Expression of an Unusual Isozyme of Lactate Dehydrogenase in the Serum of Cancer Patients and Comparison with Carcinoembryonic Antigen

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ABSTRACT

Two studies reported here demonstrate a statistically significant association between metastatic cancer and the appearance of the k isozyme of lactate dehydrogenase in serum of affected patients. The first study included 190 coded samples from three types of cancer patients and matched controls; the second included 155 preoperative and 200 postoperative colorectal cancer patients. In the second, plasma carcinoembryonic antigen was compared with serum k isozyme of lactate dehydrogenase as an indicator of the presence of metastatic cancer. This comparison showed that both markers were independently useful for assessing patient status and predicted that a combination of the two should be a better discriminator for the presence of metastases than either marker alone.

INTRODUCTION

The k isozyme of lactate dehydrogenase (LDHk) is an anoxic stress response protein which is also found at high levels in cells transformed by the Kirsten murine sarcoma virus and in many human cancers (1, 2). LDHk is distinguished from other LDH isozymes by electrophoretic mobility, subunit molecular weight, subunit peptide composition, and biochemical properties. We have reported that LDHk is also found in the serum of a wide variety of cancer patients (3–7). In this paper, we extend those earlier findings with results from three types of cancer patients.

In addition, to better define the clinical significance of serum LDHk expression, we have selected colorectal cancer as a model system. This disease is particularly well suited for study in that colorectal carcinomas can be effectively staged by surgical and pathological techniques. In addition, a predictable number of patients will develop recurrence following surgical resection of tumors (8).

CEA is currently in use as a marker of colorectal cancer, and its limitations due to false positives are well known (9–13). Our studies indicate that LDHk is a useful marker for metastatic colorectal cancer (Stage D). When determination of serum LDHk is combined with the measurement of CEA, a more accurate indication of patient status is obtained than with either marker alone.

MATERIALS AND METHODS

Serum Samples. Two sets of sera were used in the following studies. The first was a coded set of 190 frozen human sera from cancer patients, noncancer patients, and healthy volunteers obtained from the Mayo/National Cancer Institute serum diagnostic bank. The groups were all matched for sex (all female) and age. The National Cancer Institute provided us with diagnostic and other clinical information on the patients after receiving our assay results. The second set of sera was collected from patients admitted to Roswell Park Memorial Institute for treatment of colorectal cancer. Most of these patients received surgical treatment. Serum was collected the day before surgery, the day after surgery, 1 wk after surgery, and thereafter at quarterly follow-up examinations. The sera were stored at -80°C until assay; previous results have shown that LDHk activity is stable under these storage conditions (4).

Purified LDHk Standard. LDHk derived from rat muscle was prepared for use as a standard by a method to be documented elsewhere. Rat muscle was homogenized in a cold Sorval tissue grinder for 1 min in 2 volumes of 10 mM Tris-HCl (pH 7.5)-10 mM KCl-5 mM MgCl2-1 mM dithiothreitol. The homogenate was centrifuged for 10 min at 1,000 × g to remove debris, and the supernatant was centrifuged further for 60 min at 40,000 rpm (100,000 × g) in a Beckman T-65 rotor. LDHk was then purified from this supernatant by the following three steps of column chromatography. (a) The extract was applied to a column of Affigel Blue (Bio-Rad Laboratories) in 0.01 M Tris-HCl-0.001 M dithiothreitol (pH 8.4). The column was then washed with Buffer A, with Buffer A containing 1 mM NAD and 1 mM lithium lactate, and with Buffer A containing 1 mM dihydro-NAD (Buffer C). (b) LDHk activity was then eluted from the column with a 0 to 0.3 M NaCl gradient in Buffer C. The peak fractions, which contained LDHk and LDH-5, were pooled and further purified by molecular sieve chromatography on Ultrogel AcA34 (LKB) in 0.01 M Tris-HCl-0.5 mM dithiothreitol (pH 8.4). (c) The peak fractions from this column, which still contained both LDHk and LDH-5, were applied to a hydroxylapatite column (Hypatite C; Clarkson Chemical Co.) in 10 mM Tris-HCl (pH 7.4)-10 mM KCl-1 mM MgCl2-1 mM dithiothreitol. LDHk was eluted free of LDH-5 activity by a 0.05 to 0.2 M gradient of sodium borate containing 0.5 mM dithiothreitol.

Electrophoretic Assay of LDHk. The LDHk isozyme was separated from others by electrophoresis in a polyacrylamide gel as described by Polonis et al. (4). LDHk migrates cathodally under these conditions, other isozymes do not enter the gel.

Detection of LDH Activity in Electropherograms. Polyacrylamide gels were stained for activity by a modification of the method of Dietz and Lubrano (14). The staining mixture contained 0.1 M Tris-HCl (pH 8.0), 100 mM lithium lactate, 1 mM NAD, 0.5 mM nitroblue tetrazolium, 0.04 mM phenazine methosulfate, and 2% glycerol. Polyacrylamide gels were stained 4 h at 37°C with constant nitrogen sparging to avoid oxygen inhibition of LDHk. After staining, polyacrylamide gels were fixed with 45% methanol-10% acetic acid and scanned with an integrating densitometer (Quick Scan Jr.; Helena Laboratories, Beaumont, TX) to measure the amount of stain. One cycle of the integrator pen was taken as 1 unit of LDHk activity; this unit corresponds to about 10^14 IU LDH activity.

Normalization of Data from LDHk Assays. Assays for LDHk were done using duplicate slab gels, each of which included two samples of a standard preparation of partially purified rat muscle LDHk. The variability of the standard assays was analyzed by analysis of variance as shown in Table 1. This showed that the variability between gels run on different days accounted for a large part of the total. This variability is most easily attributed to variations in the nitrogen sparging, which affects both mixing of the staining solution and protection from oxygen inhibition. Accordingly, we pooled the values for the four standard
duplicate samples on the same electropherogram.

The variance of the data was separated into that derived from variation between different electropherograms done on the same day and variation between duplicate samples on the same electropherogram.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between days</td>
<td>37,179</td>
<td>21</td>
<td>1,770</td>
</tr>
<tr>
<td>Residual</td>
<td>25,661</td>
<td>66</td>
<td>389</td>
</tr>
<tr>
<td>Total</td>
<td>62,840</td>
<td>87</td>
<td>722</td>
</tr>
</tbody>
</table>

where daily standard is the sum of the 4 SDs on the day of the assay, and av. standard is the average of all such sums for all assays performed.

Assay of Carcinoembryonic Antigen. Plasma samples were assayed for CEA by one of two methods. Most samples were assayed with a commercial radioimmunoassay test kit (CEA-ROCHE; Roche Diagnostics, Nutley, NJ), using a specific polyclonal antibody. The most recent samples, constituting about 20% of those analyzed, were assayed with an enzyme immunoassay test kit (CEA-ROCHE EIA; Roche Diagnostics), using monoclonal antibody.

Stage of Colorectal Patients. Patients were staged according to the following modified Dukes' system: Stage A, tumor within intestinal mucosa; B1, tumor within bowel wall; B2, tumor penetrating serosa; C1, 1 to 4 lymph nodes involved; C2, more than 4 lymph nodes involved; D, distant metastases.

Records and Data Analysis for Colorectal Patients. Assay results and clinical data for colorectal cancer patients were maintained on an Apple microcomputer using the General Manager database management program (Sierra On-Line; Coarsegold, CA). The data were transferred to the R:base 5000 relational database running on a Sperry microcomputer for analysis. Data were selected from the database using the R:base query commands and then transferred to the Lotus 1-2-3 spreadsheet program and to a Univac 90/80 mainframe for calculations.

Linear Logistic Regression Analysis. The linear logistic model (15) for the probability of metastases was

\[ P(\text{mets}) = \frac{\exp[a + b(\text{LDH}k) + c(\text{CEA})]}{1 + \exp[a + b(\text{LDH}k) + c(\text{CEA})]} \]

where \( P(\text{mets}) \) is the probability of metastases in a patient; \( a, b, \) and \( c \) are regression coefficients; \( \text{LDH}k \) is \( \ln[1 + (\text{serum LDH}k \text{ activity})] \); and \( \text{CEA} \) is \( \ln[1 + (\text{serum CEA activity})] \).

RESULTS

Serum LDHk in Cancer Patients and Matched Control Populations. A coded set of sera was obtained from the Mayo-National Cancer Institute serum diagnostic bank. This set included sera from patients with colon cancer, breast cancer, and gynecological cancer, as well as benign tumor and nontumor patients. These samples were assayed, and the results were normalized using the average of the four standard samples done on each day. The colon and gynecological cancer patients included many with metastases, whereas the breast cancer group did not. We therefore decided to test both diagnostic group and stage of disease for a significant association with serum LDHk. Since we had had some indication for another group of patients of an association between the age of the patient and serum LDHk, we also tested for that association in these patients.

Each variable was first tested individually for an association with serum LDHk. In each case the dependent variable was log(1 + LDHk activity), because this variable had a more uniform variance over the range of LDHk activities. The nominal variables, diagnostic group and stage of disease, were tested by one-way analysis of variance. The test of diagnostic group included all 190 samples, but the test of stage included only the 88 samples from cancer patients. The association of serum LDHk with stage was quite significant \( (P = 0.001) \); that with diagnostic group was marginally significant \( (P = 0.069) \). The continuous variable, age, was tested by linear regression analysis; the association between age and serum LDHk was not significant \( (P = 0.15) \).

To further test the significance of the association of LDHk with metastasis we used a three-factor linear regression analysis, testing simultaneously for the association with diagnostic group, stage of disease, and age. Such an analysis was more informative than the single-factor analysis because the factors, diagnostic group and stage of disease, were not independent. This analysis confirmed a highly significant association between serum LDHk and stage of disease (presence of metastases).

Serum LDHk in Colorectal Cancer Patients. The above results suggested an association between the presence of metastatic cancer and elevated serum LDHk activity. To test this association further, we chose to examine a patient population which could be followed throughout the course of treatment. Serum samples were collected from patients admitted for treatment of colorectal cancer, most of whom were primarily treated surgically. These samples were collected before surgery, 2 days after surgery, 7 days after surgery, and at quarterly follow-up examinations. These were assayed electrophoretically for LDHk. Multiple assays were normalized and averaged to obtain the results presented here. Assays of plasma for carcinoembryonic antigen were also performed as a routine part of the patient evaluation.

Serum LDHk in Preoperative Patients. At the time of surgery, patients were staged according to the modified Dukes' system described in "Materials and Methods." If serum LDHk were an indicator of metastases, we would expect a correlation between the Dukes' stage and the serum LDHk value. Table 2 shows the mean preoperative serum LDHk and CEA values as a function of the Dukes' stage. The average serum LDHk is about 6-fold higher for Stage D patients than for those of Stage B or C. The average CEA value was about 3-fold higher for Stage D than for Stage B or C. Thus, serum LDHk does seem to be correlated with the presence of metastases.

The averages shown in Table 2 conceal a substantial amount of variation in the data. Fig. 1 shows the distribution of the LDHk data summarized in Table 2. To represent the wide range of activity values effectively, the data in the figure are presented using a logarithmic scale. Over half of the Stage B and C patients have undetectable amounts of serum LDHk. While most of the Stage D patients have detectable serum LDHk, the values are distributed over a wide range. Fig. 1 also

<table>
<thead>
<tr>
<th>Dukes' stage</th>
<th>Mean LDHk</th>
<th>Mean CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>4.3</td>
<td>13.0</td>
</tr>
<tr>
<td>C</td>
<td>3.5</td>
<td>13.2</td>
</tr>
<tr>
<td>D</td>
<td>26.5</td>
<td>35.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Av. follow-up values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No evidence of disease</td>
<td>1.1</td>
</tr>
<tr>
<td>Metastatic</td>
<td>35.2</td>
</tr>
</tbody>
</table>

* Mean LDHk and CEA values for preoperative data presented in Fig. 1.

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*$^*$ Mean LDHk and CEA values for follow-up data presented in Fig. 2.

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LDHk and CEA in Serum of Cancer Patients

presence of metastases changes the distribution of LDHk values from one in which less than 15% of the samples have detectable LDHk to one in which over 70% do. The CEA values show a smaller difference. There is almost a 6-fold difference in average serum CEA between patients with metastases and those without known metastases.

Correlation between Serum LDHk and CEA. The data in Figs. 1 and 2 suggest that both LDHk and CEA are associated with metastases in colorectal cancer patients. If so, we might expect LDHk and CEA to be correlated with each other. This would be the case if both were controlled by the same mechanism. However, the correlation between LDHk and CEA is actually quite low. For the preoperative samples for which both LDHk and CEA assays were available, the LDHk-CEA correlation coefficient ranged from -0.02 for the Dukes’ Stage C data to 0.22 for the Dukes’ Stage D data. The data from which these correlation coefficients were derived are displayed in Fig. 3. For postoperative samples, the coefficients were -0.01 for patients without metastases and 0.16 for those with metastases. This lack of correlation suggests that LDHk and CEA are not controlled by a common mechanism.

According to the previous results, both serum LDHk and plasma CEA are correlated with metastatic disease, but they are poorly correlated with each other. This observation suggests

unpublished results.

Unpublished results.

Fig. 1. Serum LDHk and serum CEA in preoperative patients with colorectal cancer. LDHk and CEA values are displayed on a logarithmic scale to accommodate the wide range of values. The leftmost bar in each panel includes all values less than 1. The value for N indicates the number of patients represented by each panel. On the abscissa, the dividing value between normal and abnormal is 2 \[\log(4 \text{ units})\] for LDHk and also 2 \[\log(4 \text{ ng/ml})\] for CEA (Ref. 4, Footnote 5).

Fig. 2. Serum LDHk and serum CEA in postoperative colorectal cancer patients. Nonmetastatic patients were those patients with no evidence of disease after removal of the primary cancer. Metastatic patients were those with distant metastases; in some cases the primary cancer was also still present.

Serum LDHk in Postoperative Patients. Postoperative patients were classified according to the presence of known metastases. Follow-up serum samples were obtained from these patients at quarterly examinations. Samples taken on the last sample date for each patient were used to analyze the correlation between the presence of metastases and serum LDHk or plasma CEA. These samples provided 229 LDHk determinations and 132 CEA determinations, the results of which are presented in Table 2 and Fig. 2. There is a 30-fold difference between the average serum LDHk in patients with metastases and those without known metastases. Fig. 2 shows that the

Fig. 3. Lack of correlation between serum LDHk and serum CEA in preoperative colorectal cancer patients. The normalized values plotted correspond to \[\log(X + 1)/\log(X_{max} + 1)\], where X is either the LDHk or CEA value, and X_{max} is the corresponding maximum observed value. The maximum LDHk value was 245; the maximum CEA values were greater than 50 and were given a value of 100.
that both may be able to make independent contributions to an estimate of patient status. If so, then some function of both markers should give a better estimate of patient status than either marker separately.

This possibility was tested by analyzing the data according to a linear logistic regression model (8), as described in “Materials and Methods.” For consistency with previous analyses, the variables tested were ln(1 + LDHk) and ln(1 + CEA). The postoperative data, having two patient categories (metastatic and no-evidence-of-disease), were tested most simply. When each variable was examined separately, both were found to be significantly associated with the presence of metastases (P < 0.0001 for each variable). When both variables were tested simultaneously with a multivariate model, both were still significantly associated with the presence of metastases (P = 0.0003 for LDHk and P < 0.0001 for CEA). This result indicates that both were significantly related to the presence of metastases even after adjusting for the effect of the other. In other words, both LDHk and CEA have independent prognostic value for detection of metastases.

This type of analysis was extended to the preoperative data, where three patient classes were available (Dukes' Stages B, C, and D). In this case, a polychotomous logistic regression model was used. Both LDHk and CEA individually discriminate between Dukes' Stages B and D and between Dukes' Stages C and D but not between Dukes' Stages B and C. Multivariate analysis of both LDHk and CEA simultaneously revealed the same pattern. Both distinguished between Dukes' Stages B and D (P = 0.011 for LDHk and P < 0.0001 for CEA) and between Dukes' Stages C and D (P = 0.0017 for LDHk and P = 0.0006 for CEA) but not between Dukes' Stages B and C (P = 0.34 for LDHk and P = 0.20 for CEA).

**DISCUSSION**

Our previous work (4) suggested that serum LDHk was associated with cancer and especially with metastatic cancer. This study, an extension of that work, provides two further tests of that association. The first was performed "blind" with sets of sera from patients matched for age and sex. It indicates that LDHk is not correlated with primary cancer but is highly associated with metastatic cancer. The second test was performed with patients with a single type of cancer (colorectal cancer) in a population which could be followed over the course of treatment and follow-up evaluation. This test confirmed the association between the presence of metastases and an elevated level of serum LDHk.

The association of serum LDHk with metastatic colorectal cancer was demonstrated with two separate sets of data. The first set was data obtained from preoperative serum samples. These were compared with the standard staging of the patients. LDHk was significantly associated with Dukes' Stage D, those patients with distant metastases. The second set of data was obtained from postoperative serum samples and was compared with the current clinical status of the patient. Here, too, serum LDHk was significantly associated with the presence of metastases.

Plasma CEA values were analyzed in the same way, showing that it was also associated with metastases. However, although CEA and LDHk were both associated with metastases, they were not correlated with each other. This observation suggested a simultaneous analysis of the association of both markers with metastases. This analysis, by linear logistic regression, showed that both markers were significantly associated with metastases, even after adjusting for the effect of the other marker. Both markers, therefore, are independently useful for distinguishing metastatic patients by postoperative data and for distinguishing either Dukes' Stage B or C patients from Dukes' Stage D patients by preoperative data. This fact implies that there is at least one function of LDHk and CEA which is a better discriminator for the presence of metastases than either marker alone.

The regression equation used in the analysis (see “Materials and Methods”) itself provides one such function of LDHk and CEA. The regression coefficients estimated from the multivariate analysis were \( a = -1.98 \), \( b = 0.15 \), and \( c = 0.30 \). The function using these coefficients is illustrated in Fig. 4. This function and a cutoff value of 0.67 for the value of the function define a criterion which correctly classifies 81% of the patients in the data set. This is an optimistic assessment of its discriminatory power, since these same patients were used to estimate the probabilities in the first place. Using the estimates to classify an independent data set would probably not yield the same accuracy of classification.

Our results indicate that LDHk may be a useful marker for metastatic colon cancer, particularly when used in conjunction with CEA. It is important to note that serum expression of LDHk is widespread among a number of cancer types (4) and may prove of particularly great value in those cancers where no other marker currently exists.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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