Increased Activity in Serum of an Alkaline Phosphatase Isoenzyme in Cancer: Analytical Method and Preliminary Clinical Studies

Lawrence Kahan,¹ Vay Liang W Go,² and Frank C. Larson²,⁴

Serum of cancer patients often contains high activities of a homoaarginine-sensitive isoenzyme of alkaline phosphatase with high electrophoretic mobility, which is present in relatively low activities in sera from most normal persons and persons with benign disease. In earlier studies of this isoenzyme, in a semi-quantitative assay, electrophoresis was used in the separation step. This report describes a column separation procedure, which provides quantitative data. We tested the revised procedure by assaying a mixed panel of 192 sera (cancer, benign disease, and normal). The organ sites of cancer and benign disease in this study were: lung, pancreas, uterus, and ovaries. The isoenzyme showed moderate sensitivity for lung (0.6) and pancreas (0.8) cancer and high specificity for all cancers tested. Thus the assay may be useful for discriminating between cancer sera and non-cancer sera for these cancer types.

We reported the appearance in serum of an electrophoretically fast, homoaarginine-sensitive isoenzyme of alkaline phosphatase (EC 3.1.3.1; FHAP) that bears a relationship to the presence of cancer (1). In the initial studies, we used an electrophoretic separation technique and a semiquantitative assay procedure which were described in detail (2). A procedure for the column-chromatographic separation of FHAP was described earlier (3). High FHAP values for presumably normal persons, which varied with total alkaline phosphatase concentration, led us to conclude that FHAP was incompletely separated from other serum alkaline phosphatase activity. Here, we describe an improved column separation procedure, together with an analysis of the FHAP activity in a mixed panel of cancer and non-cancer sera provided in a blind manner by the Mayo Clinic Serum Bank.

Materials and Methods

Source of Test Sera

The Mayo Clinic sera were stored frozen for various intervals at -80 °C. They were shipped in solid CO₂ and allowed to thaw just before analysis. The sera were analyzed in random order. Those responsible for the analysis (L.K., F.C.L., and My. M.) were not aware of the diagnoses until after the test results had been submitted to the Laboratory for Immunodiagnosis, NCI, NIH, Bethesda, MD. It was then learned that the sources of the sera included: 30 normal persons, 30 smokers without cancer, 30 patients with pancreatic carcinoma, 30 patients with lung carcinoma, 30 patients with cancer of the female reproductive system, 30 patients with benign gastrointestinal disease, and 12 patients with benign disease of the female reproductive system. Diagnostic information was collated with FHAP data by both groups inde-

pendently, with identical results. The statistical analysis was performed by the Biometry Branch, Division of Cancer Cause and Prevention, NCI, NIH.

Analytical Methods

Column separation technique. Equilibrate DEAE Sephadex-A50 with a solution containing, per liter, 0.15 mol of NaCl, 1.0 mmol of MgCl₂, 0.2 mmol of ZnCl₂, and 20 mmol of triethanolamine acetate, adjusted to pH 7.4 (Buffer I).

Prepare columns at room temperature on the day of assay by plugging a 5-mL syringe barrel with a glass-fiber filter circle (1.2 cm diameter) and attaching a 22-gauge 1-inch needle. Pipet 6 mL of a 1:3 slurry of DEAE Sephadex into the syringe. The final bed volume should be 1.8-2 mL. Twenty-four columns are run at a time. Pipet 0.1 mL of serum onto the column and allow to stand for 5-10 min. Place 1-cm glass-fiber filters, wetted with Buffer I, on top of the column beds and wash the columns with 30 mL of Buffer I, delivered by a 25-channel peristaltic pump at a flow rate of 36 mL/h. Place the rack of columns over a rack of tubes and elute the FHAP with 4 mL of a solution containing, per liter, 0.4 mol of NaCl, 1.0 mmol of MgCl₂, 0.2 mmol of ZnCl₂, and 20 mmol of triethanolamine acetate buffer, pH 7.4 (Buffer II), delivered at a flow rate of 36 mL/h.

FHAP fractions isolated from serum of cancer patients were subjected to electrophoretic analysis according to our previous procedure (2) and showed only the fast-mobility isoenzyme.

A pool of FHAP fractions from ion-exchange column analysis of 125 individual sera having less than 2 U of FHAP per liter was concentrated 300-fold by ultrafiltration (Amicon PM-10 membrane). The alkaline phosphatase isoenzyme profile of this pooled concentrated sample showed only FHAP and no detectable liver (α₂) alkaline phosphatase, demonstrating that the column separation method yields a FHAP fraction containing the same fast-mobility isoenzyme previously detected by electrophoretic separation methods (2), with <0.25 U of liver alkaline phosphatase contaminant per liter of original serum.

Enzyme assay. Assay FHAP by adding 1 mL of eluate to 4 mL of assay cocktail of the following composition, per liter: 378 mg of 2',4'-dimethyl-3-dihydroxy-2-naphthylidene phosphate (naphthol AS-MX phosphate, Sigma Chem. Co.); 1 mol of 2-amino-2-methylpropanol - HCl, pH 9.9 at 37 °C; 1.25 mmol of MgCl₂; 0.25 mmol of ZnCl₂; 1.8 mL of Triton X-405 surfactant.

Incubate the samples at 37 °C for 0.5 h and measure the fluorescence by aspirating an aliquot into the flow cell of a Farrand Ratio-2 fluorometer (standardized with 10⁻⁵ mol/L quinine sulfate). Excitation is at 313 nm, with use of a 20-nm bandwidth interference filter, and emission in the range 470-580 nm is measured with use of a combination of secondary filters. Continue the incubation an additional 1.5 h and again measure the fluorescence. Divide the net relative change in fluorescence (F₂₉₅ - F₀₅₅) in the unknown sample by the net relative change in fluorescence observed for a known activity of standard alkaline phosphatase as measured in a secondary standard control for each assay, in order to calculate the activity of FHAP. Express activity as units per liter, where 1 unit

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is 1 U in the 4-nitrophenyl phosphate assay of Bowers and McComb (4). In this paper we express all values in U, i.e., in terms of the 4-nitrophenyl phosphate assay, to facilitate comparison with published values, which customarily are expressed in these units. (One unit is $2.7 \times 10^{-2}$ U in the naphthol AS-MX phosphate hydrolysis assay). Correct for changes in fluorescence resulting from nonenzymic hydrolysis by reading fluorescence vs a blank containing 1 mL of Buffer II and 4 mL of cocktail.

For quality control we used high and normal FHAP "column control" serum samples separated and analyzed with each assay and analyzed a diluted serum secondary standard in each assay. The within-assay CV was 6% for samples between 2 and 30 U/L FHAP and the between-assay CV is 20% for the 2 U/L and 9% for the 30 U/L standards. The assay curve is linear between 1 and 64 U of FHAP per liter: slope = 0.55 arbitrary fluorescence units · (L/h) · U (SD = 0.0043), intercept = 0.022 arbitrary fluorescence units/h (SD = 0.121), standard error of estimate = 0.42 arbitrary fluorescence units/h.

Determination of Normal Range

A previous study of the FHAP activity of 1000 presumably normal blood donors suggested that 95% of normal persons had values less than 2.10 U/L. This figure was used as the cut-off value for distinguishing abnormality in this study.

Statistical Methods

Chi square analysis (2 × 2 tables) was used to assess the discrimination power of the assay. The pairs analyzed were: lung cancer vs smokers, pancreatic cancer vs benign disease of the gastrointestinal tract, cancer of the uterus and ovaries vs benign disease of these organs, and all cancers vs all non-cancer persons. The sensitivity and specificity, as well as the probability of misclassification, were determined in each of the paired groups.

The Gail–Green procedure (5) was used to locate the optimal cut-off value. This proved to be 2.09 U/L, which was so near the previously accepted value of 2.10 U/L that we used only the latter in the chi square analyses.

Results

Table 1 lists the FHAP assay results for each group.

Normal persons. None of this group had values exceeding the 2.10 U/L cut-off value.

Smokers. No values exceeded the 2.10 U/L cut-off.
Table 2. Sensitivity and Specificity: Chi Square Analysis

<table>
<thead>
<tr>
<th></th>
<th>Lung Cancer</th>
<th>Pancreas</th>
<th>Gyn</th>
<th>All *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos b</td>
<td>18</td>
<td>0</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>Neg</td>
<td>12</td>
<td>30</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>18/30 = 0.60</td>
<td>23/30 = 0.767</td>
<td>13/30 = 0.433</td>
<td>54/90 = 0.600</td>
</tr>
<tr>
<td>± 0.089 c</td>
<td>± 0.077</td>
<td>± 0.091</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>30/30 = 1.00</td>
<td>22/30 = 0.733</td>
<td>11/12 = 0.917</td>
<td>93/102 = 0.912</td>
</tr>
<tr>
<td>± 0.000</td>
<td>± 0.081</td>
<td>± 0.080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chi-square: d</td>
<td>22.936 (p &lt;0.0001)</td>
<td>14.141 (p &lt;0.0001)</td>
<td>3.281 (p &lt;0.0694)</td>
<td>54.503 (p &lt;0.0001)</td>
</tr>
</tbody>
</table>

* This analysis pairs all cancer patients with all normal test subjects, smokers, and patients with non-malignant diseases. b Cut-off value: 2.1 U FHAP per liter. c Values of specificity or sensitivity ± standard error. d Value of chi square (with probability) for the indicated comparison.

Lung cancer. Eighteen patients with lung cancer had values exceeding the 2.10 U/L cut-off.

Gastrointestinal disease, benign. There were eight patients in this group with values exceeding the 2.10 U/L cut-off. The high mean value reflects to a large extent one extremely high FHAP value, obtained for a patient with pancreatic pseudocyst.

Pancreatic cancer. There were 23 patients in this group who had values exceeding the 2.10 U/L cut-off.

Benign disease of the uterus and ovaries. Only one patient had a value exceeding the 2.10 U/L cut-off.

Cancer of the uterus and ovaries. There were 13 patients in this group with values exceeding the 2.10 U/L cut-off. Twenty one of these patients had uterine cancer (mostly cervical). Of these, 12 had FHAP values exceeding 2.10 U/L. Only two patients with ovarian cancer had FHAP values exceeding the cut-off.

Table 2 shows the sensitivity and specificity of the test. Sensitivity characterizes the incidence of positive test results, i.e., FHAP scores of 2.10 U/L or above among patients known to have cancer (true positives). Specificity characterizes the incidence of negative test results, i.e., FHAP scores ≤2.10 U/L (true negatives) in patients presumed not to have cancer.

These two test variables were examined by organ site and as groups. The sensitivity of FHAP for lung cancer was 0.60, for pancreatic cancer, 0.77, and for cancer of the uterus and ovaries (combined) it was 0.43. Because there were no FHAP values ≤2.10 U/L among the normal or smoker groups, the specificity was 1.0. However, lower values were obtained for benign diseases of the gastrointestinal tract (0.73) and for the benign gynecologic group (0.92).

The pooled cancer data were compared with the data for all non-cancer persons, including those with benign disease. The sensitivity of the test was 0.60. The specificity for the normal group was 1.0, while for the combined group it was reduced to 0.91.

The chi-square analysis demonstrated that it was highly unlikely (p <0.0001) that the difference between the groups compared had occurred by chance, except in the case of gynecologic cancer vs benign disease of the female reproductive system, where the probability was <0.069.

Discussion

We previously reported the association of a serum alkaline phosphatase enzyme with various cancers. This enzyme, now denoted as FHAP, was initially distinguished from other serum alkaline phosphatase enzymes by its rapid mobility on electrophoresis. It was later found to be homoarginine sensitive, phenylalanine insensitive, and inactivated by heat (65°C for 5 min) (2). In the initial clinical studies we used a semiquantitative electrophoretic separation assay procedure. Initial attempts to develop a quantitative ion-exchange chromatographic method provided more quantitative data but higher-than-expected FHAP values for normal persons, owing, we believe, to contamination of the FHAP fraction with non-FHAP alkaline phosphatase. This prompted the improvement in the separation procedure that we report here. The FHAP isolated by the improved separation procedure is identical in properties and electrophoretic mobility to the isoenzyme previously described (2).

The correlations of serum FHAP with cancers of the lung, pancreas, uterus, and ovaries reported here are similar to the correlations of FHAP with cancers of breast and colon reported elsewhere (6). This marker appears to be enhanced in cancers of a wide range of sites (and cell types). It appears that the substitution of a column separation procedure for the electrophoresis procedure used previously and the quantitative measurement of isoenzyme concentrations has resulted in improved specificity, even though FHAP is present in low concentration in all sera.

FHAP is similar to or identical with the α1 liver alkaline phosphatase isoenzyme (2) which has recently been correlated with the presence of hepatic metastasis in cancer patients (7). With this in mind, we reviewed the medical records of 72 patients for evidence of hepatic metastasis. Nearly all had had liver scans and many had been autopsied. Table 3 shows the results of this survey. Of 72 patients with cancer in this study, 17 were known to have hepatic metastasis. Of these, eight had FHAP values less than the cut-off score of 2.1 U/L. Thirty-nine had no evidence of metastasis. Of these, 17 had above-normal FHAP values. There appears to be no strong relation in our study between increased FHAP and hepatic metastasis. Some correlation might be expected, because we have observed a correlation between serum FHAP concentration and tumor load (unpublished observations).

Table 3. Results for Cases of Metastasis to the Liver

<table>
<thead>
<tr>
<th>FHAP, U/L</th>
<th>Yes</th>
<th>Unknown</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2.1</td>
<td>8</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>≥2.1</td>
<td>9</td>
<td>13</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 4. Relative Incidence of Increased Alkaline Phosphatase and Increased FHAP

<table>
<thead>
<tr>
<th>FHAP b</th>
<th>Total alk. phosphatase a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased</td>
<td>Normal</td>
</tr>
<tr>
<td>FHAP increased</td>
<td>19</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
</tr>
</tbody>
</table>

* Mayo Clinic normal ranges were: men over 18 years of age, 90–239 U/L; women under 45 years of age 76–196 U/L; women over 45 years of age 87–250 U/L. b Values >2.10 U/L were classified as increased.
We have considered the possibility that increased FHAP activity is a direct consequence of increased total alkaline phosphatase activity. Our previous studies have not shown a high correlation between these markers. We analyzed the relationship between total alkaline phosphatase and FHAP among the 71 cancer patients in this study for whom total serum alkaline phosphatase values were available. As shown in Table 4, only 26% of these patients had increases in both markers, an additional 25% had increased FHAP and normal total alkaline phosphatase, 8% had increased total alkaline phosphatase but not FHAP, and 40% had normal values for both markers. A linear regression of FHAP vs total alkaline phosphatase gave a correlation coefficient of 0.49. Thus high FHAP activity is not a direct consequence of high total serum alkaline phosphatase activity.

We conclude that the activity of an alkaline phosphatase enzyme, FHAP, is increased (far more commonly) in the serum of cancer patients than in the serum of normal persons or persons with benign diseases. FHAP can be detected and quantified rapidly by a simple technique involving an ion-exchange resin and a fluorometer. In this study the value of FHAP assay in detecting cancer of the lung, the pancreas, and the female reproductive system was examined. FHAP assay showed a moderate sensitivity for lung and pancreatic cancer (0.6-0.8) and a very high specificity. The assay seems to have merit for discriminating between cancer and non-cancer sera for certain types of cancer.

We are indebted to Ms. Mary Mohlke for her role in preparing and submitting the test sera and in helping with clinical data evaluation, and to Ms. Myunghee Moon for performing the FHAP analyses. Credit is due to Ronald B. Herberman, Chief, Laboratory of Immunodiagnosis, DCBD, NCI, NIH, Bethesda, MD, who enabled us to obtain the sera and who arranged for the statistical analysis. This work was supported in part by contract CB-74173 awarded by the National Cancer Institute.

References