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Evaluation of Galactosyltransferase Isoenzyme II in a Human Colon Carcinoma-derived Cell Line, HCT–8*

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Abstract—*Polyacrylamide gel electrophoresis of galactosyltransferase (GT) extracted from a human colon adenocarcinoma cell line, HCT-8, demonstrated the presence of two peaks of activity: a slow-moving peak, referred to as GT-II, and a more anodally migrating peak, designated as GT-I, which was also found for normal human serum. However, if GT solubilized from HCT-8 cells was separated by isoelectric focusing, no unique isoenzymes could be detected. Total GT activity from HCT-8 cells was purified by α-lactalbumin–Sepharose affinity chromatography followed by ion exchange chromatography on either DEAE-cellulose or FPLC using a Mono Q anion exchange resin. Three major peaks of activity were resolved from anion exchange chromatography. Electrophoresis of each peak revealed a GT pattern identical with that originally observed for the crude (detergent) solubilized homogenate. No enrichment of either GT-I or GT-II was observed in the three enzyme fractions. The data suggest that GT-II may be an artifactual activity of cancer cells composed of GT-I associated with some contaminating protein.*

INTRODUCTION

ALTERATIONS in cell surface glycoconjugates have been reported which are believed to be characteristic or obligatory for cancer and metastases. Glycosyltransferases are enzymes that catalyze the transfer of monosaccharides from nucleotide-sugars to oligosaccharide chains of glycoproteins and glycolipids, and hence the significance of these enzymes to malignant behavior is, in part, related to changes in the carbohydrate structure of these molecules upon transformation. Indeed, alterations in these compounds may be indicative of a more fundamental enzymatic change.

Glycosyltransferases, which are membrane-bound enzymes within the cell, are also detected as soluble activities in the bloodstream and body fluids [1–3]. Elevated glycosyltransferase activities have been reported in malignant cells and tissues [4, 5] as well as in the sera of cancer patients [4–8]. In particular, serum elevations of both sialyltransferase and galactosyltransferase activities have been demonstrated to correlate with tumor burden, raising the possibility that these enzymes might be useful diagnostic tools for detecting the presence of tumor tissue.

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Podolsky and Weiser [9] reported that a unique isoenzyme of galactosyltransferase was present in sera of cancer patients and could be separated by electrophoresis from galactosyltransferase found in healthy individuals. The cancer-associated isoenzyme, referred to as galactosyltransferase II (GT-II), has been reported by these investigators to be detectable in approximately 70% of all cancer patients tested (for review, see [10]).

In view of the potential significance of GT-II as a prognostic indicator for neoplasia and its proposed role in metastasis [11], we endeavored to isolate this isoenzyme for further evaluation of its utility as a cancer marker and to develop specific inhibitors which might be useful in elucidating the function of GT-II in cancer. We demonstrated the presence of GT-II and GT-I in a human colon tumor cell line and utilized this source of enzyme to carry out our isolation procedures.

MATERIALS AND METHODS

Solubilization of membrane-associated galactosyltransferase activity.

HCT-8 human colon adenocarcinoma cells [12] (generously provided by Dr J. Bertino, Yale University) were grown in 40 Corning 150-cm^2 tissue culture flasks in RPMI-1640 medium supplemented with 10% fetal calf serum and 25 mM

HEPES, pH 7.0. When cells reached a confluent monolayer, the culture medium was removed and discarded, and cells were detached from the plate with a rubber policeman into 0.02 M Tris–HCl buffer, pH 7.0, and 2 mM EDTA. The cells were washed two times in buffer and the resulting cell pellet was resuspended in 2 vol of a medium consisting of 20 mM Tris–HCl buffer, pH 7.0, 40 mM DTT, 0.5 mM TPCK, 0.5 mM TLCK, 2 mM EDTA and 1% (3[3-cholamidopropyl] dimethylamino)-1-propane sulfonate (CHAPS). The suspension was then sonicated to disrupt the plasma membrane and the homogenate was incubated at 37°C for 1 hr to ensure virtually complete solubilization of GT activity from the membranes. The homogenate was centrifuged at 12,000 ***g*** for 10 min at 4°C, the pellet extracted two additional times with the above medium and the combined supernatants centrifuged at 100,000 ***g*** for 60 min at 4°C. The pellet was discarded and to the supernatant material 0.5% streptomycin sulfate (w/v) was added in order to precipitate free nucleic acids. The material was stirred for 30 min at 4°C prior to centrifugation at 12,000 ***g*** for 10 min at 4°C. The resulting supernatant was assayed for GT activity and this material was used for further studies.

Assay of galactosyltransferase activity

GT activity was measured as previously described [13] with the following modifications. The assay medium (total volume, 160 μl) consisted of 80 μl 1 M HEPES, pH 7.0, 5 μl of 1 M manganese acetate, 20 μl of 20 mg/ml ovalbumin, 5 μl of UDP[^{14}C]galactose (6.7 μCi/ml sp. act., 337 mCi/mmol, New England Nuclear, Boston, MA) and 50 μl of sample. The assay mixture was incubated at 37°C for 1 hr.

Electrophoresis

Polyacrylamide gel electrophoresis (using 8% gels) was carried out following procedures described by Podolsky and Weiser [9]. Electrophoresis was performed at a constant current of 8 mA/gel for 2 hr at 4°C. After electrophoresis, gels were sliced into 2.5-mm fractions with the aid of a Gilson Gel Slicer (Gilson Electronics, Middleton, WI) and homogenized in 150 μl of 0.1 M sodium cacodylate buffer, pH 5.3, and 0.5 mM *N*-acetylglucosamine (GlcNAc). The fractions were incubated for 1 hr at 37°C prior to assay of GT activity.

Isoelectric focusing

Isoelectric focusing was carried out in granulated dextran gels and 5% ampholine (LKB) over a pH gradient of 3.5–10 in an LKB multiphor 2117 following procedures described by the manufacturer. Sera from six normal healthy individuals and three GT preparations from HCT-8 cells were examined. Samples were electrofocused for 18 hr at 10°C at a constant power of 8 W. Following the run, the pH was measured across the gel using a surface pH electrode. A 30-slot grid was pushed into the gel to prevent diffusion of the focused bands and to facilitate recovery of the samples. Gel from each slot was placed in a plastic elution column with a fine mesh covering the bottom. Distilled water (2 ml) was used to elute the protein from the gel and each sample was assayed for GT activity.

Purification of galactosyltransferase

GT extracted from HCT-8 cells was affinity chromatographed on an α-lactalbumin–Sepharose column following procedures of Podolsky and Weiser [14]. The single peak of GT activity eluted from the column was concentrated 10-fold with a Millipore CX-30 ultrafiltration unit and the concentrate chromatographed using a Pharmacia FPLC system equipped with a Mono Q anion exchange prepacked column. The sample was eluted with 20 mM Tris–HCl buffer, pH 7.5, 20% glycerol, and a NaCl gradient of 0–750 mM. Each fraction (1 ml) was monitored for protein at a wavelength of 280 nm and assayed for GT activity.

RESULTS

Electrophoresis of GT solubilized from HCT-8 cell membranes revealed an enzyme activity profile strikingly similar to that reported by Podolsky and Weiser [9] for GT from human malignant effusions (Fig. 1a). A slow moving band of enzyme activity was consistently detected in gel slices 4 and 5. Like GT-II, this activity was only found in extracts of tumor cells; in this case, the HCT-8 colon tumor cells. GT activity from HCT-8 cells was also observed in that region of the gel corresponding to the normal isoenzymes of galactosyltransferase collectively referred to as GT-I. Electrophoresis of normal human serum revealed enzyme activity only in the GT-I region of the gel (Fig. 1b). In contrast to that reported by Podolsky and Weiser, however, the GT-I activity from both samples appeared to be comprised of at least two and possibly three isoenzymes (Fig.1). Isoelectric focusing also revealed the presence of several GT isoenzymes (Fig. 2). Five major isoenzyme forms of GT from either normal human serum or HCT-8 cells were observed with isoelectric points at pH 4.25, 4.90, 5.20, 5.75 and 6.44. Isoenzyme activities from HCT-8 cells (Fig. 2a) were elevated at lower pH values (4.25, 4.90 and 5.20) when compared to normal human serum (Fig. 2b) but no unique isoenzymes could be detected, although their presence cannot be ruled out since recovery of GT activity from these gels was only 45%. Unlike GT

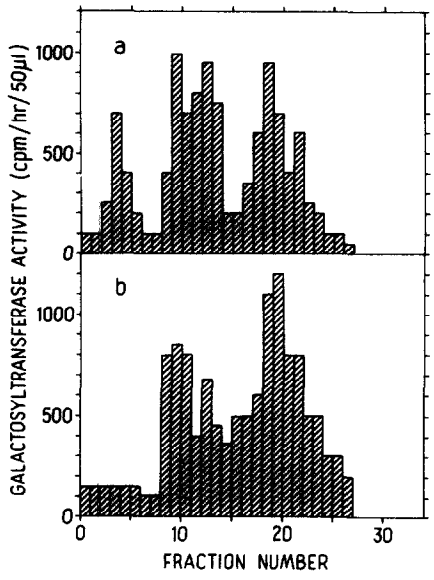

Fig. 1. Electrophoresis of GT solubilized from HCT-8 cells (a) and normal human serum (b).

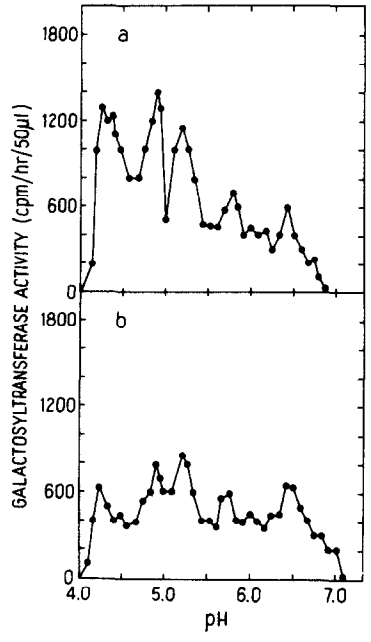

Fig. 2. Representative profiles of GT from HCT-8 cells (a) and normal human serum (b) on preparative isoelectric focusing using a granulated dextran gel.

isoenzymes studied by Podolsky and Weiser [15], soluble enzyme activity from HCT-8 cells was not contaminated with immunoglobulins. Double immunodiffusion analysis of soluble enzyme activity failed to demonstrate the presence of either IgG or IgA (data not shown).

The procedures used to purify GT-I and GT-II isoenzymes from HCT-8 cells are summarized in Table 1. Virtually all of the GT activity was solubilized by CHAPS detergent from HCT-8 membrane, with less than 2% of the total GT activity associated with the 100,000 ***g*** membrane pellet. This activity was then applied to an α-lactalbumin–Sepharose column. Essentially all of the GT activities bound to the column in the presence of 5 mM GlcNAc (Fig. 3). When GlcNAc was omitted from the buffered medium, the GT activity was recovered as a single peak. This GT activity was pooled, concentrated 10-fold and applied to an anion exchange FPLC Mono Q column with a linear salt gradient of 0–750 mM. Three major areas of GT activity were observed from the FPLC column (Fig. 4). The enzyme activity from each of these areas was pooled, concentrated and electrophoresed in order to determine if GT-I and GT-II had been separated by anion exchange chromatography as reported by Podolsky and Weiser [14]. In addition, individual fraction tubes

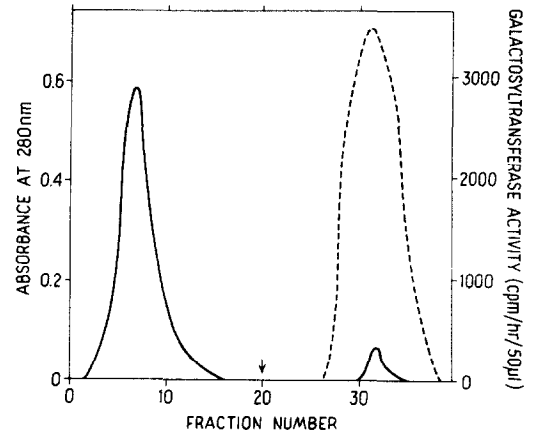

Fig. 3. Chromatography of GT solubilized from HCT-8 cells on α-lactalbumin–Sepharose 4B. Solubilized GT was dialyzed against 25 mM sodium cacodylate buffer, pH 7.2 + 40 mM KCl, adjusted to 5 mM GlcNAc, and applied to the column (0.9 × 30 cm). The column was washed with the above buffer + 5 mM GlcNAc until absorbance (——) was near zero. GT activity (- - -) was recovered by eluting with buffer only (at arrow).

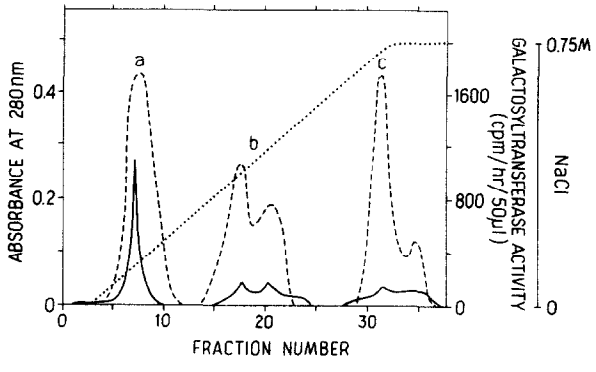

Fig. 4. FPLC of concentrated GT fraction from α-lactalbumin–Sepharose column. GT activity (- - -) was eluted in a linear NaCl gradient (·····) of 0–750 mM in 20 mM Tris–HCl buffer, pH 7.5 + 20% glycerol on a prepacked Mono Q anion exchange column. Three peaks of GT activity (a–c) were isolated from the column. Absorbance (——).

Table 1. *Purification of galactosyltransferase from HCT-8 cells*

Step	Sp. act. (units*/mg)	Total protein† (mg)	Purification (fold)	Yield (%)
Homogenate	5.5	2025	1	100
12,000 ***g*** supernatant	8.25	1350	1.5	100
100,000 ***g*** supernatant	17.82	540	3.24	86.4
Streptomycin precipitation	23.04	383	4.19	79.2
α-Lactalbumin–Sepharose	642.4	10.9	116.8	63.0
FPLC in exchange‡	528.2	9.7	96.0	46.0

*1 unit = 1 nmol of galactose incorporated/60 min.
†Protein was determined by the method described by Bradford [30].
‡The three major fractions eluted from the column were pooled and protein and enzyme activity determined.

from each area were also electrophoresed in non-denaturing polyacrylamide gels. In both instances we observed GT activity in areas of the gels representative of GT-I and GT-II (Fig. 5). Numerous FPLC separations as well as chromatography on DEAE–cellulose (data not shown) resulted in the same activity profile from the columns and on electrophoretic gels.

DISCUSSION

Numerous studies have examined the potential value of serum glycosyltransferases as prognostic indicators for cancer (for review, see [10]). Studies by Podolsky and Weiser [9] demonstrated that total serum galactosyltransferase from cancer patients was not statistically elevated beyond that activity found in disease controls. With the possible exception of ovarian cancer, subsequent studies tend to support this finding, although exceptions have been noted [6–8, 16].

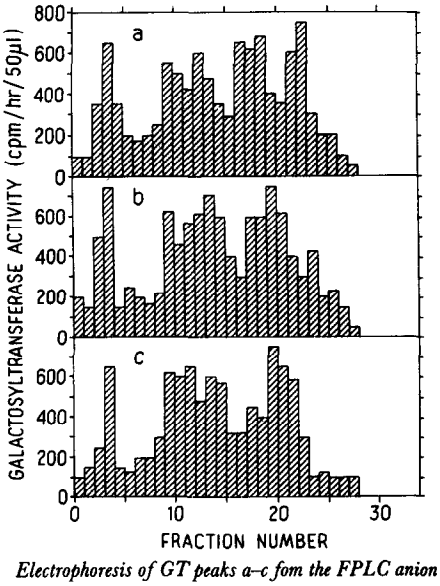

Fig. 5. *Electrophoresis of GT peaks a–c fom the FPLC anion exchange column.*

Podolsky and Weiser also observed a unique isoenzyme of galactosyltransferase (designated GT-II) in the sera of cancer patients which was absent from sera of normal and most disease controls. In their clinical studies [17–19] GT-II was detected in the sera of over 70% of all cancer patients tested. Both GT isoenzymes have been isolated to homogeneity and extensively characterized [14], and recently monoclonal antibodies to these isoenzymes were also isolated [20].

In spite of the potential clinical value of GT-II, only three other laboratories have reported detecting this isoenzyme [21–23]. All three reports, however, were in abstract form, and none of these investigators have reported further on GT-II. All other studies of GT isoenzymes have failed to detect the presence of GT-II in sera or body fluids of cancer patients [24–28].

Kim *et al.* [26] were unable to detect GT-II from human malignant effusions although they did detect a small amount of another isoenzyme associated with cancer by anion exchange chromatography. Davey *et al.* [28] were also unable to detect GT-II in the sera from cancer patients using high resolution agarose isoelectric focusing. Gerber *et al.* [24] purified GT activities from human milk, pooled amniotic fluid and malignant ascites by affinity chromatography. They found by SDS gel electrophoresis only one protein staining band, with a molecular weight of 55,000 daltons, which closely agrees with that reported by Podolsky and Weiser for isolated GT-I [14].

In the present study, we examined GT activity from HCT-8 colon adenocarcinoma cells. Similar to that reported for the human leukemia CCRF-CEM cells [29], GT was not released as soluble enzyme activity into the tissue culture medium (data not shown). GT activity from these cells could only be detected when disrupted cells were first treated with detergent. Polyacrylamide gel electrophoresis of this solubilized enzyme activity

revealed the presence of a slow moving peak of GT not found in normal human serum which appears to correspond to GT-II, and a major, more anodally moving area of activity also found in normal human serum which appears similar to GT-I. Unlike the GT-I gel pattern reported by Podolsky and Weiser [9], the electrophoretic pattern of GT for HCT-8 cells and normal human serum suggest that the normal GT is comprised of several isoenzymes (Fig. 1). In support of this, isoelectric focusing of the same samples also reveals the presence of several GT isoenzymes (Fig. 2). However, in accord with previous studies described above [24, 27], we were unable to demonstrate any unique GT isoenzymes from HCT-8 cells by isoelectric focusing (Fig. 2). Moreover, purification of GT by affinity chromatography and anion exchange chromatography, was unable to achieve any separation of GT-II from GT-I. Our inability to obtain any degree of chromatographic separation of GT isoenzymes is difficult to understand. However, it is intriguing that the study of human GT by Wilson *et al.* [15] failed to report any separation of GT-II from GT-I using procedures similar to Podolsky and Weiser [14], and, for that matter, ours. We believe this is a significant omission, particularly since the second author of that study was Weiser. On the other hand, Podolsky and Isselbacher [20] have recently isolated monoclonal antibodies to GT which were isolated following procedures of Wilson *et al.* [15], and these monoclonal antibodies appear to have selective specificities to either GT-I or GT-II.

Perhaps the inability to isolate GT-II or detect any unique isoenzymes by isoelectric focusing can be explained if one proposes that a small amount of contaminating protein becomes associated with GT in cancer cells which cannot be totally separated from the enzyme by electrophoresis but is completely dissociated in isoelectric focusing. This would explain the discrepancies between GT studies employing electrophoresis and those with isoelectric focusing. It is also conceivable that the association of this contaminating protein with GT is strong enough to follow GT through several purification steps. Depending on the amount of contaminating protein and the nature of its association with GT, both of which may be a function of the tissue source, it may or may not be possible to purify GT-I and GT-II. In this regard, Wilson *et al.* [15] discovered that IgA and IgG were associated with purified GT isolated from human malignant effusions by affinity chromatography. However, in the present study, GT from HCT-8 cells does not appear to be complexed with either immunoglobulin.

In conclusion, we have detected the cancer-associated isoenzyme of GT, GT-II, in homogenates of a human colon adenocarcinoma cell line, HCT-8, but have been unable to separate this enzyme activity from the normal isoenzyme(s), GT-I. We suggest that GT-II may represent GT-I with a contaminating protein associated with it, causing the enzyme to migrate slower in non-denaturing gel electrophoresis. Our data as well as others point to the need to re-evaluate and recharacterize GT-II in an effort to determine the nature of this enzyme and its importance in cancer.

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