Development of a Dual Monoclonal Antibody Immunoassay for Total Human Kallikrein 2

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Background: Human kallikrein 2 (hK2) shares 80% sequence identity with prostate-specific antigen (PSA). Because both hK2 and hK2-α1-antichymotrypsin (hK2-ACT) complexes have been identified in patient sera, we devised an immunoassay for total hK2 (thK2; hK2 and hK2-ACT) and evaluated it in healthy subjects and patients with prostate disease.

Methods: We developed monoclonal antibodies (mAbs) with high specificity for hK2 and hK2-ACT and minimal cross-reactivity to PSA. Using these mAbs, a sandwich assay was developed and its specificity for forms of hK2 was assessed. Serum samples (n = 1035) from healthy volunteers, patients with increased PSA, and men who had undergone radical prostatectomy were assayed for thK2. We also measured thK2 in samples before and after storage under common laboratory conditions.

Results: The minimum detectable concentration in the thK2 assay was 0.008 μg/L, and PSA cross-reactivity was <0.001%. The assay detected prohK2 and three different hK2–serum protease complexes. The median serum concentration of thK2 in control samples (0.013 μg/L) was significantly lower than the median in samples from patients with increased PSA concentrations (0.085 μg/L). Immunoreactive hK2 changed little in samples stored for up to 1 month at −70 °C.

Conclusions: The thK2 assay recognizes all forms of hK2 that have been found in bodily fluids to date.

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Prostate-specific antigen (PSA)3 is a serine protease, secreted from prostatic epithelial cells, that has been used extensively as a serum prostate cancer marker. The properties and use of PSA have been extensively reviewed (1–5). PSA exists as two major immunologically distinct forms in serum: (a) as uncomplexed, commonly referred to as free PSA (FPSA), and (b) as PSA-α1-antichymotrypsin (PSA-ACT). PSA is a member of the human kallikrein protein family. Three well-known human kallikreins exist, and many new human kallikreins have recently been discovered (6). One of the well-known family members is human kallikrein 2 (hK2), which shares 80% sequence identity with PSA, as well as the important properties of organ localization and hormonal regulation (1, 3, 5, 7–9).

Both hK2 and PSA have been found in other biological fluids besides the prostate, and a good correlation has been established for these two proteins in these tissues (10–12). Immunohistochemical studies of prostate tissue sections using a monoclonal antibody (mAb) specific for hK2 show that the number of cells staining positively for hK2 is highest in prostate cancer, less in both prostatic intraepithelial neoplasia and benign hyperplastic tissue, and lowest in healthy tissue (9). In contrast, PSA stains more weakly in section areas containing prostate cancer cells than in those containing benign disease. These data indicate that hK2 is more associated with tumors than PSA.

Several hK2 forms are known to exist in bodily fluids (13–17), and we have constructed an immunoassay and tested its ability to recognize a variety of these forms. Because the assay has the ability to recognize all forms of

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3 Nonstandard abbreviations: PSA, prostate-specific antigen; FPSA, free PSA; PSA-ACT, PSA-α1-antichymotrypsin; hK2, human kallikrein 2; mAb, monoclonal antibody; thK2, total hK2; CG, cathepsin G; PRP, post-radical prostatectomy; phK2, mutant prohK2 with alanine changed to valine; hK2-PCI, hK2-protein C inhibitor; hK2-PAI-1, hK2-plasminogen activator inhibitor type 1; hK2-PAI-6, hK2-protease inhibitor 6; rPSA, recombinant PSA; and MDC, minimum detectable concentration.
hK2 currently known to us, we have named it the total hK2 (thK2) assay. Whether hK2 is useful as a tumor marker for prostate cancer in conjunction with PSA and FPSA is currently the subject of intensive study. Measurement of serum hK2, including measurement of thK2 using the assay we describe here, improves discrimination of benign disease from prostate cancer in conjunction with PSA and percentage of FPSA (18–21). Provided here are the details of the performance and characteristics of the thK2 assay. This analysis and the knowledge of the forms recognized by the assay may help in the interpretation of the clinical results found thus far and aid in establishing the clinical utility of hK2.

Materials and Methods

CHEMICALS, REAGENTS, BUFFERS, AND IMMUNOASSAY REAGENT SETS

GammaBind Plus Sepharose was purchased from Pharmacia Biotech. Streptavidin microtiter plates were purchased from Wallac Oy or Labsystems. Tandem®-R PSA Zero Calibrator/Specimen Diluent (zero diluent), Tandem-R, and Tandem-MP PSA and FPSA assay reagent sets were obtained from Hybritech Inc., a subsidiary of Beckman Coulter, Inc. ACT and cathepsin G (CG) were purchased from Athens Research and Technology, Inc. N-acetyl-d,l-homocysteine thiolactone and o-phenylenediamine were purchased from Sigma. Goat anti-mouse γ-specific-horseradish peroxidase conjugate was purchased from Jackson ImmunoResearch Laboratories, Inc. The europium labeling reagent set, assay buffer, and enhancement solution were purchased from Wallac Oy. NHS-LC-biotin II was purchased from Pierce Chemical Co. mAbs were labeled with N-hydroxysuccinimidyl-long chain-biotin II and europium according to recommendations from the manufacturer. Poly Mak 33 was obtained from Boehringer Mannheim. Protein C inhibitor was a generous gift from Drs. Mary Jo Heeb and John Griffin (The Scripps Research Institute, La Jolla, CA). All human serum samples were obtained with institutional review board approval. Serum samples from healthy males and females, post-radical prostatectomy (PRP) patients, or patients with increased PSA were obtained from our serum sample collection or were provided by Dr. William Catalona of Washington University in St. Louis, MO. The samples were collected with the approval of each institution’s review board, and all subjects gave informed consent. Serum samples from healthy males (mean age, 35 years) had a mean PSA of 0.6 μg/L. Samples were measured with Tandem-R or Tandem-MP PSA and FPSA assays (Hybritech).

PREPARATION OF PURIFIED PROTEIN CALIBRATORS

Purification of recombinant wild-type hK2 and the mutant prohK2, prohK2A217V (phK2), from conditioned media has been described previously (22, 23). The phK2 is a mutated form of prohK2 with alanine 217 changed to valine. PSA was purified from seminal fluid by established methods (24, 25). Recombinant PSA was prepared as described previously (26). CG-ACT was prepared by reacting 100 μg of CG with 1 mg of ACT, confirming complex formation with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and assigning values from the molecular weight of the complex assuming 100% recovery of CG as final CG-ACT complex. The concentration of hK2 was determined by absorbance at 280 nm (absorbance of 1.97 = g/L hK2) (27). hK2-protein C inhibitor (hK2-PCI) and hK2-plasminogen activator inhibitor type 1 (hK2-PAI-1) were prepared as described previously (28).

Matched hK2 and hK2-ACT, hK2-PCI, hK2-PAI-1, and hK2-P16 calibrators were prepared as described previously (29) by the incubation of equal amounts of hK2 with buffer or a 2- to 10-fold excess of the indicated protease inhibitor for 3 h at 37 °C. At the end of the incubation, the inhibitor mixtures typically displayed <2% of starting enzymatic hK2 activity and >90% complexed hK2 when assessed by size exclusion chromatography. Because the same amount of hK2 was added to both the hK2 and the hK2 complex tubes, the samples were, by definition, matched on a molar basis. Matched hK2 and hK2-ACT stocks for calibrator preparation and other hK2-protease complexes were diluted to 10 g/L into 0.2 mol/L Tris (pH 8) and 5 mmol/L EDTA containing 1 g/L BSA. Autodigested hK2 was prepared by incubating active hK2 at 0.119 g/L in pH 8 Tris buffer at 37 °C for 60 h. The hK2 was 70% cleaved at amino acids 102, 145, and 226.

PREPARATION AND VALUE ASSIGNMENT OF hK2 CALIBRATOR lots

One lot of hK2 was value assigned by amino acid analysis and laser desorption mass spectrometry as described previously (27). The molecular weight of hK2 was 28 460. This lot of hK2 was used in the thK2 microplate assay to value assign all subsequent lots of hK2 that were used as calibrators.

PREPARATION OF mAbs to hK2-act

Murine mAbs to hK2-act were obtained using standard protocols of immunization and hybridoma production with some modifications (30). Hybridomas were screened with biotinylated antigen ELISAs. mAbs were selected based on reactivity to both hK2 and hK2-act and on lack of reactivity to PSA. Antibodies were purified from ascites or culture supernatant with GammaBind Plus Sepharose.
washing, color was developed for 30 min with o-phenylenediamine solution, the color reaction was stopped with H$_2$SO$_4$, and the color intensity was then measured at 490 or 540 nm.

**Protocol for thK2 Assay**
The biotinylated capture mAb (100 µL; 5 mg/L) was incubated with the calibrator or sample (50 µL) for 2 h with shaking in a streptavidin microtiter plate. The plate was washed and the europium-labeled detection mAb (100 µL, 1 mg/L) was added and allowed to incubate with shaking for 2 h. Heat-treated mouse sera (0.05 g/L IgG) and Poly Mak 33 (0.1 g/L) were included in the incubation mixtures to minimize interference with human-anti-mouse antibodies and heterophilic antibodies that may have been present in the samples. The plate was washed again, and enhancer solution was added and allowed to incubate for 5 min. Time-resolved fluorescence was measured in a 1234 Delfia® research fluorometer (Wallac, EG&G).

**Determination of Reactivity to hK2 Forms and Autodigested hK2 and Cross-reactivity to Potential Interfering Proteins**
Different concentrations of hK2, autodigested hK2, phK2, hK2-ACT, hK2-PCI, hK2-PAI-1, hK2-protease inhibitor 6 (hK2-PI6), PSA, recombinant PSA (rPSA), PSA-ACT, CG-ACT, and ACT were measured in the assay to determine the relative reactivity ratios among the forms. Several dilutions of the indicated analytes were assayed in triplicate. The slope of the test analyte divided by the slope of hK2 across the concentration range was the mean reactivity ratio reported for the hK2 forms. The slope ratio of the cross-reacting analytes divided by hK2 multiplied by 100 was reported as the percentage of cross-reactivity for the given non-hK2 analyte. The concentrations for the highest dilution tested for each analyte tested were as follows: phK2 and hK2-ACT, 3 µg/L; autodigested hK2, hK2-PCI, hK2-PAI-1, 100 µg/L; PSA, rPSA, PSA-ACT, and CG-ACT, 10 000 µg/L; and ACT, 100 000 µg/L. The hK2-ACT/hK2 ratio was also measured at 0.1 µg/L in five separate assays, and the mean ratio was calculated.

**Determination of Detection Limit**
The minimum detectable concentration (MDC) was assessed by assaying five samples (0.387–32.4 µg/L thK2) that were serially diluted 1:2 three times in zero diluent. Linearity was assessed by diluting 2.3 and 7.6 µL of a serum sample, which had a high concentration of thK2 (31.9 µg/L), into 0.2 mL of 5 different serum samples with concentrations of thK2 that were 0.000–0.152 µg/L. The measured and expected values of the high thK2 sample were compared.

**Determination of thK2 Stability**
A stability study was performed on serum samples. We also assayed the same samples with thK2, Hybritech’s Tandem-R PSA, and Tandem-R FPSA assays. Blood was drawn from 12 men who had signed an informed consent form that was approved by the Institutional Review Board. The blood was allowed to clot for 30 min. The serum was removed and aliquots were immediately placed on wet or dry ice. The fresh, unfrozen serum samples were assayed within 4.5 h of the blood draw (time zero). We assayed the freshly prepared serum and compared it with serum stored at the following temperatures for the following lengths of time: (a) room temperature for 1 day; (b) 4 °C for 1 day and 1 week; and (c) −70 °C for 1 day, 1 week, and 1 month.

**Regression Analysis**
The regression line drawn in the Fig. 1 was calculated with the Deming method. The Deming slope and intercept and the correlation coefficient (±95% confidence interval) are reported in the legend of Fig. 1.

**Results**

**Development of the thK2 Assay**
The optimum mAb pair was selected for the thK2 sandwich assay as described previously (29), except that the mAbs were tested for the signal produced in the presence
of both hK2 and hK2-ACT. The mAb pair that gave the highest sensitivity while still having similar reactivity for hK2 and hK2-ACT was chosen. This assay was then tested on different concentrations of hK2.

RECOGNITION OF hK2 AND PSA
The calibration curves were similar with both hK2 and hK2-ACT (Fig. 2A), and the ratios of hK2-Pl6/hK2, hK2-PCI/hK2, and hK2-PAI-1/hK2 were 1.0, 1.2, and 1.3, respectively. The ratio of hK2-ACT/hK2 with the thK2 assay was 0.5–0.7 with a mean of 0.6. The ratio of phK2/hK2 was 1.1. The autodigested hK2/hK2 ratio was 0.5–0.6. The cross-reactivities of relevant analytes tested in the assay were as follows (Fig. 2B): PSA, 0.52%; rPSA, <0.001%; PSA-ACT, 0.07%; CG-ACT, <0.001%; and ACT, <0.001%.

EDUCATION OF PRECISION, LINEARITY AFTER DILUTION, AND RECOVERY
The mean value after dilution was 116% of expected (range, 48–149%). The high and low values were seen at the highest dilution (1:8). The mean recovery was 74% (range, 49–99%). The precision for pool 1 was evaluated over 14 assays over 4 months with 1 lot of calibrators and 3 different lots each of biotinylated and europium-labeled mAb. The precision for pool 2 was evaluated over nine assays over 2.5 months with one lot of calibrators, two different lots of biotinylated mAb, and three different lots of europium-labeled mAb. The within-run imprecision (SD) was 0.020 μg/L for pool 1 (mean concentration, 0.107 μg/L) and 0.014 μg/L for pool 2 (mean concentration, 0.047 μg/L). The total imprecision (SD) for pool 1 was 0.022 μg/L and for pool 2 was 0.015 μg/L.

MEDIAN thK2 CONCENTRATIONS ARE INCREASED IN PATIENTS WITH INCREASED PSA
Healthy males and females and serum from men PRP who had undetectable PSA gave a much lower signal in the assay than samples from men with increased PSA (Table 1). The median thK2 value for 975 patients was 0.008 μg/L, whereas the median PSA value for these same patients was 3.78 μg/L. These two medians and the slope from Fig. 1 indicate that among the patients with increased PSA, on average, the thK2 concentration was 2–4% of the concentration of PSA. The concentration of the two kallikreins in serum was significantly (P <0.0001) correlated.

SERUM thK2 IMMUNOREACTIVITY DOES NOT DECREASE IN SAMPLES STORED AT BELOW –70 °C FOR UP TO 1 MONTH
The median serum concentration of thK2 in the 12 stability study patients at time zero was 0.221 μg/L (range, 0.108–3.23 μg/L). The ratio of thK2/PSA in the samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>thK2, μg/L</th>
<th>Median</th>
<th>Range</th>
<th>n</th>
<th>Significantly different from PRP patients?</th>
</tr>
</thead>
<tbody>
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<td>PRP patients</td>
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<td>0</td>
<td>20</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Control females</td>
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<td>0–0.038</td>
<td>20</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Control males</td>
<td>0.013</td>
<td>0–0.064</td>
<td>20</td>
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<td>No</td>
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<tr>
<td>Men with PSA 2–10 μg/L</td>
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<td>0–1.160</td>
<td>929</td>
<td></td>
<td>Yes</td>
</tr>
</tbody>
</table>

a Total hK2 is increased only in patients with increased PSA.
b Dunnett’s method of means comparisons (α = 0.05).
was ~2% on both fresh samples and samples frozen at below ~70 °C for 1 week. Dunnett’s method (32) was used to determine whether the percentage of recovery from time zero for all samples for each of the three assays was statistically significant. The α used was 0.05. The data for PSA and FPSA were consistent with previously published data (33). Conditions significantly different from time zero for thK2 were as follows: 1 day at room temperature, 1 week at 4 °C, 1 week at below ~70 °C, and 1 month at below ~70 °C. Although significant, the changes were not very great (Table 2).

**Discussion**

hK2 can exist in the body in a variety of forms. Human prostate tumor tissue is known to contain hK2-Pf6 (13), human serum is known to contain the pro form of hK2 (14) and hK2-ACT (15), human seminal fluid has been shown to contain hK2-PCI (16, 17), and it has been documented that hK2 can readily form a complex with PAI-1 (28). Furthermore, free hK2 is theoretically composed of prohK2 and noncomplexable (inactive) hK2. Because several types of complexes and forms of hK2 may exist in the various bodily fluids, measurement of any of these forms has the potential of improving PSA and FPSA predictive values.

Because of the variety of potentially useful forms of hK2, we constructed an immunoassay that could recognize the many different forms of hK2. To achieve this, we raised mAbs against the hK2-ACT complex and screened the mAbs for similar reactivity on hK2 and hK2-ACT, while at the same time, eliminating clones that had cross-reactivity to PSA.

We tested our assay and found that it had very similar reactivity for hK2, phK2, hK2-ACT, hK2-PCI, hK2-Pf6, and hK2-PAI-1. Because the assay recognized all the forms of hK2 currently known by us to exist in bodily fluids, we have named it the thK2 assay.

The cross-reactivity of the thK2 assay for rPSA, which has no hK2 contamination, was <0.001%. We also measured the cross-reactivity of the assay for PSA purified from seminal fluid by standard methods and found a cross-reactivity of 0.52%. We believe the difference between the cross-reactivity of the rPSA and the PSA purified from seminal fluid is attributable to a small amount of hK2 that contaminates PSA preparations from seminal fluid. This hK2 contamination of PSA derived from seminal fluid has been reported previously (34).

We tested autodigested hK2 in the assay to determine how sensitive the assay would be to hK2 that was proteolytically degraded. We found that the recognition of hK2 was decreased by ~50% when hK2 was ~70% proteolytically cleaved. Therefore, if hK2 is proteolytically cleaved, it may also no longer be recognized by immunoassays developed against intact hK2. We do know that degraded forms of hK2 have been detected by Western analysis in seminal fluid and tissue homogenates (16) although clipped forms of hK2 have not been detected in serum (17). Nevertheless this is an issue that needs further attention to be assured that full recovery of all forms of hK2 is realized.

The concentration of thK2 was increased in patients in the clinically relevant PSA range of 2–10 µg/L compared with PRP patients with undetectable PSA concentrations, healthy males, and healthy females (Table 1). Furthermore the thK2 concentration was ~2–4% of the PSA concentration in 975 men with a PSA range of 2–20 µg/L. The correlation of thK2 to PSA was statistically significant.

Three other specific immunoassays for hK2, in addition to ours, have been reported in the scientific literature. An indirect immunoassay, reported by Becker et al. (36) uses one hK2 mAb (with 5% cross-reactivity to PSA) to capture hK2 and one mAb with equal reactivity to PSA and hK2 to detect hK2 while using 4 mAbs specific to PSA to inhibit cross-reaction of PSA in the assay. This assay described by Becker et al. (36), recognizes hK2 and hK2-ACT similarly. Two other assays for hK2 have been reported that use simple mAb formats (37, 38). The assay reported by Klee et al. (37) measures primarily free hK2. hK2-ACT is recognized to a lesser degree in the assays from Black et al. (38) (A. Magklara and E. Diamandis, personal communication) and Klee et al. (37). The clinical correlation of hK2 assays may be affected by the degree of recognition of complexed hK2 forms.

Preliminary studies performed with the Black et al. (38) hK2 assay on calibrators prepared in our laboratory have detected an approximately sixfold higher signal than our thK2 assay on the same calibrators (A Magklara and E Diamandis, unpublished results). If the median and percentage values of hK2/PSA are divided by 6 for the assay by Black et al. (38), the values are fairly close to the same parameters measured with hK2 assays from the other three laboratories. These data imply that the reason for a large portion of the discrepancy between the Black et al. (38) assay and the other three assays may be a calibration difference.

Data from clinical studies show that measurement of the different forms of PSA add specificity in distinguishing prostate cancer from benign prostatic disease and is

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**Table 2. Stability of serum thK2.**

<table>
<thead>
<tr>
<th>Length of storage</th>
<th>Temperature</th>
<th>thK2 recovery</th>
<th>FPSA recovery</th>
<th>PSA recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>Room temperature</td>
<td>115a</td>
<td>96</td>
<td>95</td>
</tr>
<tr>
<td>1 day</td>
<td>4°C</td>
<td>111</td>
<td>93</td>
<td>91a</td>
</tr>
<tr>
<td>1 day</td>
<td>Below ~70°C</td>
<td>110</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td>1 week</td>
<td>4°C</td>
<td>120a</td>
<td>74a</td>
<td>96</td>
</tr>
<tr>
<td>1 week</td>
<td>Below ~70°C</td>
<td>118a</td>
<td>93</td>
<td>98</td>
</tr>
<tr>
<td>1 month</td>
<td>Below ~70°C</td>
<td>120a</td>
<td>101</td>
<td>97</td>
</tr>
</tbody>
</table>

*a* Recovery significantly different from time zero by Dunnett’s method (32) of means comparisons (α = 0.05).
clinically useful in prostate cancer diagnosis and management (39–42). Human hK2 exists both free and complexed to ACT in human serum (15), analogous to the complex formation of PSA with ACT first described by Stenman et al. (43). Therefore, it is reasonable to expect that the ratio of the hK2 forms may also be important clinically. Although recognition of complexed hK2 forms may be important, precise recognition of free hK2 compared with hK2-ACT may not be as important as precise recognition of FPSA compared with PSA-ACT because of the relative amounts of the two kallikrein complexes that are found in the serum of relevant patients. The median ratio of hK2-ACT/hK2 with the thK2 assay was 0.6. Although the assay does not have a perfect 1.0 ratio of hK2-ACT/hK2, this has a minimal effect on the values in the current assay because the vast majority of the hK2 in serum appears to be uncomplexed. Even with an hK2-ACT/hK2 ratio of 0.6, a 100 ng/L sample that contained 75% complexed PSA would read 0.7 µg/L in the assay (a 30% decrease).

Both Black et al. (38) and Becker et al. (36) reported that hK2 is primarily free. Both groups assayed fractions from a total of eight patient samples after separation by size exclusion chromatography to discriminate free hK2 from hK2 complexes. If hK2 in serum is primarily free and only a small (<20%) portion is complexed, the forms recognition of hK2 assays may not be as important clinically as is the recognition of different PSA forms where the majority of PSA is complexed (80–90%).

Most patient samples we tested with the thK2 assay were serum samples stored at below −70 °C for 1–4 years. We began a stability study of thK2 with the assay and found that thK2 is stable in serum stored at below −70 °C for at least 1 month when compared with freshly drawn serum. Therefore samples can be measured soon after blood draw or stored frozen and the similar clinical results should be realized. It is still important to carry out the stability for longer periods of time to determine whether the immunoactivity of serum samples changes over months or years.

The thK2 assay described here has recently been used to test serum from 937 patients from two institutions (19). This study indicates that the ratio thK2FPSA may add additional specificity for cancer detection over PSA and %FPSA. In this type of scheme all three markers can be used to make the best decision about whether or not to biopsy particular patients. Therefore this assay for thK2 that has shown promise in aiding the discrimination of benign disease from prostate cancer should be helpful in further exploring this idea, as well as assessing the clinical utility of hK2 forms in serum and other bodily fluids.

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References


