Highly Sensitive Automated Chemiluminometric Assay for Measuring Free Human Glandular Kallikrein-2

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Background: Human glandular kallikrein (hK2) is a serine protease that has 79% amino acid identity with prostate-specific antigen (PSA). Both free hK2 and hK2 complexed to α1-antichymotrypsin (ACT) are present in the blood in low concentrations. We wished to measure hK2 in serum with limited contribution from hK2-ACT for the results.

Methods: We developed an automated assay for hK2 with use of a select pair of monoclonal antibodies. The prototype assay was implemented on a Beckman Coulter ACCESS® analyzer.

Results: The detection limit of the assay was 1.5 ng/L, the “functional sensitivity” (day-to-day CV <15%) was <4 ng/L, cross-reactivity with PSA and PSA-ACT was negligible, and cross-reactivity with hK2-ACT was 2%. After surgical removal of prostate glands, serum hK2 was <7 ng/L and was <15 ng/L in most healthy women. The median serum concentration of hK2 in healthy men without prostate cancer was 26 ng/L. The median concentration of hK2 was 72 ng/L for men having prostate cancer with lower Gleason scores compared with 116 ng/L for men with more advanced cancer. The concentration of hK2 correlated weakly with PSA, with the mean hK2 concentrations generally 30- to 80-fold lower than PSA concentrations.

Conclusion: The availability of a robust, high sensitivity automated assay for hK2 should facilitate further investigations of the role of hK2 measurements in the management of patients with prostate disease.

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Human glandular kallikrein (hK2)5 is a serine protease similar to prostate-specific antigen (PSA) (1, 2). There is ~79% identity in the amino acid sequences of these two molecules (3, 4). Like PSA, hK2 is localized in prostate tissue and seminal fluid and detected in serum and urine (5). Also like PSA, multiple forms of hK2 exist, including free forms of pro and mature and complexes with protease inhibitors such as protein C inhibitor and α1-antichymotrypsin (ACT). In common with hK1 and PSA, hK2 is initially produced with a 24-amino acid activation peptide (pphK2), which is cleaved proteolytically into prohK2 with a 7-amino acid leader sequence (phK2) and subsequent activation to the 237-amino acid mature protein (hK2). Serum concentrations of hK2 are substantially higher in patients with prostate disease compared with healthy controls; however, the role of this new marker in the management of men with prostate disease requires further investigation (6, 7). Preliminary studies indicate that serum hK2 concentrations are not proportional to PSA concentrations and, therefore, should provide additive information to PSA in managing patients with prostate disease.

The initial development of assays for hK2 posed an intriguing challenge: how to measure a compound that was postulated to exist on the basis of molecular sequencing of mRNA found in prostate disease, but whose protein form had not been isolated (1). Our initial steps were to synthesize peptides to the regions of the predicted hK2 molecule that were least homologous with the PSA molecule. Antisera raised from these peptides were used to develop a competitive assay that did not cross-react with PSA (8). However, this assay was not sensitive enough to measure hK2 in most human serum samples. Insertion of the cDNA of pphK2 into transformed cells led

5 Nonstandard abbreviations: hK2, human kallikrein 2; PSA, prostate-specific antigen; and ACT, α1-antichymotrypsin.
Materials and Methods

MONOCLONAL ANTIBODIES

We developed 10 monoclonal antibodies specific for hK2 by immunizing A/J mice with peptides to hK2, recombinant pHK2, recombinant phK2, and mature hK2 proteolytically activated from recombinant pHK2 (8–11, 13). Fusions were performed using P3.653 myeloma cells, and the clones were selected on the basis of strong binding to mature hK2 and negligible reactivity with PSA. Each of these 10 antibodies was both immobilized on polystyrene beads (Clifton) and labeled with acridinium C2 NHS ester obtained from Assay Designs (product no. 90600), using the method of Weeks et al. (14). Each of the 10 immobilized antibodies was incubated with hK2 at 0 and 215 μg/L, washed, reacted with the remaining nine sets of labeled antibodies, washed, and measured in a luminometer. The 24 antibody pairs with the highest signals were tested for linearity of hK2 dose–response and cross-reactivity to PSA. The two antibody pairs with the best signals and specificity were further evaluated for reproducibility.

ASSAY FORMAT

An automated immunometric assay was developed using the Beckman Coulter ACCESS® immunoassay system. Monoclonal antibody H449 was used to capture hK2, and monoclonal antibody H599 was used as the detection antibody. Both antibodies were purified using protein G-Sepharose and elution with 10 mmol/L glycine, pH 2.5. The capture antibody was conjugated to biotin at a 27:1 molar ratio of biotin to antibody (EZ-Link™ kit; Pierce). The detection antibody was labeled with alkaline phosphatase by the method of Ngo (15) using succinimidyl 4-(N-maleimidomethyl) 3-(2-pyridyldithio)-propionate.

In the optimized assay, paramagnetic particles coated with streptavidin (0.57 μg/L) were used to capture the bound antigen-antibody complexes on the ACCESS system. A two-step sequential assay format was used. In the first step, either 50 or 75 μL of serum was reacted with 100 ng of biotinylated H499 and 50 μL of paramagnetic particles at 37 °C for 30 min. Nonimmune mouse sera concentrate and Poly Mak33 concentrate (Boehringer Mannheim) were added to incubation mixtures to minimize interference with human anti-mouse antibodies. After washing, the paramagnetic particle-H449 antibody-hK2 complex was reacted with alkaline phosphatase-conjugated H599 detection antibody for an additional 30 min. The complex was again washed and reacted with substrate (dioxetane phosphate) to produce a chemiluminescent signal, which is quantified in a luminometer in terms of relative light units. Six calibrators (0, 10, 100, 1000, 3000, and 9000 ng/L) were made by the addition of recombinant mature hK2, prepared as described previously (16), into the zero diluent from the Tandem®-R PSA kit (Hybritech Incorporated, San Diego, CA).

ANALYTIC VALIDATION

We assessed linearity by serially diluting sera from nine patients individually with Tandem-R PSA kit zero diluent and plotting the product of the dilution ratio times the concentration vs the dilution ratio. We assessed recovery by adding 300 and 1000 ng/L hK2 from a high-concentration patient serum sample to five patient serum samples with low concentrations of hK2 and comparing the results with their baseline concentrations. Precision was assessed by repetitive measurement of three serum pools over 13 days. Cross-reactivity was assessed by measurement of Tandem-R zero diluent with added ACT (100 mg/L), PSA (10 mg/L), PSA-ACT (5 mg/L), cathepsin G-ACT (10 mg/L), prohK2 (670 μg/L), and hK2-ACT (10 μg/L). Purified ACT was obtained from Athens Research and Technology. PSA-ACT (17) and hK2-ACT (11, 18) were prepared as described previously. We prepared cathepsin G-ACT by reacting 100 μg of the enzyme with 1 mg of ACT, confirming complex formation with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the values assigned for the molecular weight of the complex assuming 100% recovery. The concentrations were assigned using the following specific absorbances at 280 nm for 1 g/L: 0.12 for ACT, 0.18 for PSA, and 0.20 for hK2 (13, 19, 20). The specific absorbances for hK2-ACT and PSA-ACT were calculated from the specific absorbances of hK2, PSA, and ACT. The low-end precision profile was determined by 20 measurements of pools with low concentrations of hK2. The minimum analytic detection limit was calculated as the dose corresponding to the mean + 2.5 SD of the zero calibrator. The functional sensitivity was calculated as the minimum concentration with imprecision <15%.

HUMAN STUDIES

Permission for the testing of specimens for these studies was approved by the Mayo Clinic Institutional Review Board (IRB no. 0015-92). The concentration of hK2 was measured in 13 healthy female volunteers 19–42 years of age and 26 female blood donors. Informed consent to collect serum for PSA and hK2 measurements was obtained from 70 men who subsequently had prostate cancer confirmed histologically and 39 men with a history of radical prostatectomy for prostate cancer and no evidence of tumor recurrence. The men with prostate cancer were divided into two groups; group I included 31 men in with Gleason scores of 3 + 3 or less and no evidence of metastasis; group II included 39 men in with Gleason scores greater than 3 + 3 and/or metastatic cancer. For group I, the age range was 50–88 years, with a mean age of 67.3 years; for group II, the age range was 52–85 years, with a mean age of 68.3 years. The concentrations of total
PSA and free PSA for these specimens were measured using the Hybritech Tandem-R PSA and Tandem-R free PSA kits. The concentration of hK2 was measured on the ACCESS instrument.

The distribution for hK2 in men without prostate cancer was determined by the measurement of a random sample of 256 men stratified by age from Olmsted County, MN (21). Each of these men had a digital rectal examination, transrectal ultrasound, and urine flow studies. All men with potential signs or symptoms of prostate cancer were further evaluated, and positive cases were eliminated from the study. The 95th percentiles (upper reference limits) for hK2 were determined both nonparametrically for the whole group and parametrically as a function of age. The age-dependent reference limits were determined by adding the residual variances to the linear regression curve of the logarithmically transformed hK2 values vs age (22). Both the logarithms of the hK2 values (P <0.001) and the residual scatter from this regression curve (P <0.003) were linearly dependent on age. Therefore, a linear model was used to estimate the residual variances as a function of age before adding them to the log-transformed regression line.

Spearman correlation coefficients between hK2 and maximum urine flow rate, ultrasound estimates of prostate volume, and the American Urologic Association prostate symptoms scores also were calculated.

We assessed the association between the concentrations of hK2 and PSA in 218 specimens for which PSA measurements had been clinically requested at the Mayo Clinic. These PSA concentrations were measured with the AxSym™ analyzer (Abbott Laboratories). The specimens remained frozen at −20 °C for 4 years between the measurements of PSA and hK2. These specimens were chosen to represent 10 ranges of PSA (Table 3). Because informed patient consent was not obtained for the use of these specimens, our Institutional Review Board required that the patient identification on these specimens be removed before hK2 testing; therefore, no further clinical information is available on these cases.

## Results

### Analytic Performance

The characteristics of the 10 monoclonal antibodies are summarized in Table 1. The two antibodies raised against hK2 peptides did not pair with any of the other antibodies. Similarly, the antibody raised against phK2 expressed in Escherichia coli did not pair well.

The signal-to-background ratios for the pairing of the other seven antibodies are shown in Table 2. Monoclonal antibodies H365 and H449 were the best capture antibodies, and antibody H599 was selected as the detection antibody. The H449 (capture)/H599 (detection) pair was chosen for assay development because of better reproducibility.

A two-step analytic procedure was developed because the extra wash step provided better analytic sensitivity. A typical dose–response curve for the assay is shown in Fig. 1. The signal-to-background ratio progressively increases from 1.3 at 10 ng/L to 624 at 9000 ng/L. The assay was calibrated with recombinant hK2. The assay performed well in terms of linearity, recovery, precision, cross-reactivity, and detection limit. The dilution linearity curves for nine patients are shown in Fig. 2. Seven of the nine patients showed excellent linearity, whereas two had an increase in relative concentration with dilution. Recovery of endogenous serum hK2 added to five patient samples at two concentrations was 87–114%, with a mean of 97%. The between-run imprecision (CV) for 13 analytical runs over 2 weeks was 4.3% at 47 ng/L, 5.3% at 520 ng/L, and 2.4% at 922 ng/L. Cross-reactivity was <0.0005% with ACT and cathepsin G-ACT on a weight basis. The cross-reactivities with PSA and PSA-ACT were 0.008% and 0.0009%, respectively. Our assay detected hK2-ACT at ~9% on a weight basis, which corresponds to

### Table 1. Characteristics of the monoclonal antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Immunogen</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>A523</td>
<td>IgG2a</td>
<td>hK2(41–56)</td>
<td>phK2, hK2</td>
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<td>B104</td>
<td>IgG1</td>
<td>ppK2</td>
<td>phK2, ppK2, hK2</td>
</tr>
<tr>
<td>D106</td>
<td>IgG1</td>
<td>hK2(17–71)</td>
<td>hK2</td>
</tr>
<tr>
<td>G586</td>
<td>IgG1</td>
<td>phK2</td>
<td>phK2, hK2</td>
</tr>
<tr>
<td>H247</td>
<td>IgG1</td>
<td>hK2</td>
<td>hK2</td>
</tr>
<tr>
<td>H286</td>
<td>IgG1</td>
<td>hK2</td>
<td>phK2, hK2</td>
</tr>
<tr>
<td>H365</td>
<td>IgG1</td>
<td>hK2</td>
<td>phK2, hK2</td>
</tr>
<tr>
<td>H449</td>
<td>IgG1</td>
<td>hK2</td>
<td>phK2, hK2</td>
</tr>
<tr>
<td>H463</td>
<td>IgG1</td>
<td>hK2</td>
<td>phK2, hK2</td>
</tr>
<tr>
<td>H599</td>
<td>IgG1</td>
<td>hK2</td>
<td>phK2, hK2</td>
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</table>

### Table 2. Paired antibody signal-to-background response at 215 μg/L hK2.

<table>
<thead>
<tr>
<th>Signal antibody</th>
<th>G586</th>
<th>H247</th>
<th>H286</th>
<th>H365</th>
<th>H449</th>
<th>H463</th>
<th>H599</th>
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<tr>
<td>G586</td>
<td>1</td>
<td>45</td>
<td>192</td>
<td>194</td>
<td>135</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>H247</td>
<td>20</td>
<td>1</td>
<td>275</td>
<td>187</td>
<td>71</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H286</td>
<td>250</td>
<td>2</td>
<td>769</td>
<td>605</td>
<td>448</td>
<td>302</td>
<td></td>
</tr>
<tr>
<td>H365</td>
<td>415</td>
<td>3</td>
<td>357</td>
<td>5</td>
<td>3</td>
<td>431</td>
<td></td>
</tr>
<tr>
<td>H449</td>
<td>549</td>
<td>5</td>
<td>624</td>
<td>2</td>
<td>2</td>
<td>872</td>
<td></td>
</tr>
<tr>
<td>H463</td>
<td>33</td>
<td>1</td>
<td>478</td>
<td>364</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H599</td>
<td>436</td>
<td>2</td>
<td>479</td>
<td>1275</td>
<td>1140</td>
<td>861</td>
<td></td>
</tr>
</tbody>
</table>
3% on a molar basis. The cross-reactivity with prohK2 is ~81% in this assay. The limit of detection was 1.5 ng/L, based on the signal 2.5 SD above zero. The low-end precision showed CVs of 4.6% at 40 ng/L, 13% at 4 ng/L, and 240% at 0.4 ng/L. Therefore, the functional sensitivity (with <15% CV) is <4 ng/L.

CLINICAL PERFORMANCE

Very low concentrations of hK2 were found in the female subjects. Twelve of the 13 healthy subjects had values ≤4 ng/L. The other subject had a value of 10 ng/L. Similarly, in 23 of 26 of the healthy blood donors, hK2 was ≤9 ng/L, and in the remaining 3, the values were 13, 14, and 37 ng/L.

Very low hK2 also was found in the 39 post-radical prostatectomy patients. All of these men had undetectable PSA (<0.1 µg/L) and undetectable free PSA (<0.1 µg/L). Most (34 of 39) of these men had undetectable hK2 (<4 ng/L), with the remaining 5 exhibiting values of 4, 5, 6, 7, and 7 ng/L.

The distribution of hK2 in the sera of men with prostate cancer is illustrated in Fig. 3. The men without metastatic disease and with lower Gleason scores as a group had lower hK2 concentrations compared with the men with more advanced cancer. The median hK2 concentration in group I was 72 ng/L (range, <4 to 352 ng/L) compared with a median of 116 ng/L (range, <4 to 2675 ng/L) in group II; however, the two groups had considerable overlap. The median PSA concentrations in the two groups were 7.4 and 9.3 µg/L, respectively.

Fig. 4 shows the hK2 values plotted against age for the sample of 256 healthy men without prostate cancer from Olmsted County. The age distribution and clinical characteristics of this subgroup were not statistically different from the larger sample used for the PSA reference interval study (21). For the overall group, the median hK2 concentration was 26 ng/L, the nonparametric upper reference limit was 81 ng/L, and the range was <4 to 246 ng/L. The mean serum hK2 concentration increased with age, as shown by the regression line of the logarithmically
transformed data \((P < 0.001)\). The parametric upper reference limit, depicted by the dashed line, also increases with age. For men without prostate cancer (but including men with benign prostatic hypertrophy), the upper reference limit increased from 59 ng/L at age 45 years to 201 ng/L at age 85 years.

The hK2 concentrations correlated weakly with maximum urine flow rates, prostate volume, and American Urologic Association symptom scores with Spearman correlation coefficients of \(-0.17, 0.39,\) and \(0.07,\) respectively. The corresponding correlation values for PSA and free PSA were slightly higher: \(-0.21, 0.60,\) and \(0.10\) for PSA, and \(-0.24, 0.63,\) and \(0.12\) for free PSA.

The relationship between hK2 and PSA is depicted in Fig. 5 and Table 3. The top panel in Fig. 5 shows that for men with PSA concentrations \(<4 \mu g/L\), hK2 correlates weakly with PSA, with a linear correlation coefficient of 0.564. There is a progressive increase in the mean value of the hK2 concentration from \(<4\) to 47.2 ng/L as the PSA group mean increases from \(<0.15\) to 3.42 \(\mu g/L\) (Table 3). Similarly, for the entire data set, PSA concentration is associated only weakly with hK2 concentration \((\rho = 0.551;\) Fig. 5). Throughout the range of PSA concentrations, the PSA values are \(-30\)- to 80-fold higher than the hK2 values, but PSA and hK2 are not highly correlated. Based on an \(r^2\) value of 0.30, only \(-30\)% of the variation of hK2 concentrations are predictable from the PSA variations. Therefore, hK2 may provide additional clinical information beyond that provided by PSA.

**Discussion**

The biosynthesis and tertiary structure of hK2 are similar to PSA \((2, 23)\). Both are produced intracellularly with prepro signal sequences and activation peptides, and both are secreted with activation peptides that serve to make the proforms of the molecules enzymatically inactive until the peptides are cleaved. The enzymatically active forms of both hK2 and PSA rapidly and irreversibly bind to serum protease inhibitors such as ACT and \(\alpha_2\)-macroglobulin \((16, 24)\). Endogenous complexes of PSA-\(\alpha_2\)-macroglobulin have been detected in human serum; however, it has been difficult to generate sensitive immunoassays that recognize PSA-\(\alpha_2\)-macroglobulin \((25, 26)\). This is because when proteases bind to the \(\alpha_2\)-macroglobulin inhibitor, they are then shielded from antibody binding \((22)\). We expect the case to be similar for complexes of hK2-\(\alpha_2\)-macroglobulin.

In human sera, the relative concentration of PSA-ACT is 3- to 10-fold higher than the concentration of free PSA and the ratio of free PSA to total PSA provides additional specificity for prostate cancer. Therefore, it is important

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Range (PSA, (\mu g/L))</th>
<th>Mean (PSA)</th>
<th>Range (hK2, ng/L)</th>
<th>Mean (hK2)</th>
<th>PSA/hK2, mass ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>15</td>
<td>(\leq 0.15)</td>
<td>(\leq 0.15)</td>
<td>(&lt;4)</td>
<td>(&lt;4)</td>
<td>30.7</td>
</tr>
<tr>
<td>II</td>
<td>24</td>
<td>0.15-0.99</td>
<td>0.57</td>
<td>(&lt;4-12)</td>
<td>(&lt;4-36)</td>
<td>30.0</td>
</tr>
<tr>
<td>III</td>
<td>26</td>
<td>1.0-1.9</td>
<td>1.43</td>
<td>5-107</td>
<td>32.9</td>
<td>43.5</td>
</tr>
<tr>
<td>IV</td>
<td>16</td>
<td>2.0-2.9</td>
<td>2.43</td>
<td>6-123</td>
<td>46.5</td>
<td>52.3</td>
</tr>
<tr>
<td>V</td>
<td>15</td>
<td>3.0-3.9</td>
<td>3.42</td>
<td>7-101</td>
<td>47.2</td>
<td>72.5</td>
</tr>
<tr>
<td>VI</td>
<td>21</td>
<td>4.0-6.9</td>
<td>5.03</td>
<td>14-154</td>
<td>66.2</td>
<td>76.0</td>
</tr>
<tr>
<td>VII</td>
<td>29</td>
<td>7.0-9.9</td>
<td>8.38</td>
<td>10-275</td>
<td>102.2</td>
<td>82.0</td>
</tr>
<tr>
<td>VIII</td>
<td>23</td>
<td>10.0-19.9</td>
<td>13.07</td>
<td>13-1264</td>
<td>199.6</td>
<td>65.5</td>
</tr>
<tr>
<td>IX</td>
<td>34</td>
<td>20.0-199</td>
<td>122.0</td>
<td>145-24 450</td>
<td>3575</td>
<td>34.1</td>
</tr>
<tr>
<td>X</td>
<td>15</td>
<td>200-1000</td>
<td>392.3</td>
<td>626-25 073</td>
<td>9672</td>
<td>40.6</td>
</tr>
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</table>
that total-PSA assays recognize free PSA and PSA-ACT equally (27). On the other hand, the concentration of hK2-ACT appears to be much lower than the concentration of free hK2 (24). The assay described here has a very low (3%) cross-reactivity with hK2-ACT, making this assay essentially an assay for “free hK2”.

The very low concentration of hK2 in female sera and in sera from males, after radical prostatectomy provide strong evidence for the specificity of hK2 as a marker for the prostate. However, like PSA, hK2 is not a marker that is unique for prostate cancer, in that the concentrations in men with benign prostate disease are substantially higher than the concentrations in subjects without prostate disease. In addition, the age dependency of hK2 suggests that, like PSA, this marker increases with hypertrophy of the prostate gland.

The median concentration of hK2 in the sera of men with prostate cancer is higher than the median concentration in age-matched men without prostate cancer. In addition, on average, the concentrations of hK2 in sera from men with high-grade and/or metastatic cancer are higher than the hK2 concentrations in men with lower Gleason scores. However, there is considerable overlap among these populations. In this small study, hK2 did not discriminate the cancer cases from the non-cancer cases as well as PSA, but larger studies are needed to fully define the receiver operator characteristics of these analytes. The low correlation between the hK2 and PSA values suggests that they may be complementary.

The physiologic role of hK2 is unknown. Biochemically, it is an active protease with arginine-selective specificity. This activity suggests that hK2 could have a role in the activation and/or degradation of proteins, and the prostate localization of this enzyme suggests that the proteolytic role of hK2 probably is focused in the prostatic milieu. In vitro studies have shown that hK2 can cleave proPSA to generate PSA (28).

Recently, Tremblay et al. (29) have shown that measurement of hK2 complex with protein C inhibitor in human seminal plasma may have a role in the diagnosis of obstructive azoosperma. A group from Europe recently reported preliminary data showing that the hK2/free PSA ratio may improve the discrimination between prostate cancer and benign hyperplasia for men having total PSA concentrations between 4 and 10 μg/L (30).

The full role of hK2 in medical practice will require extensive investigation. The availability of a reliable highly sensitive automated assay should help these investigations.

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