Clinical Studies of a Fast Homoarginine-sensitive Alkaline Phosphatase in Patients with Cancer


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ABSTRACT

The activity of an isoenzyme of alkaline phosphatase (FHAP) was measured in serum samples obtained from 1692 individual subjects. The median FHAP concentration in patients with untreated or recurrent cancer (2.73 IU/liter) was two-fold higher than in hospitalized control patients with illnesses other than cancer (1.17 IU/liter) and three-fold higher than in healthy control subjects (0.93 IU/liter). Among patients with either breast or colorectal cancer who were clinically disease free following their initial therapy, the median FHAP concentration (1.54 IU/liter) was intermediate between the median FHAP concentration in patients with untreated or recurrent cancer and that of healthy control subjects. In order to illustrate the potential clinical application of FHAP as a diagnostic cancer marker, we have selected a serum FHAP concentration of 2.22 IU/liter as a reference value above which only 3% of healthy control subjects would have a positive test. Utilizing this reference value, 58% of the patients in the present study with untreated or recurrent cancer would have a positive FHAP test, whereas only 11%, of hospitalized patients with illnesses other than cancer would have a positive test. These data suggest that FHAP may be equivalent to the carcinoembryonic antigen as a diagnostic cancer marker.

INTRODUCTION

We previously identified and characterized an isoenzyme of alkaline phosphatase, FHAP, which was detected more frequently in the sera of cancer patients than in the sera of healthy blood donors or of patients with benign diseases (2, 5). In our initial studies of FHAP, the isoenzyme was separated by electrophoresis on cellulose polyacetate strips. The presence and relative concentration of the isoenzyme were determined subjectively by visual assessment of α-naphthol ASMX-phosphate hydrolysis.

In this communication, we are reporting the development of improved quantitative methods for the separation and assay of FHAP and the results of a study utilizing these methods in 1692 subjects. We have found that FHAP is present at low concentrations in the sera of all normal individuals and at significantly elevated concentrations in the sera of patients with a variety of cancers.

MATERIALS AND METHODS

Chemicals. NaCl, α-naphthol ASMX-phosphate, triethanolamine, 2-amino 2-methylpropanol, and Triton X-405 were obtained from Sigma Chemical Company, St. Louis, Mo. MgCl2, ZnCl2, and acetic acid were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J.

Separation Procedure. Five ml of blood were drawn and were allowed to clot. The serum was harvested and stored at 4°, —20°, or —70° until assayed. The variations in storage temperature were found to have no influence on the results of the assay.

Thirty g of DEAE-Sephadex A-50 were equilibrated for at least 4 hr at room temperature with 6 liters of 0.15 M NaCl, 1.0 mM MgCl2, 0.2 mM ZnCl2 and 20.0 mM triethanolamine, adjusted to pH 7.4 with acetic acid (Buffer 1). This equilibration was repeated 3 times. The exchange agent was stored as a 1:3 slurry in Buffer 1 at 4°.

Columns were prepared at room temperature on the day of assay by plugging a 5-ml plastic syringe barrel (inside diameter, 1.2 cm) with a glass-fiber filter (Reeve Angel 934AH). A 22-gauge 1-inch needle was attached. Six ml of the 1:3 slurry which had been degassed for 10 min at room temperature were pipetted into the syringe barrel. The column was allowed to drain, and the final bed volume was 1.8 to 2.0 ml. One-tenth ml of serum was pipetted onto the column and allowed to stand for 5 min. A 1-cm wet glass-fiber filter was placed on top of the bed, and the column was then washed with 30 ml of Buffer 1 at a flow rate of 34 ml/hr, which removed all non-FHAP alkaline phosphatase. FHAP was eluted with 4 ml of 0.4 M NaCl, 1.0 mM MgCl2, 0.2 mM ZnCl2, and 20.0 mM triethanolamine, adjusted to pH 7.4 with acetic acid (Buffer 2) at a flow rate of 34 ml/hr.

Assay Method. A Farrand Ratio-2 fluorometer equipped with a flow cell was used. Excitation was at 313 nm using a 20-nm-bandwidth interference filter. Emitted light between 470 and 580 nm was measured using Farrand 5-56 and 3-71 glass secondary filters.

FHAP was measured in the Buffer 2 eluate. One ml of eluate was added to 4 ml of an assay mixture of the following composition: α-naphthol ASMX-phosphate (378 mg/liter); 1 mM 2-amino 2-methylpropanol-HCl, pH 9.9, at 37°; 1.25 mM MgCl2; 0.25 mM ZnCl2; and Triton X-405 (1.8 ml/liter). This sample was then incubated at 37° and was aspirated through the flow cell by a solenoid-controlled vacuum. The net relative fluorescence of the sample was determined at 30 and 120 min of incubation against a blank composed of 1 ml of Buffer 2 and 4 ml of assay mixture. Duplicate 1-ml alkaline phosphatase standards (0.56 IU/liter by DuPont automated clinical analyzer) were used as references.
were measured with each assay.

The change in net relative fluorescence ($F_{120} - F_{30}$) of the test sample was divided by the change in net relative fluorescence of the alkaline phosphatase standard in order to calculate the concentration of FHAP:

$$\text{FHAP} = \frac{\text{Sample} (F_{120} - F_{30})}{\text{Standard} (F_{120} - F_{30})} \times 0.56 \text{ IU/liter} \times 40$$

**Populations Studied.** The serum concentration of FHAP was determined in 1692 individual subjects. Five separate populations were studied: (a) healthy control subjects; (b) hospitalized control patients with illnesses other than cancer; (c) pregnant females; (d) hospitalized patients with untreated or recurrent cancer; and (e) patients with cancer who had received primary therapy within the previous 6 months (surgery and/or irradiation) and who had no clinical evidence of recurrent or residual disease.

The 931 healthy control subjects were selected from adult donors at a regional blood center. Among this group, 466 were male and 465 were female. The age distribution of these 931 healthy control subjects is shown in Chart 1A.

The 393 hospitalized control patients with illnesses other than cancer were selected from patients admitted to the inpatient wards of the University Hospitals in Madison, Wis. The medical records of these patients were reviewed to determine the final diagnoses. Patients with a previous diagnosis of cancer except basal cell carcinoma of the skin or in situ carcinoma of the uterine cervix were excluded from this study. The age distribution of these 393 hospitalized control patients is shown in Chart 1B.

The 73 pregnant females were selected from patients in the outpatient clinics or the inpatient wards. Since the total number of pregnant females in this study is small, no attempt has been made to analyze this group on the basis of age or duration of pregnancy.

The 198 patients with untreated or recurrent cancer all had a histologically confirmed diagnosis of cancer. Most of these patients had widespread metastatic disease and were hospitalized for palliative therapy. The age distribution of these 198 cancer patients is shown in Chart 1C.

The final group consisted of 97 patients with either breast cancer or colorectal cancer who had received definitive local therapy within the previous 6 months. All of these patients were clinically free of recurrent or residual disease at the time the serum concentration of FHAP was determined. The patients with breast cancer all had histological evidence of axillary lymph node involvement at the time of their original diagnosis, thereby rendering them at high risk for subsequent recurrence. Similarly, all of the patients with colorectal cancer were at high risk for recurrence based upon extension of their primary tumor to the serosa and/or the presence of histologically positive regional lymph nodes.

Differences between groups were assessed using the Wilcoxon rank sum test, stratified by age where appropriate.

**RESULTS**

**Separation and Assay Procedures.** The column separation conditions were found to give a complete separation of the FHAP and liver alkaline phosphatases as determined by cellulose polyacetate electrophoresis at pH 7.0 (2). The assay of alkaline phosphatase activity was based on the method described by Johnson (4), with the addition of ZnCl₂ to improve stability and a non-ionic detergent to improve flow characteristics. In addition, the stronger excitation peak for α-naphthol ASMX at 313 nm was used in the fluorescence measurement.

The separation and assay procedures used in this study provided a reliable quantitative measurement of FHAP activity in the sera. Product formation was found to be a linear function of the enzyme concentration over the range of 1 to 64 IU/liter (Chart 2). The within-assay coefficient of variation for the combined separation and assay procedures was 6% for both high-concentration (30 IU/liter) and low-concentration (2.3 IU/liter) samples. Control samples containing high FHAP activity and low FHAP activity were included with each set of assays. The between assay coefficient of variation for the combined separation and assay procedures ranged from 9% for the high-activity control samples to 20% for the low-activity control samples.
**Healthy Control Subjects.** The mean and median serum FHAP concentrations in the 931 healthy control subjects (blood donors) is shown in Table 1. The frequency distribution of FHAP values within this group is illustrated in Chart 3A. There was no significant difference in the serum FHAP concentrations between healthy male subjects and healthy female subjects. However, regression analysis of variance did demonstrate a slight but statistically significant increase (0.07 IU/liter/decade) in the FHAP levels with increasing age \((p < 0.001)\).

**Hospitalized Control Patients and Pregnant Females.** The mean and median serum FHAP concentrations in these groups are shown in Table 1. The frequency distribution of FHAP values in the 393 hospitalized control patients is illustrated in Chart 3B. There was no significant difference in the serum FHAP concentrations between males and females. There also was no significant increase in the FHAP levels with increasing age in this group of patients.

There were 33 patients with overt diabetes mellitus in the group of hospitalized control patients. The FHAP levels in these diabetic patients were significantly higher than in the remaining 360 hospitalized control patients \((p < 0.001)\).

Although the difference in the serum FHAP concentrations between the hospitalized control patients and the healthy control subjects was small, this difference was significant \((p < 0.001)\). The group of pregnant females also had significantly higher FHAP values than did the corresponding group of healthy female subjects \((p < 0.001)\).

**Cancer Patients.** The mean and median serum FHAP concentrations for the patients with cancer are shown in Table 1. The frequency distribution of FHAP values in the 198 patients with untreated or recurrent disease is illustrated in Chart 3C. The median FHAP value in these patients was 2-fold higher.
Comparisons based upon the percentage of patients with elevated FHAP values

<table>
<thead>
<tr>
<th>FHAP (IU/liter)</th>
<th>Healthy subjects</th>
<th>Hospitalized patients</th>
<th>Breast cancer (&lt;6 mos. post-operative)</th>
<th>Colon cancer (&lt;6 mos. post-operative)</th>
<th>Untreated or recurrent cancer</th>
</tr>
</thead>
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<tr>
<td>2.77</td>
<td>1.0</td>
<td>7.8</td>
<td>17.8</td>
<td>32.0</td>
<td>49.5</td>
</tr>
<tr>
<td>2.42</td>
<td>2.0</td>
<td>6.6</td>
<td>17.8</td>
<td>36.8</td>
<td>53.5</td>
</tr>
<tr>
<td>2.22</td>
<td>3.0</td>
<td>10.9</td>
<td>24.7</td>
<td>40.0</td>
<td>57.6</td>
</tr>
<tr>
<td>1.93</td>
<td>5.0</td>
<td>15.7</td>
<td>31.5</td>
<td>52.0</td>
<td>64.6</td>
</tr>
</tbody>
</table>

than the median value in hospitalized patients with benign disorders ($p < 0.001$) and 3-fold higher than in healthy control subjects ($p < 0.001$).

In the 97 patients with either breast or colorectal cancer who were clinically disease free following their initial therapy, the mean and median FHAP values were intermediate between those of the 198 patients with untreated or recurrent cancer and those of the 931 healthy control subjects. A comparison of FHAP values between the following groups revealed that the differences in all instances were significant ($p < 0.001$): (a) healthy female subjects versus patients with breast cancer who were clinically disease free; (b) healthy female subjects versus patients with untreated or recurrent breast cancer; and (c) patients with breast cancer who were clinically disease free versus patients with untreated or recurrent breast cancer.

In order to better illustrate the potential clinical application of FHAP as a diagnostic cancer marker, we have arranged our data somewhat differently in Table 2. A serum FHAP concentration of 2.22 IU/liter was selected as a reference value because that is the concentration above which only 3% of the healthy control subjects would have a “positive” test. Among the patients in the present study with untreated or recurrent cancer, 58% would have a positive FHAP test ($>2.22$ IU/liter), whereas only 11% of those with benign disorders had elevated levels of FHAP.

The results of our present study demonstrate that the FHAP values in patients at high risk for recurrent breast or colorectal cancer are significantly higher than those of healthy control subjects. This observation suggests that the serum concentration of FHAP following primary therapy may be a useful prognostic indicator. In order to confirm this hypothesis, additional follow-up of the patients reported in this series will be required. We are also conducting long-term follow-up studies to assess the correlation between the serum levels of FHAP and more conventional measures of response in patients receiving systemic therapy for recurrent breast or colorectal cancer.

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