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Studies on Sialyltransferase Isoenzymes in Plasma of Patients With Breast Cancer

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Abstract—*An elevated level of sialytransferase was often found in plasma of patients with breast cancer, but the extent of this elevation was highly variable, as was the enzyme level in plasma of normal donors. Electrofocusing studies indicated the presence of sialyltransferases, focusing at pH* 5.2 *and* 5.6, *in all plasmas examined. Elevated enzyme activity in plasma of normal donors was associated with appearance of an 'inflammatory response' isoenzyme with pI* = 7.5. *In plasma of the individuals with untreated primary or recurrent breast cancer, we found a new sialyltransferase isoenzyme with pI* = 4.7. *This isoenzyme was not detected in plasma of patients with benign breast disease.*

INTRODUCTION

SEVERAL laboratories have reported elevated levels of plasma sialyltransferase activity in patients with metastatic breast cancer[1–5], other neoplastic diseases[3,4,6,7] and non-neoplastic pathologic disorders[8]. However, an elevated level of plasma was not uniformly associated with tumour progression[1,3,6,9]. In this report, we describe studies on total plasma sialyltransferase and on enzyme electrofocusing patterns using donors free from neoplastic disease, and patients with breast cancer, or with benign breast disease.

MATERIALS AND METHODS

Blood samples were anti-coagulated with EDTA and stored at 0°C until centrifugation, which occurred within 30 min of collection. Red cells and platelets were removed by successive centrifugations at 1000 and 10,000 *g* for 5 min each (all at 4°C): plasmas were stored at −70°C. No sample was stored for more than 15 days, except those described in Fig. 3. Total enzyme levels were measured by modification of an earlier procedure[4]. This involved a 60 min incubation of 50 μl of plasma, 0.5 mg of desialated fetuin[10], 20 mM HEPES buffer, pH 7.0, and 42 nmoles of CMP [^{14}C] sialic acid (0.8 Ci/mole, New England Nuclear Corp., Boston, MA). The total volume was 200 μl. The CMP-sialic acid concentration was 0.2 mM, the approx. K_m for this plasma enzyme activity[7]. Incorporation of label into the fetuin-derived acceptor was measured either by acid precipitation[4], or by elution[11] of the reaction mixture through 0.5 × 1 cm columns of Dowex 1 (OH form). In the latter case, the lightly-sialated glycoprotein product appears in the eluate, while free sialic acid and CMP-sialic acid are retained by the column. Total enzyme activity is reported in terms of counts/min of radioactivity incorporated into glycoprotein acceptor during 60 min incubations, using 50 μl of plasma.

Electrofocusing studies on 2 ml plasma samples were carried out for 16 hours (constant power: = 7 watts) on a chilled flat-bed gel of ultrafine Sephadex[11], using a pH range of 4–8. The LKB Ampholines (LKB, Silver Spring, MD) were employed at a 2.5% concentration. The gel bed was then divided into 30 equal fractions and each eluted with 3 ml of cold 20 mM HEPES buffer (pH 7.0). We analyzed fractions which focused between pH 4.2 and 7.6. The four or five samples at the ends of the plate were not used, since preliminiary

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studies showed no detectable enzyme activity in these fractions.

Incubations were carried out for 4 hr at 37°C in mixtures containing 0.5 ml of enzyme fraction, 10 mM of Mg acetate, 20 mM of HEPES buffer, pH 7.0, 2 mg of desialated fetuin[11] or 10 mM phenyl-galactoside[12], 2 mg of bovine serum albumin and 4.2 nmoles of CMP-[^{14}C] sialic acid (8 Ci/mole). The use of high specific-activity material was required because of the losses in enzyme activity associated with electrofocusing, as noted in the Discussion. Incorporation of sialic acid into the desialated fetuin acceptor was measured by elution of incubation mixtures through Dowex 1 columns, as described above. Levels of radioactive CMP-sialic acid and of radioactive degradation products were monitored by paper chromatography[13] of the reaction mixtures after incubation.

Studies utilizing phenyl galactoside as acceptor were terminated by the addition of 2 volumes of ethanol. The precipitate was discarded, the soluble fraction concentrated *in vacuo* and the products separated by thin-layer chromatography on cellulose (solvent: = 3 parts 1 M ammonium acetate, 7 parts ethanol).

Figures 4–6 show the pH at which individual fractions focused, and incorporation of radioactivity into acceptor for each fraction. For the study shown in Fig. 4 (bottom), a set of focused fractions was concentrated 10-fold by ultrafiltration[11] before enzyme levels were measured.

RESULTS

Conditions specified for collection, processing and storage of plasma samples are critical for resolution of enzyme activities by electrofocusing. Delays in processing, or exposure of blood samples to temperatures of more than 4°C, resulted in altered focusing patterns.

All studies described here were carried out on plasma samples which were fresh, or had been stored for less than 14 days at −70°C, except for those shown in Fig. 3, where storage of 0.5–2 years was involved. In another study, we found a progressive loss of enzyme activity during storage of plasma samples at −70°C. A loss of 35 ± 5% would be expected during a 2-year storage interval. The data shown in Fig. 3 were corrected for enzyme loss during storage.

We found that either the column elution or the acid precipitation procedure afforded results which did not significantly differ. The former procedure was employed for all results shown here. Mg^{++} promoted the enzyme activity in fractions which focused at pH > 7.0 by 10–20%, but did not affect activity in other fractions. The 10 mM level was therefore routinely included in electrofocusing assays. Addition of 10 mM Mg^{++} did not significantly alter the level of total sialytransferase, measured with 50 μl plasma samples, and therefore was not included in these incubation mixtures. In the measurement of total plasma enzyme activity, incorporation of label into acceptor was proportional to the amount of plasma protein present, and was linear, under the conditions defined above, for at least 2 hours.

In studies of sialyltransferase activity in electrofocused fractions, chromatographic analysis showed that at least 60% of the initial concentration of radioactive CMP-sialic acid remained after 4 hr incubations of electrofocused fractions. The rate of incorporation of radioactivity into acceptor was proportional to the amount of enzyme protein present, and was linear for at least 4 hours. Substantial loss of the nucleotide–sugar substrate occurred only in fractions which focused a pH 3.5–4.0. In other studies, we found that plasma sialidases focused in these fractions.

When electrofocused samples were concentrated by dialysis prior to assay, activity in fractions with pH > 6.5 was lost, (Fig. 4, bottom). All other results shown here were therefore obtained without prior concentration of focused fractions.

In a series of studies of sialyltransferase activity in plasma from normal donors, we observed a substantial variation when we examined enzyme levels in plasma samples obtained from a single donor at 4 hr intervals (Fig. 1A), from three donors daily for three successive days (Fig. 1B) and in a group of 13 different individuals (Fig. 1C).

When the data from this normal group were compared with results obtained from 10 patients with benign breast disease and 20 individuals with primary or recurrent breast cancer, approx. 1/2 of the latter group showed a level of plasma sialyltransferase activity above the normal range (Fig. 2). Because of the variability encountered in all sample groups, we could not, however, distinguish patients with breast cancer solely on the basis of the total level of plasma sialyltransferase.

We also measured the level of total plasma sialyltransferase in a group of women at intervals following primary surgery for breast

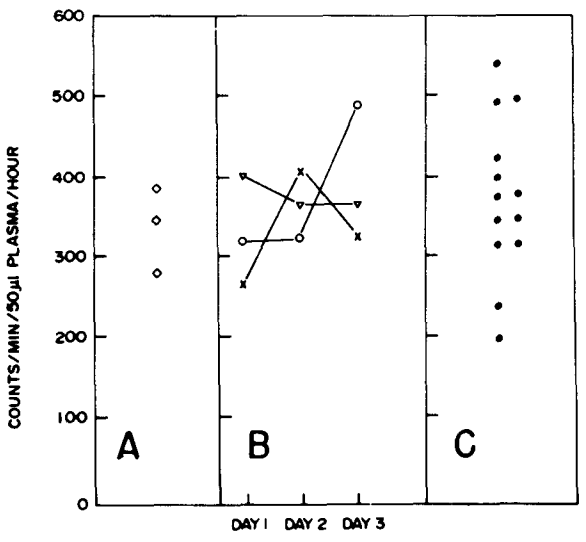

Fig. 1. Levels of sialyltransferase in plasma of normal donors. (A) Single donor, samples taken at 4 hr intervals; (B) Multiple daily samples taken from 3 donors; (C) Single samples obtained from 13 normal donors. Units: counts/min of CMP-[^{14}C]-sialic acid incorporated into desialated fetuin/60 min/50 µl plasma.

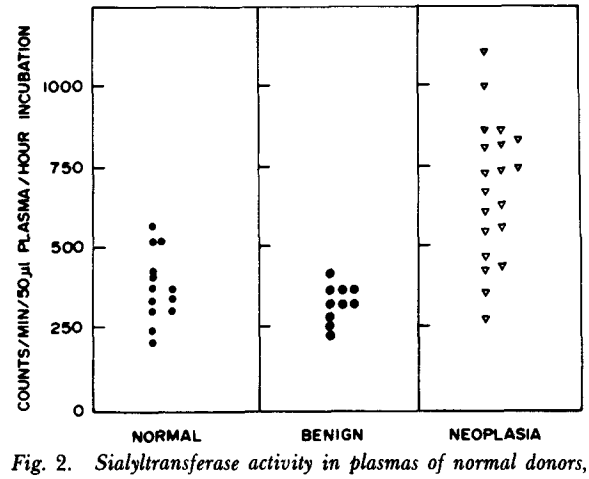

Fig. 2. Sialyltransferase activity in plasmas of normal donors, patients with benign breast disease and patients with primary or recurrent untreated breast cancer. Units are defined in the legend to Fig. 1.

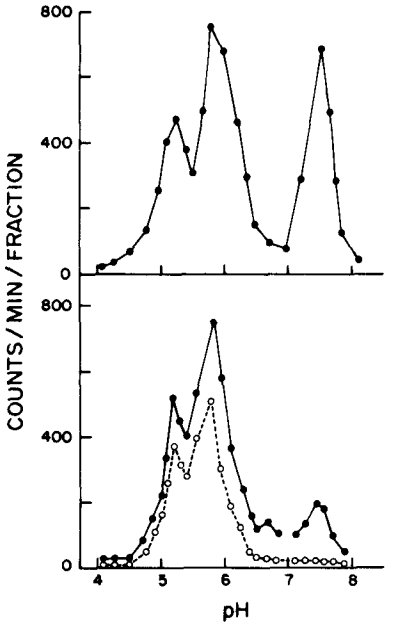

Fig. 4. Electrofocusing paterns of sialyltransferase in plasma of two normal donors. Top:-●-, samples assayed directly after electrofocusing; -○-, samples concentrated by membrane filtration prior to enzyme assays. Bottom: plasma from a patient with a mild upper respiratory infection. Data represent incorporation of radioactive sialic acid into acceptor during 4 hr incubations by enzyme fractions which focused at the indicated pH values.

cancer. The data shown in Fig. 3 do not indicate a progressive rise in enzyme level prior to the appearance of clinically-detectable metastatic disease.

Electrofocusing studies indicated presence of two major sialyltransferase isoenzymes in normal plasma (Fig. 4, bottom). These enzyme activities focused at or near pH 5.1 and 5.5. The level of a third isoenzyme, which focused at pH 7.5, was highly variable. An elevated level of total plasma sialyltransferase activity in normal donors was generally associated with an elevated level of the latter activity (Fig. 4, top).

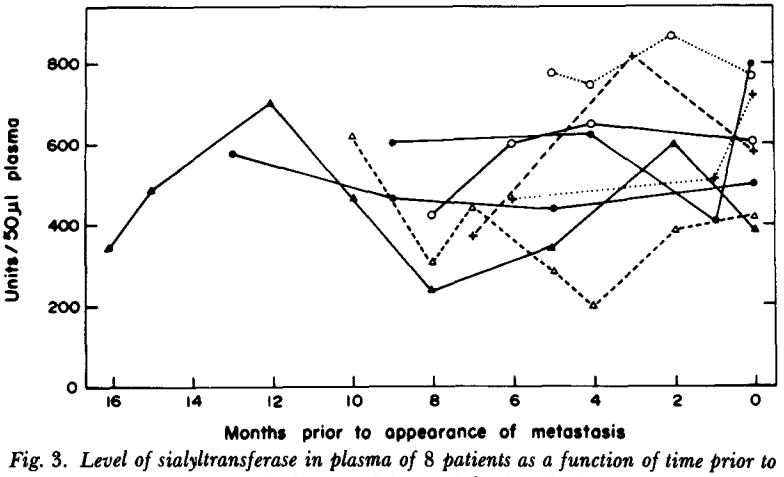

Fig. 3. Level of sialyltransferase in plasma of 8 patients as a function of time prior to appearance of metastatic disease. Units are defined in the legend to Fig. 1.

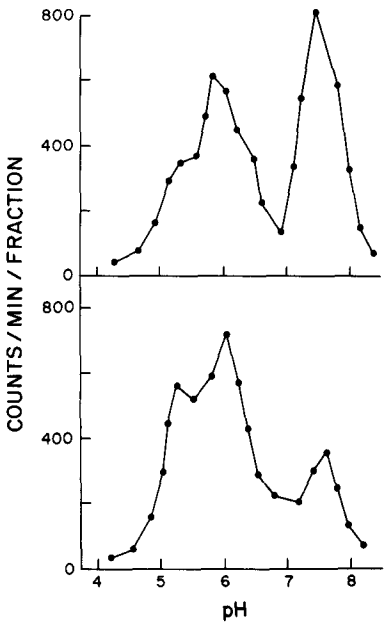

Fig. 5. Electrofocusing patterns from patients with rheumatoid arthritis (top) and benign breast disease (bottom).

An elevated enzyme activity with pI = 7.5 was generally found when we examined plasma samples from donors with respiratory infections or allergic reactions, suggesting that an inflammatory response may be a determinant of the level of this isoenzyme.

The focusing patterns shown in Fig. 5 are from patients with non-neoplastic pathologic status: rheumatoid arthritis (top) and benign breast disease (bottom). The high level of isoenzyme with pI > 7 in the former sample is in agreement with the hypothesis that this activity reflects an inflammatory process. The focusing pattern shown in Fig. 5, bottom, is typical of results found with four other such plasmas, indicating no significant enzyme activity at pI < 5 in benign breast disease.

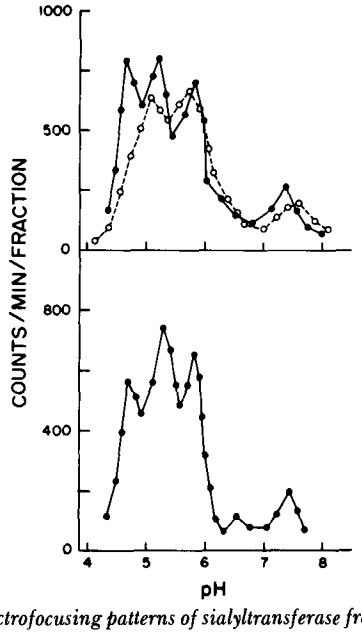

Fig. 6. Electrofocusing patterns of sialyltransferase from plasma of patients with breast cancer. Top:-●-, sample obtained prior to, and -○-, 6 months post surgery for primary tumor. Bottom: sample obtained from a patient with recurrent metastatic disease prior to initiation of drug therapy.

Electrofocusing studies on plasmas obtained from 12 patients with primary or recurrent breast cancer prior to initiation of any therapeutic programs showed presence of new enzyme activity, which focused at approx. pH 4.7. Typical patterns are shown in Fig. 6, top (primary tumor) and bottom (recurrent disease). In these plasmas, we also generally found enzyme activity which focused at pH 7.5 (10 out of 12 samples examined). The effect of surgery on one pattern is shown in Fig. 6 (top). A second sample (open circles) was obtained 6 months post-surgery when there was no clinical evidence of recurrent disease.

We found that all sialyltransferases studied here required acceptors terminating in galactose residues. Either desialated fetuin or phenyl-galactose[12] could be employed, the latter being an unambiguous acceptor for enzymes requiring a terminal galactose residue. Acceptors terminating in *N*-acetylglucosamine were not suitable. All enzyme activity was inhibited by 10 mM dithiothreitol.

DISCUSSION

In this study, we report data suggesting the presence of a new sialyltransferase isoenzyme in plasma associated with presence of primary or metastatic breast cancer. This activity shows a pI of approx. 4.7 and can be distinguished from isoenzymes present in normal plasma, which focus at pH 5.2 and 5.6. An additional enzyme, with pI = 7.5, appears to be associated with inflammatory processes.

It must be emphasized that a study of a larger patient population will be required before the general utility of plasma sialytransferase isoenzyme analysis can be assessed. Furthermore, the technique of electrofocusing is poorly adapted to screening of large numbers of plasma samples in a prognostic test procedure. A substantial loss of total enzyme activity is involved, requiring use of high specific-activity CMP-sialic acid. Removal of sialidase and other hydrolases during electrofocusing doubtless accounts for the presence of unaltered CMP-sialic acid after incubation of electrofocused fractions.

There have been several reports of elevated

levels of sialyltransferase activity in plasma or serum of animals [14–17] and humans [1–6,9] with neoplastic disease. But correlations between this elevation vs the extent of disease were generally found to be poor [1,9]. The present data provides an explanation for these observations, i.e. the cancer-associated isoenzyme represents, at most, only a fraction of the total plasma sialyltransferase. Moreover, the variable level of the enzyme activity focusing at pH 7.5 may be responsible for many false positives encountered. The finding of substantial variation in total enzyme activity among normal donors, and during serial samplings of given individuals, also argues against the potential use of total plasma sialyltransferase as a sensitive prognostic factor.

Plasma sialyltransferase activity has been partly characterized [12,18,19]. This activity apparently derives from host liver [8] and, perhaps, from erythrocytes [18]. Klohs *et al.* had reported that the level of sialyltransferase was higher in serum than in plasma samples [19]. This result might be related to the 'leakage' of enzyme from erythrocytes, as described by Kim *et al.* [18], during the clotting process.

In the rat mammary tumors, an association was found between capacity for metastasis vs level of plasma sialyltransferase activity [14,16,17]. These data were interpreted to indicate extensive 'shedding' of enzyme activity by rapidly-metastasizing cell lines. The finding that plasma sialyltransferase was elevated in plasma of patients with malignant but not benign breast disease is in agreement with the animal data, i.e. appearance of additional sialyltransferase activity in plasma is associated with a neoplastic process. The development of a rapid means of detecting the presence of new sialyltransferases in plasma could provide a useful procedure for assessing patient prognosis after initial surgery.

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