the *KLK4* cDNA sequence cloned in-frame with the *P. pastoris* α-factor secretion signal and the 3'-end myc epitope and polyhistidine (His)₆ tag.

Protein production. The construct was transformed into yeast strain KM71H, and a stable transformant was selected as prescribed by the manufacturer. The selected

yeast clone was then grown in a 30 °C shaking incubator for 4 days in BMMY medium (10 g/L yeast extract; 20 g/L peptone; 100 mmol/L potassium phosphate, pH 6.0; 13.4 g/L yeast nitrogen base; 40 mg/L biotin; and daily additions of methanol to the medium to yield a final concentration of 5 mL/L). The supernatant was recovered from the culture by centrifugation. To test for the presence of recombinant hK4, we assayed the supernatant by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred the resultant protein samples to a Hybond-C membrane (Amersham Pharmacia Biotech). Subsequently, the membrane was probed with either a hK4-specific rabbit antiserum [diluted 1:500 in Tris-buffered saline–Tween (TBS-T) buffer] that had been raised in-house against a 15-amino acid-long synthetic hK4 peptide sequence or a mouse monoclonal anti-His tag antibody (Invitrogen). After extensive washes with TBS-T and application of horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG (Amersham), fluorescence was detected with a SuperSignal® West Pico chemiluminescent substrate (Amersham).

Protein purification. Recombinant hK4 protein was purified from the medium on Ni-NTA nickel ion-affinity spin columns (Qiagen). We adjusted 18 mL of supernatant to pH 8.0 and applied it (in 600- μ L aliquots) successively to the spin column. The column was washed and eluted according to the manufacturer's instructions. To assess the purity and molecular weight of the protein, the purified, recombinant hK4 was assayed by SDS-PAGE and stained with Gelcode blue reagent (Pierce Chemical Co.). The total protein concentration was determined by the Bradford method, with bovine serum albumin (BSA) as the calibrator (Pierce). The identity of hK4 was confirmed by tandem mass spectrometry (MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described previously (14).

PRODUCTION OF ANTI-hK4 POLYCLONAL ANTIBODIES

We diluted 100 μ g of the purified, recombinant hK4 protein with an equal volume of complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injections) and injected it subcutaneously into female BALB/c mice and female New Zealand White rabbits every 3 weeks for a total of six injections per animal. Blood samples taken from the animals 2 weeks after every injection were tested for immunoreactivity by an immunofluorometric method. In this procedure, sheep anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch) was immobilized on 96-well, white, polystyrene microtiter plates. The mouse or rabbit serum samples were prepared in dilutions ranging from 1:500 to 1:100 000. One hundred microliters of each dilution was added to the plates; the plates were incubated and washed, and then biotinylated recombinant hK4 (5–10 ng/well) was added to each plate. After incubation, washes, the addition of streptavidin-alkaline phosphatase

(ALP) conjugate, further incubation, and more washes, ALP activity was measured by time-resolved fluorescence (see next section for more details).

IMMUNOFLUOROMETRIC ASSAY FOR hK4 PROTEIN

Sheep anti-mouse IgG, Fc fragment-specific antibody (diluted 1:400 in 50 mmol/L Tris buffer, pH 7.8; Jackson ImmunoResearch) was used as the coating antibody. One hundred microliters of the coating antibody (500 ng) was added to each well of a 96-well, white, polystyrene microtiter plate, and the plate was incubated overnight at room temperature. On the following day, the plate was washed three times with the wash buffer (9 g/L NaCl and 0.5 g/L Tween 20 in 10 mmol/L Tris buffer, pH 7.4). The anti-hK4 mouse serum, prediluted 1:10 in 10 mmol/L Tris buffer, pH 7.4, was further diluted to 1:500 in BSA solution (60 g/L BSA in 50 mmol/L Tris, pH 7.8, containing 0.5 g/L NaN₃), and 100 μ L was applied to each well. The plate was then incubated for 2 h or overnight at room temperature. After the plate was washed three times with wash buffer as before, 100 μ L each of the hK4 calibrators and samples was added to each well. The plate was incubated for 2 h with shaking, followed by six washes. After the washes, 100 μ L of the anti-hK4 rabbit serum, diluted 1:3000 in 60 g/L BSA solution, was pipetted into each well and incubated for 1 h with shaking. The plate was washed six times, and 100 μ L of the ALP-conjugated, Fc fragment-specific goat anti-rabbit IgG (Jackson ImmunoResearch), diluted 1:3000 in assay buffer (50 mmol/L Tris buffer, pH 7.8, containing, per liter, 60 g of BSA, 0.5 mol of KCl, 10 g of bovine immunoglobulin, 25 mL of normal mouse serum, and 100 mL of normal goat serum) was added to each well and incubated for 45 min with shaking. This step was followed by six washes as described earlier. For the detection of ALP activity, 100 μ L of 1 mmol/L diflunisal phosphate diluted in substrate buffer (1 mmol/L MgCl₂, 0.1 mol/L NaCl, 0.1 mol/L Tris, pH 9.0) was added to each well and incubated for 10 min with shaking. Finally, 100 μ L of developing solution (2 mmol/L TbCl₃, 3 mmol/L EDTA, 0.4 mol/L NaOH, 1 mol/L Tris base) was pipetted into each well and mixed briefly with shaking. The resulting fluorescence was measured with a Cyberfluor 615 Immunoanalyzer, a time-resolved fluorometer (MDS Nordion). Calibration and data reduction were performed automatically as described previously (15). This optimal assay configuration was obtained after we tested various dilutions and diluents of antibodies, the order in which the antibodies were added, and the incubation times at each step of the procedure.

BIOLOGICAL FLUIDS AND TISSUES

Prostatic tissues. Matched healthy–cancerous prostatic tissue pairs were obtained from 21 prostate cancer patients who had undergone radical retropubic prostatectomy for prostatic adenocarcinoma at the Charite University Hospital (Berlin, Germany). The patients had not received

hormonal therapy before surgery. The tissue samples were dissected from cancerous and noncancerous (healthy) portions of the prostate immediately after surgical removal. The samples were stored in liquid N₂ until needed. To determine the malignant or benign nature of the tissue samples, a histologic analysis was performed as described previously (16). These samples were approved for research purpose use by the Ethics Committee of the Charite Hospital.

Fluids. Biological fluids (cerebrospinal fluid, amniotic fluid, milk, and seminal plasma samples) were leftovers of samples submitted for routine biochemical testing that had been stored at -80 °C. To test the recovery of hK4 from serum, we added 0, 50, or 100 µL of the 1000 µg/L hK4 calibrator to 400 µL of serum samples from two men and two women. In each case, the volume was adjusted to 500 µL by addition of 60 g/L BSA. As a control, the hK4 calibrator was added to a 60 g/L BSA solution in a similar manner. These sample preparations were left undisturbed at room temperature for 30 min before being assayed for hK4.

PROTEIN EXTRACTION

Extracts for the matched prostate tissues. Frozen prostate tissues (30–50 mg) were thawed, chopped, and homogenized on ice in 100 µL of protein extraction buffer (2.5 mL/L Triton X-100 in 10 mmol/L sodium phosphate, pH 7.5) with a Wheaton glass homogenizer. After sonication, the extracts were transferred to 1.5-mL Eppendorf tubes; extracts that still remained in the homogenizer were recovered with two rinses each of 100 µL of extraction buffer. The 300-µL extraction mixture was then centrifuged at 4 °C for 15 min at 23 000g followed by recovery of the supernatants. On the basis of data obtained from procedure optimization (17), the extraction procedure was repeated twice more for each tissue, and the three aliquots of supernatant were pooled for analysis. All extracts were stored and shipped at -80 °C.

Cytosolic extracts for all other tissues. To prepare human tissue cytosolic extracts, 0.2 g each of various frozen human tissues was pulverized on dry ice. One milliliter of extraction buffer (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, and 1 g/L each of aprotinin and leupeptin) was added to the pulverized tissue samples, which were then incubated on ice for 30 min with repeated shaking and vortex-mixing every 10 min. After centrifugation of the mixtures at 14 000g at 4 °C, the supernatants (cytosolic extracts) were collected and stored at -80 °C until needed.

OTHER ASSAYS

Total protein concentrations were assessed in all tissue extracts by the bicinchoninic acid method, with BSA as the calibrator (Pierce). In five prostate cancer samples

(unmatched series), we also determined the PSA concentrations with a PSA-specific immunoassay (Diagnostic Systems Laboratories) as described previously (18).

SIZE-EXCLUSION LIQUID CHROMATOGRAPHY

Amniotic fluid fractionation. An amniotic fluid sample with a relatively high hK4 content (as determined by the hK4-specific immunoassay) was concentrated 20-fold in a Centricon YM 10 concentrator (Amicon Inc.). Five hundred microliters of the concentrated amniotic fluid was then separated on a silica-based gel-filtration column, as described previously (19), at a flow rate of 0.5 mL/min. Gel-filtration fractions were then analyzed with the developed hK4 immunoassay, and fluorescence values were plotted vs fraction number.

Fractionation of prostate cancer extracts. Among the prostate cancer tissue extracts, two samples (samples 7 and 10) with the highest hK4 concentrations were separated on the gel-filtration column as described previously.

IMMUNOHISTOCHEMISTRY

A rabbit polyclonal antibody was raised against the full-length hK4 recombinant protein produced in yeast cells as described above. For the negative control slides, this antibody was substituted with the preimmune rabbit blood obtained before the animal immunizations with hK4. Immunohistochemical staining for hK4 was performed according to a standard immunoperoxidase method. In brief, paraffin-embedded prostatic tissue sections (4 µm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with aqueous H₂O₂ (30 mL/L) for 15 min. The sections were then treated with 4 g/L pepsin, pH 2.0, for 5 min at 42 °C and blocked with 200 g/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 sections were washed, biotinylated anti-rabbit antibody (Signet Laboratories) was added after being diluted fourfold in antibody dilution buffer (Dako). After incubation and washing, streptavidin-tagged horseradish peroxidase was added for 30 min at room temperature. The sections were washed again, and detection was by reaction with aminoethylcarbazole for 5–10 min. The slides were counterstained with hematoxylin and covered with coverslips.

Results

RECOMBINANT hK4 PROTEIN PRODUCTION AND PURIFICATION

The yeast supernatants, which were recovered after induction of *KLK4* stable transformants for 4 days with 50 mL/L methanol, contained secreted, recombinant hK4 protein, as shown in Fig. 1. Probing this immunoblot with anti-hK4 peptide rabbit serum or with an anti-His tag mouse monoclonal antibody detected recombinant pro-

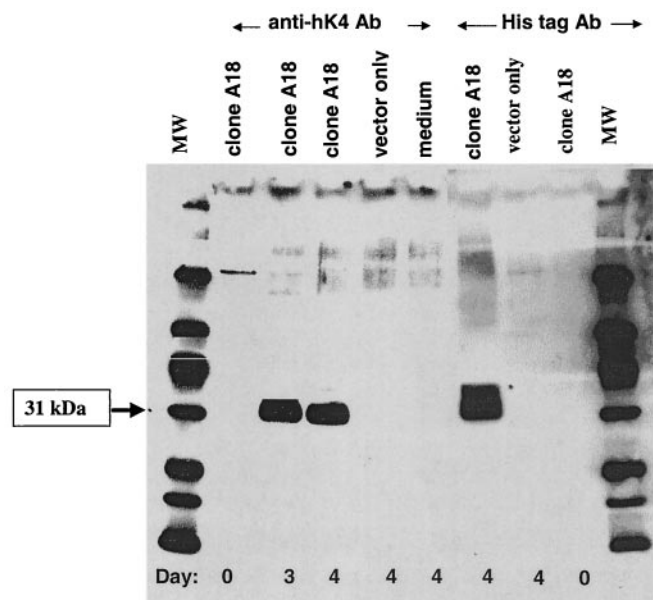


Fig. 1. Western blot of supernatants before and after induction of stably transfected yeast clones with 50 mL/L methanol.

Protein bands are visible with both the anti-His tag and the anti-hK4 peptide antibodies (Ab) at ~30 kDa (reduced samples) after 3 and 4 days of induction of the hK4-positive clone A18. No bands were observed with either of the antibodies before induction (day 0) or after 4 days of induction in vector-containing clones. MW, molecular mass marker.

tein in the supernatant of induced cells but not in the uninduced cells or in the induced, stable transformants that carried the vector-only construct.

Using nickel ion-affinity chromatography, we successfully purified the recombinant protein after the supernatant was passed through the Ni-NTA affinity columns several times. The protein was essentially pure and had a molecular mass of 31–33 kDa on a 4–12% bis-Tris polyacrylamide gel as visualized by Gelcode® blue staining (Fig. 2). MS determined the *myc*/His-tag recombinant hK4 protein (which has a calculated molecular mass of 28 kDa) to have a molecular mass of 33 kDa. Positive identification of the tryptic peptides from this band by nanoelectrospray MS verified that this protein was indeed hK4; most notably, the product ion spectrum identified 13 distinct peptide fragments from 7 to 21 amino acids long that had 100% identity to the known hK4 sequence (data not shown).

PRODUCTION OF ANTI-hK4 ANTIBODIES

The mouse and rabbit antisera demonstrated increasing hK4 immunoreactivity (higher titers) with an increasing number of injections (data not shown). No immunoreactivity significantly higher than background (the 60 g/L BSA solution) was noted when preimmune rabbit or mouse serum was substituted for the respective immune serum. We used the final mouse and rabbit blood samples (after six immunizations) to establish the hK4 immunoassay as described below.

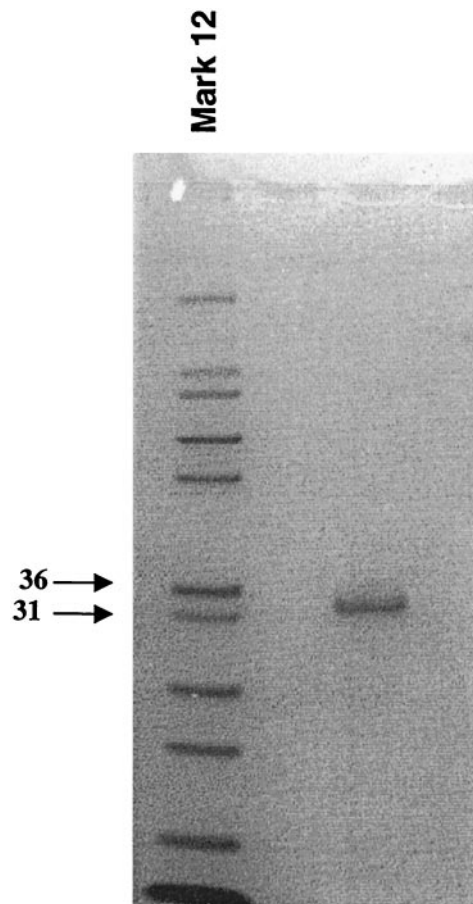


Fig. 2. Gelcode blue staining of concentrated hK4 recombinant protein, purified on nickel ion-affinity spin columns.

A single band is visible at ~33 kDa (nonreduced sample). Mark 12 is a molecular weight ladder.

hK4-SPECIFIC IMMUNOASSAY

The hK4 immunoassay was optimized as described in *Materials and Methods*. The purified, recombinant hK4 protein stock solution (24 mg/L) was diluted further with a 60 g/L BSA solution to various concentrations, and the six calibrators chosen were 0, 0.1, 0.5, 1, 5, and 20 μ g/L. Over this range, the assay showed a strong, linear relationship (Fig. 3). The imprecision of the assay (CV) was <10% within the measurement range, as assessed with quality-control materials prepared by addition of recombinant hK4 protein to a 60 g/L BSA solution ($n = 12$). The lower limit of detection, defined as the concentration that can be distinguished from zero with 95% confidence (mean + 2 SD of zero calibrator), was 0.1 μ g/L.

The cross-reactivity of our assay with other kallikreins was determined by the use of purified, recombinant hK2 (250 μ g/L), hK3 (PSA; 1000 μ g/L), hK5 (1000 μ g/L), hK6 (2500 μ g/L), hK7 (1000 μ g/L), hK8 (1000 μ g/L), hK9 (1000 μ g/L), hK10 (1000 μ g/L), hK11 (1000 μ g/L), hK12 (1000 μ g/L), hK13 (1000 μ g/L), and hK14 (1000 μ g/L). In all cases, cross-reactivity was <1%. In addition, we used the supernatant of the fusion protein hK5 with the *myc*/

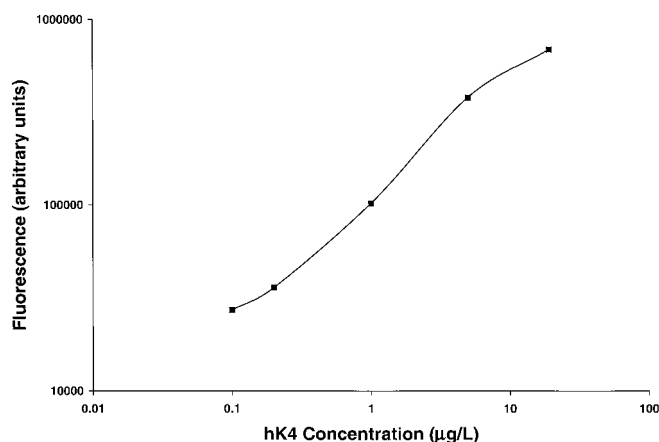


Fig. 3. Calibration curve of the hK4-specific immunoassay.

hK4 concentrations are 0, 0.1, 0.2, 1, 5, and 20 $\mu\text{g/L}$. The detection limit is 0.1 $\mu\text{g/L}$. The identity of the band as hK4 was verified by nanoelectrospray MS.

His tag ($\sim 5000 \mu\text{g/L}$) and the supernatant that contained His-tagged heat-shock protein 90 to assess the His-tag cross-reactivity. We did not detect significant cross-reactivity from either the His or the *myc* epitopes (all readings were $<0.1 \mu\text{g/L}$). We also tested recombinant hK4, without the *myc*/His epitopes, which was produced in yeast by similar techniques; the assay was able to detect hK4 with or without the *myc*/His epitope equally well.

hK4 IN TISSUE EXTRACTS AND BIOLOGICAL FLUIDS

Tissue extracts. Eight adult tissue extracts (urether, lung, spleen, spinal cord, kidney, liver muscle, and thyroid) contained trace amounts of hK4 immunoreactivity (0.10–0.13 $\mu\text{g/L}$), whereas the other tissues (cerebellum, adrenal gland, skin, pituitary, testis, heart, trachea, small intestine, seminal vesicle, stomach, pancreas, colon, and bone) had hK4 concentrations that were below the detection limit of our assay. Similarly, most fetal tissue extracts (liver, thymus, kidney, stomach, lung, heart, intestine, cartilage, bone, adrenal gland, colon, muscle, ovary, pancreas, spleen, and urether) were negative for hK4; only fetal skin extract showed some positive reaction (0.15 $\mu\text{g/L}$). However, all three placental extracts tested positive for hK4 (range, 0.6–1.0 $\mu\text{g/L}$) compared with the corresponding umbilical cord extracts, in which hK4 was below the detection limit. Furthermore, hK4 was undetectable in all tested ovarian extracts (8 healthy, 7 benign, and 27 cancerous). Prostatic extracts are described in detail below.

Prostate cancer tissues. hK4 concentrations were assayed in 18 (unmatched) prostate cancer extracts. Among these extracts, 9 were negative for hK4, 3 were weakly positive at 0.06 $\mu\text{g/g}$ of total protein (TP), and 6 were clearly positive for hK4 (0.42–1.72 $\mu\text{g/g}$ of TP). Two samples had substantially higher concentrations of hK4: prostate cancer samples 7 (1.72 $\mu\text{g/g}$ of TP) and 10 (0.62 $\mu\text{g/g}$ of TP).

For these two samples, we performed gel-filtration liquid chromatography to establish the molecular mass of the immunoreactive protein. As shown in Fig. 4, peak hK4 concentrations were measured around a molecular mass of 30 kDa. We also measured PSA concentrations in five prostate cancer extracts and compared their values with the hK4 concentrations (Table 1). The results showed that PSA and hK4 concentrations do not correlate, which suggests that the measured hK4 concentrations are not the result of cross-reaction with PSA (known to be present at high concentrations in prostatic tissue extracts).

Matched healthy–cancerous prostatic tissues. The comparative hK4 concentrations per gram of total protein in the 21 matched prostate sample pairs are shown in Table 2. In 5 of the 21 pairs (24%), hK4 was undetectable in the cancerous tissues but detectable in their healthy tissue counterparts. In 52% of the pairs (11 of 21), there was no measurable hK4 in either the healthy or the cancerous extracts. On the other hand, in 14% of the pairs (3 of 21),

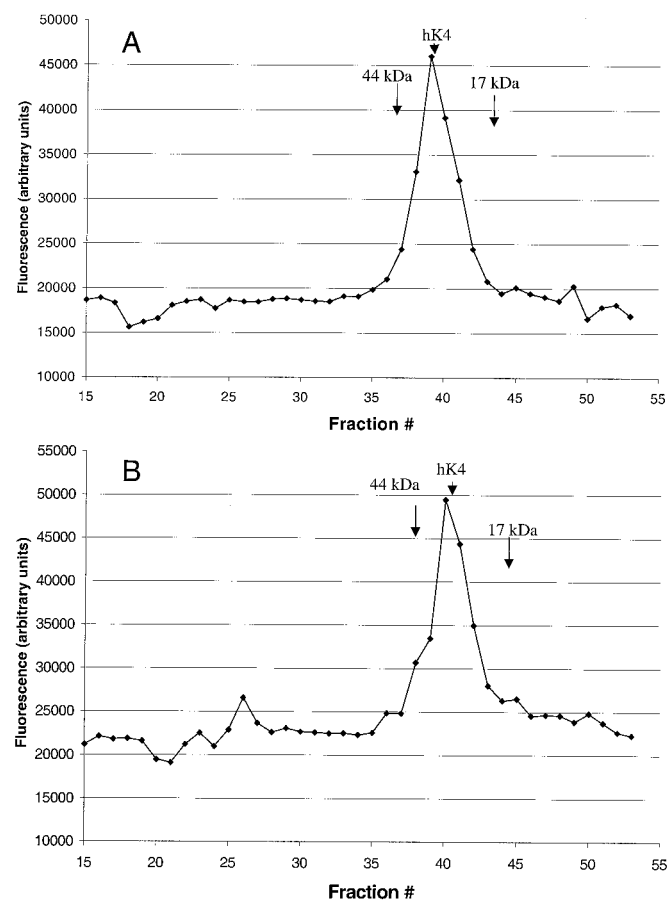


Fig. 4. Fractionation of hK4-positive prostate cancer cytosolic extracts 7 (A) and 10 (B).

The fluorescence measured in each fraction was plotted against the fraction number (Fractions 15–60). Major immunoreactive peaks eluted at a molecular mass of ~ 30 kDa. Arrows indicate elution of the molecular-mass standards.

Table 1. Comparison of PSA and hK4 expression in five prostate cancer tissue extracts.

Prostate cancer extract (unmatched series)	PSA, $\mu\text{g/L}$	hK4, $\mu\text{g/L}$
7	1.0×10^5	6.7
10	0.2×10^5	2.4
16	0.2×10^5	0
17	0.9×10^5	0
18	1.9×10^5	0

hK4 was expressed in both the healthy and the cancerous prostatic extracts, whereas in only 2 of the 21 pairs was hK4 protein found in cancerous tissue but not in its healthy-tissue counterpart.

Biological fluids. Among the biological fluids tested with this assay, hK4 was undetectable in cerebrospinal fluid ($n = 16$). Seventeen amniotic fluid samples were weakly positive for hK4 ($0.1\text{--}0.6 \mu\text{g/L}$), whereas six had hK4 concentrations below the detection limit ($0.1 \mu\text{g/L}$). Fractionation of an amniotic fluid sample with a relatively high hK4 concentration revealed a single peak at the expected molecular mass of hK4 (Fig. 5). Of six breast milk samples, four were positive ($0.2\text{--}0.75 \mu\text{g/L}$) and two were negative for hK4. Analysis of six seminal plasma samples showed weak immunoreactivity in all of them, with values from 0.10 to $0.93 \mu\text{g/L}$. The recovery of hK4 in serum was $36\text{--}37\%$ in male serum sample 1, $28\text{--}29\%$ in male serum sample 2, 0% in female serum sample 1, and $14\text{--}17\%$ in female serum sample 2.

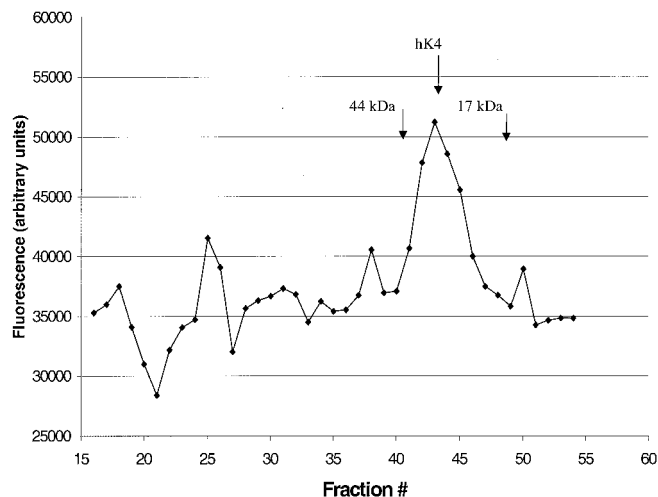


Fig. 5. Fractionation of one hK4-positive amniotic fluid sample by size-exclusion gel-filtration chromatography.

The fluorescence measured in each fraction was plotted against the fraction number (Fractions 15–60), and the flow rate was 0.5 mL/min . Other details are described in *Materials and Methods*. The major immunoreactive peak eluted at fractions 42–44, which correspond to a molecular mass of $\sim 30 \text{ kDa}$. Arrows indicate the elution position of two molecular-mass standards (44 and 17 kDa).

Table 2. hK4 protein concentrations in matched (healthy/cancer) prostate tissue extracts.

	hK4 protein presence (+) or absence (–) in healthy/cancer tissue (N/C)			
	+/+ (N/C)	–/–	+/–	–/+
hK4, $\mu\text{g/g}$ of TP	0.13/0.02 0.03/0.03 4.62/1.22	0.00/0.00 ^a (all)	0.05/0.00 0.06/0.00 0.03/0.00 0.06/0.00 0.02/0.00	0.00/0.03 0.00/0.07
n ^b	3	11	5	2

^a hK4 content as $0.00 \mu\text{g/g}$ indicates that hK4 concentration in the extract was below the detection limit of the assay ($0.1 \mu\text{g/L}$).

^b n, number of pairs in each category. Note that determination of hK4 per gram of TP was necessary because the wide range of TP concentrations in the extracts ($2.36\text{--}4.56 \text{ g}$ in healthy and $2.47\text{--}5.14 \text{ g}$ in cancer).

KLK4 mRNA IN MATCHED HEALTHY VS CANCEROUS PROSTATE TISSUES

Examples of the results of *KLK4* mRNA expression by RT-PCR in five pairs of matched prostate tissue samples are shown in Fig. 6. In 12 of 20 pairs (60%), the *KLK4* band was more intense in cancerous tissue than in its matched, healthy counterpart. In 5 of 20 pairs (25%), it was less intense in the cancerous tissues, in 2 pairs there was no difference, and in 1 pair there was no detectable RT-PCR product in either healthy or cancerous tissue. The absence of RT-PCR-detectable mRNA expression was correlated with undetectable hK4 in the protein extract of the corresponding prostate tissue. We did not detect a significant correlation between hK4 protein concentration and the intensity of the RT-PCR product band.

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We localized hK4 protein immunohistochemically in both hyperplastic (Fig. 7, A–C) and cancerous (Fig. 7D) glandular epithelial cells. The staining was cytoplasmic and most pronounced apically (Fig. 7, A and B). In negative control slides on which the primary anti-hK4 antibody was substituted with preimmune rabbit serum, no cytoplasmic staining above background was observed (not shown).

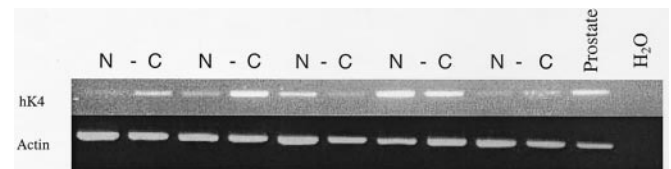


Fig. 6. *KLK4* mRNA expression by RT-PCR in five healthy (N) and cancer (C) matched prostate-tissue pairs.

A positive (prostatic cDNA; *Prostate*) and a negative (H_2O) control are also shown. $\beta\text{-Actin}$ was amplified from the same cDNA samples as a control for cDNA quality. We examined a total of 20 pairs. Higher *KLK4* expression in cancer was found in 12 of 20 pairs; lower expression in cancer was found in 5 of 20 pairs; and no difference was found in 3 of 20 pairs. For a more complete discussion, see the text.

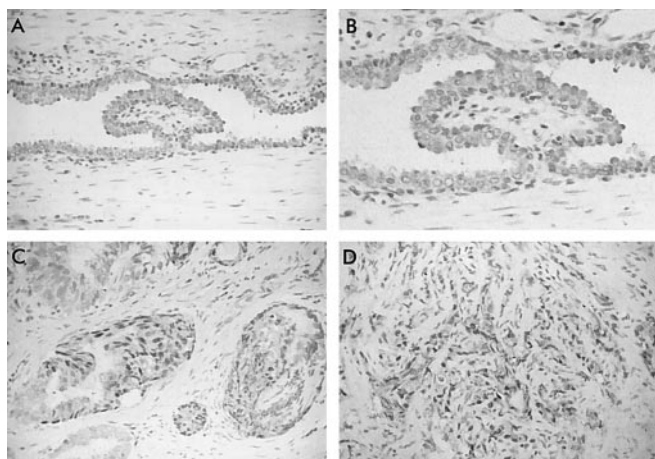


Fig. 7. Immunohistochemical staining for hK4 protein with a rabbit polyclonal antibody raised against full-length hK4 protein (dilution, 1:1000).

(A and B), stained prostatic ductal epithelial cells, most pronounced apically. (C), stained areas in hyperplastic glandular epithelial cells. (D), stained invasive, glandular epithelial cells of a prostatic adenocarcinoma. All magnifications are $\times 400$.

Discussion

KLK4 (prostase/*KLK-L1*) is one of the newest members of the human kallikrein gene family (1, 2). Despite extensive characterization of the gene and its mRNA (3, 5, 9, 10), no studies have yet examined the encoded protein, hK4. Only one group studied hK4 protein expression in response to estrogen and progesterone in endometrial cancer cell lines (20). The lack of protein studies is attributable to the unavailability of either recombinant hK4 protein or specific antibodies that would facilitate its detection. Therefore, we undertook this study to develop recombinant hK4 protein, antibodies to hK4, and a highly sensitive immunologic assay for hK4 quantification in tissues and fluids. We produced hK4 protein either as a fusion protein with *myc*/His epitopes or without any additional sequences. The protein was highly purified, and its sequence was verified by nanoelectrospray MS. Because MS determined a higher molecular mass for the *myc*/His recombinant hK4 than we had on the basis of its amino acid sequence, the protein is most likely N-glycosylated at residues 145–147 (NVS).

We used this protein to develop polyclonal mouse and rabbit antibodies suitable for immunologic studies. The assay has a detection limit of $0.1 \mu\text{g/L}$, and it appears highly specific for hK4, because BSA and other biological fluids that were augmented with relatively high concentrations of 12 highly homologous kallikreins (hK2, hK3, and hK5–hK14) did not produce any significant cross-reactivity. However, we cannot exclude the possibility that some cross-reactivity with kallikreins hK1 and hK15 may exist. In addition, an irrelevant protein that contained the same His-tag epitope as hK4 remained undetectable at a wide range of concentrations in BSA. This result indicates that our polyclonal antibodies used in this assay are highly specific for the hK4 portion of the

recombinant protein. Moreover, the assay recognizes non-tagged hK4 indiscriminately from the His-tagged version. Previous studies have demonstrated relatively high expression of *KLK4* mRNA, as detected by Northern blotting, in prostatic tissue (3). We have also reported the presence of *KLK4* mRNA in some other tissues but at lower concentrations than in the prostate.

On the basis of knowledge of other prostatic tissue kallikreins (hK2 and hK3), we expected relatively high concentrations of hK4 in prostatic tissue and in prostatic secretions (e.g., seminal plasma). Although we did find traces of hK4 immunoreactivity in some other human tissues, we observed the highest concentrations of hK4 protein in the prostate, but the amounts were relatively low. Moreover, many prostatic extracts were negative for hK4. The hK4 concentration was also very low in seminal plasma ($<1 \mu\text{g/L}$). The concentration of PSA is $>10^4$ times higher than that of hK4 in prostatic extracts, as assessed by this assay (Table 1). The seminal plasma hK4 concentration is $\sim 10^6$ - and 10^4 -fold lower than the concentration of PSA and hK2, respectively. [These comparisons are based on our data and published concentrations of hK2 and PSA in seminal plasma (21)]. These data indicate that hK4 protein concentrations in prostatic tissue, as assessed by this assay, are much lower than expected from the corresponding *KLK4* mRNA abundance. In addition, the hK4 concentration was unexpectedly low in seminal plasma. Given that the hK4 content was low in tissues and biological fluids, we cannot exclude the possibility that recombinant hK4 may be immunologically different from its native counterpart. Other possible reasons for the difference in hK4 and *KLK4* amounts include the following: (a) efficient *KLK4* gene transcription but inefficient translation; (b) efficient *KLK4* gene transcription but rapid degradation of *KLK4* mRNA; (c) rapid degradation of hK4 protein shortly after its synthesis; and (d) an intracellular location for hK4 and very low secretion rates into seminal plasma. These possibilities merit further investigation. We also identified traces of hK4 immunoreactivity in a few other biological fluids, including breast milk and amniotic fluid. Our assay is not sensitive enough to reliably quantify hK4 in sera from either healthy individuals or patients with prostate cancer (data not shown). Because the recovery of hK4 from serum was very low, it is possible that native hK4, at least in serum, is bound by serine protease inhibitors, which causes an underestimation of hK4 concentrations by our polyclonal assay. Other kallikreins, including hK2 and hK13, are already known to bind to proteinase inhibitors in serum.

Immunohistochemical localization of hK4 indicates that this protein is present in the cytoplasm of healthy, hyperplastic, and cancerous prostatic tissues, with most of the staining occurring apically (Fig. 7). At the mRNA level, we found that *KLK4* expression was higher in the majority of prostatic cancer tissues in comparison with matched healthy control tissues. In contrast, hK4 protein

concentrations were frequently higher in the matched, healthy tissue. This apparent discrepancy between mRNA and protein contents is difficult to explain; clearly, more studies are necessary.

The usefulness of PSA and hK2 testing for prostate cancer diagnosis and monitoring is well known (21–23). More studies will be necessary to assess the value of hK4 as a prostatic cancer biomarker. The data from our laboratory and another group have recently suggested that *KLK4* expression is associated with advanced ovarian carcinomas that have an unfavorable prognostic outcome (11,12). However, we could not detect hK4 protein in ovarian cancer tissue extracts. Thus, similar to our findings in prostatic tissue, *KLK4* mRNA is readily detectable in ovarian cancer, but hK4 protein is not.

In conclusion, we report for the first time the production of recombinant hK4 protein, the production of anti-hK4 antibodies, and the development of immunologic assays for quantifying hK4 protein. Although hK4 content was often higher in the healthy counterpart than in the cancer tissue of the matched healthy–cancerous prostate tissue pairs, we perceived no statistically significant difference in hK4 concentrations between healthy and cancerous tissues. Although we found the highest concentrations of hK4 protein in healthy and cancerous prostatic tissue extracts, these concentrations were unexpectedly low, and the protein was not found in appreciable amounts in seminal plasma. The physiology and pathobiology of hK4 protein and its value as a disease biomarker merit further investigation.

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