Europ. J. Cancer, Vol. 15, pp. 1351–1356
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0014-2964/79/1101-1351 $02.00/0

Glycosyltransferases in Metastasizing and Non-Metastasizing Rat Mammary Tumors and the Release of these Enzymes in the Host Sera

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Abstract—*In order to study the role of glycosyltransferases in the process of metastasis, levels of sialyltransferase and two* N-*acetylglucosaminyltransferases in three established strains of spontaneously metastasizing rat mammary tumors were compared with four non-metastasizing strains. Activity of sialyltransferase was significantly lower in the metastasizing tumor, while the levels of both the* N-*acetylglucosaminyltransferases were comparable in the two groups. Levels of five glycosyltransferases were determined in the serially drawn sera of a group of rats bearing a representative metastasizing tumor (SMT-2A), a representative non-metastasizing tumor (MT-W 9B) and a group of control female rats, from the day of tumor transplantation up to the* 6*th* week *of tumor development, at weekly intervals. In the sera of rats bearing SMT-2A, the levels of sialyl-and galactosyltransferases were significantly* ($P < 0.05$) *elevated from the* 3*rd to* 4*th* week *after tumor transplant; while no such elevation was observed for those bearing MT-W9B, compared to the control group of rats. Levels of two fucosyltransferases, one* N-*acetylglucosaminyltransferase, were not elevated significantly in any of the tumor bearing rats. No significant change in the levels of protein-bound hexose, fucose or hexosamine was observed in these tumor bearing rats compared to control group.*

INTRODUCTION

IT HAS been reported earlier that spontaneously metastasizing rat mammary tumors are non-immunogenic and readily shed their cell surface antigens into systemic circulation of the host [1]. As a result of this glycocalyx shedding, the levels of a number of plasma membrane marker enzymes were diminished in the metastasizing tumor and were inversely proportional to the metastasizing capacity of the tumor [2]. Glycoproteins and glycolipids are the 2 important constituents of the cell plasma membrane. These glycoconjugates are synthesized by specific glycosyltransferases by the step-wise transfer of monosaccharides from the nucleotide-sugar to suitable acceptors. The glycosyltransferases are specific for the donor nucleotide-sugar and the acceptor glycoconjugate. It has been proposed that together with the glycoconjugates, the glycosyltransferases reside on the outside of the cell-surfaces (ectoenzymes) and play significant role in the intercellular communication [3]. In order to study the role of glycosyltransferases in the process of cell-surface shedding and cancer metastasis, we compared the activities of one galactosyl- and two fucosyltransferases in the metastasizing tumours with those in the non-metastasizing ones and found that the levels of galactosyl- and one of the fucosyltransferases (fucosyltransferase B), were significantly higher in the metastasizing group; whereas those of a second fucosyltransferase (fucosyltransferase A) were comparable in the two groups [4, 5].

Assay of a sialyltransferase and two *N*-acetylglucosaminyltransferases in these two groups of tumors demonstrate that in contrast to galactosyl- and fucosyltransferase B, the levels of sialyltransferase were lower in the metastasizing tumors. The activities of the two *N*-acetylglucosaminyltransferases were comparable in both types of tumors. Five glycosyltransferases were assayed in the sera of tumor hosts and a group of control female rats, at weekly intervals starting from the day of

Accepted 22 March 1979.

tumor transplant to the 6th week of tumor development. Results of this follow-up are reported here.

MATERIALS AND METHODS

Materials

CMP [4-^{14}C]-sialic acid (1 Ci/mole), UDP [1-^{14}C]-*N*-acetyl D-glucosamine (43 Ci/mole), GDP [U-^{14}C]-L-fucose (174 Ci/mole), UDP [U-^{14}C]-galactose (301 Ci/mole) and triton X-100 were obtained from New England Nuclear, Boston, Mass. ATP, NAD^+, CTP, DTT, unlabeled UDP-galactose, unlabeled UDP-*N*-acetylglucosamine, fetuin (grade IV) and ribonuclease A were obtained from Sigma Chemical Co., St. Louis, Mo. Ovalbumin (3x crystalline) was obtained from Miles Laboratories, Inc., Elkhart, Indiana. All other chemicals were the purest available from commercial sources.

Preparation of sialic acid acceptor from fetuin

Acceptor for sialic acid, asialofetuin (ASF) was prepared by mild acid hydrolysis of fetuin as described earlier [4, 5].

Tumor transplantation and preparation of homogenate

Procedures for tumor transplantation have been described earlier [1, 2]. When the transplanted tumors grew to 1–2 cm in average diameter, they were removed and promptly dissected free of necrotic tissue and fat. Homogenates from the tumors were then prepared as described earlier [2].

Assay of glycosyltransferases

Incubation media for sialyltransferase contained in 100 μl, 50 mM Tris–HCl, pH 7.0; 10 mM $MgCl_2$; 0.1% triton X-100; 100 nmole of CMP [4-^{14}C]-sialic acid (200,000 dis/min); 1 mg of ASF and about 50 μg of homogenate protein. Incubation was for 1 hr at 37°C.

Assay media for *N*-acetylglucosaminyltransferase contained 50 mM Tris-maleate, pH 7.1; 0.5% triton X-100; 50 mM $MnCl_2$; 2 mM NAD^+, 5 μl of UDP [1-^{14}C]-*N*-acetylglucosamine (100,000 dis/min); 1mM unlabeled UDP-*N*-acetylglucosamine; about 100 μg of homogenate protein; and 1 mg either of ovalbumin (*N*-acetylglucosaminyltransferase 1) or ribonuclease A (*N*-acetylglucosaminyltransferase 2) in a total volume of 50 μl. The incubation was run for 2 hr at 37°C. The reaction was terminated by 1% phosphotungstic acid in 0.5 N HCl and the precipitated protein was processed for radioactivity determination as described earlier for the assay of other glycosyltransferases [4, 5].

For the assay of these enzymes in the serum similar incubation conditions were used except the omission of triton X-100, which had no effect on serum enzyme and replacement of the homogenate by 5–10 μl of serum.

Transfer of saccharides to the endogenous acceptor(s) were kept to a minimum by using low concentrations of enzyme protein. In tumor homogenates, sialyltransferase had endogenous values ranging from 3 to 10% of the radioactivity in the presence of ASF. Endogenous values in the tumor homogenate for *N*-acetylglucosaminyltransferase 1 ranged from 2 to 10% and those for *N*-acetylglucosaminyltransferase 2 were 13–36%. In the serum, the values of endogenous incorporation were as follows: sialyltransferase, 9–15%; galactosyltransferase, less than 1%; fucosyltransferase A, 9–33%; fucosyltransferase B, 4–10%; *N*-acetylglucosaminyltransferase 1, 6–11%. Endogenous values were always subtracted for the calculation of specific activity in the presence of externally added acceptor.

Collection of serum

Female rats (W/F_u) were divided into 3 groups each containing 6 rats. One group was transplanted with non-metastasizing tumor cells (MT-W9B), a second group with metastasizing tumor cells (SMT-2A), while the control group received the injection of media only. Blood was drawn before injection of tumors and at weekly intervals thereafter from the orbital veins from all the rats bearing tumors and controls. Serum was separated after clotting in cold by centrifugation at 0–2°C, and stored at −20°C or −80°C.

Determination of protein and monosaccharide contents of the sera

Total serum protein was determined by the Lowry procedure [6]. Protein-bound hexose, hexosamine, and fucose in the sera were determined by the method of Winzler [7]. All other procedures have been described earlier [4, 5].

RESULTS

Optimum conditions of assay

All the homogenates were prepared from

tumors which grew to 1–2 cm in diameter in an average period of 3–4 weeks. The assays were done under conditions when the reaction rate was linear with respect to both time of incubation and the amount of enzyme protein. Amount of enzyme protein per tube was kept as low as possible so that the endogenous glycosyltransferase activity (i.e., activity in the absence of added acceptor) and hydrolysis of the donor nucleotide sugar were both minimum. Pyrophosphatase present in the tumor homogenates marginally hydrolyzed CMP-sialic acid, however, UDP-*N*-acetylglucosamine was extensively hydrolyzed.

In order to check the hydrolysis of nucleotide-sugars by pyrophosphatases, the reaction products were analyzed by paper chromatography as described earlier [5]. One hour after incubation with the homogenate from a representative non-metastasizing tumor (MT-W9B), 80–90% of the added CMP-sialic acid remained intact, while it was only 5–10% for UDP-*N*-acetylglucosamine. In the metastasizing tumors, pyrophosphatase levels were low, and usually 60–70% of the added UDP-*N*-acetylglucosamine was left intact after the routine incubation. Inclusion of 20 mM NAD^+ protected UDP-*N*-acetylglucosamine from pyrophosphatase hydrolysis, and 2–3 fold stimulation of the *N*-acetylglucosaminyltransferases were observed for MT-W9B in the presence of this nucleotide. NAD^+ had marginal effect on these glycosyltransferases present in the SMT-2A homogenate.

Optimum pH, optimum concentrations of donor nucleotide-sugars and acceptors were established for each glycosyltransferase, and assays were done under those conditions for comparison between metastasizing and non-metastasizing tumors.

Sialyltransferase was stimulated by only 10–20% in the presence of 10–20 mM $MgCl_2$. *N*-acetylglucosaminyltransferase, on the other hand, had an absolute requirement for Mn^{2+}.

Triton X-100 stimulated the activity of *N*-acetylglucosaminyltransferase by 2–3 fold, while sialyltransferase was stimulated by 30–70% over the control value in the tissue homogenates. Triton X-100 had no effect on serum enzymes.

Neither the optimum conditions for the transfer of monosaccharides nor the ionic requirements of the enzymes differed significantly when the enzyme source was either a non-metastasizing or a metastasizing tumor. Similarly, the properties of the serum enzymes were also identical for rats bearing either types of tumor or no tumor at all. We did not investigate whether the optimum conditions of assay changed in the tumor or in the serum with the progress of tumor development.

Sialyltransferase and N-acetylglucosaminyltransferases in the metastasizing and non-metastasizing rat mammary tumors

Sialyltransferase was assayed using ASF as acceptor in homogenates from three metastasizing and four non-metastasizing strains of rat mammary tumors. Data shown in Table 1 indicate that the specific activity of this enzyme is about 2 fold higher in the non-metastasizing tumors compared to the metastasizing group.

N-acetylglucosaminyltransferase was assayed using two acceptors, ovalbumin (*N*-

*Table 1. Activities of glycosyltransferases in rat mammary tumor homogenate**

Tumor strain	Metastasizing capacity†	Activity (pmole/hr/mg protein)‡		
		Sialyl-	*N*-acetyl-glucosaminyl-1	*N*-acetyl-glucosaminyl-2
MT-W9B	0	3578±405	1221±141	156±21
MT-W9A	0	6606±361	1360±168	135±18
MT-100	0	5274±400	1160±96	133±25
MT-66	0	6210±824	1569±180	208±35
SMT-077	++	3355±396	1030±119	123±21
TMT-081	+++	2084±180	1143±150	180±31
SMT-2A	+++	2445±642	1123±180	190±29

*Incubation conditions have been described under Materials and Methods.

†Metastasizing capacity was rated arbitrarily as 0=negative, ++=marked, +++=extensive.

‡The specific activity values are mean±S.D. of at least 3 independent determinations.

acetylglucosaminyltransferase 1) and ribonuclease A (*N*-acetylglucosaminyltransferase 2). The first enzyme probably transfers *N*-acetylglucosamine to the peripheral mannose and *N*-acetylglucosamine moieties, while the second enzyme is responsible for the formation of the first carbohydrate–peptide bond by the transfer of *N*-acetylglucosamine to asparagine [8]. Results presented in Table 1 indicate that there is no significant difference in the levels of either of these two *N*-acetylglucosaminyltransferases between the two groups of tumor.

Levels of glycosyltransferases in the sera

Evidences for the shedding of plasma membrane constituents into the surrounding media have been obtained *in vitro* as well as *in vivo* [9]. Since a part of the glycosyltransferases are plasma membrane bound ectoenzymes [4, 5], it is conceivable that some of these glycosyltransferases shed from the tumor cells will appear in the host sera. Elevation of sialyltransferase in the sera of the metastasizing group of rats have been demonstrated by Bernacki and Kim [10]. Levels of five glycosyltransferases were serially determined in the sera of three groups of rats. Group 1 was injected with cells from SMT-2A, group 2 with cells from MT-W9B, while group 3 was the control which received the media in which tumor cells were suspended. Each group had six W/F_u female rats of similar age and weight. Serum was drawn from these rats before tumor transplant and at weekly intervals. The results of this experiment are summarized in Fig. 1. Data of sialyltransferase, which was assayed by a different procedure from that of Bernacki and Kim [10], were also included for comparison. It can be seen that besides sialyltransferase, galactosyltransferase was also significantly elevated ($P<0.05$) in the sera of rats bearing metastasizing SMT-2A tumor from the 3rd week after tumor transplant. No such elevation was observed for the rats bearing non-metastasizing MT-W9B tumor. Levels of *N*-acetylglucosaminyltransferase 2 were negligible in the sera of these rats. Activities of fucosyltransferase A, fucosyltransferase B and *N*-acetylglucosaminyltransferase 1 were detectable in the sera, but their values were similar in the 3 groups of rats all through the period of tumor development.

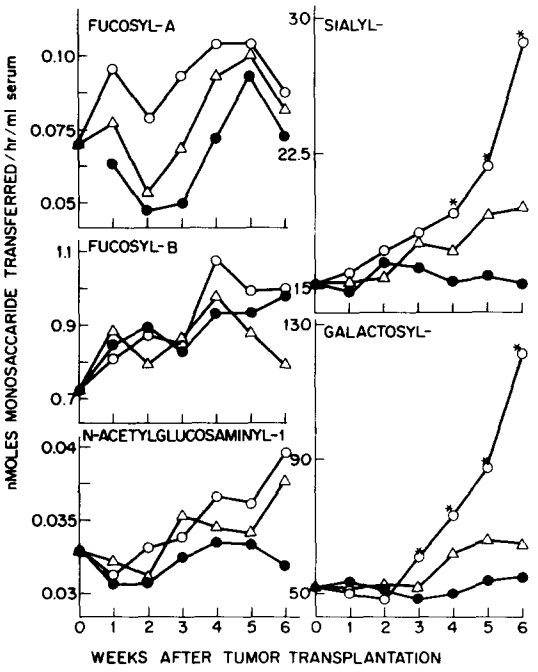

Fig. 1. Serial determination of glycosyltransferases in the sera of rats bearing nonmetastasizing and metastasizing mammary tumors and control group of rats. The procedure for the determination of glycosyltransferases have been described under Materials and Methods. Mean of at least 6 values are plotted for SMT-2A (○), MTW9B (△), control rats (●). Asterisk indicates statistical significance ($P<0.05$).

Levels of protein-bound hexose and hexosamine in the sera of tumor-bearing rats

Bernacki and Kim [10] demonstrated that in the sera of rats bearing SMT-2A tumors, the levels of sialoconjugates were elevated concomitantly with the increase in the activity of sialyltransferase. In order to establish such a relationship, amounts of protein bound hexose, hexosamine and fucose were determined in the serially drawn sera of the three groups of rats. The results summarized in Fig. 2 indicate that there was no significant difference ($P>0.05$) in the levels of these sugars in the three groups of rats. In the metastasizing group, the total protein content of the sera dropped significantly on the 6th week after tumor transplant.

DISCUSSION

Comparison of the activities of glycosyltransferases in the homogenates from metastasizing and non-metastasizing rat mammary tumors demonstrates that in the metastasizing group, the activities of galactosyltransferase [4] and fucosyltransferase B [5] were high, while the activity of sialyltransferase was low, while the levels of fucosyltransferase A [5], *N*-acetylglucosaminyltransferase 1 and *N*-acetylglucosaminyltransferase 2 were comparable in the two groups of tumor. Bernacki

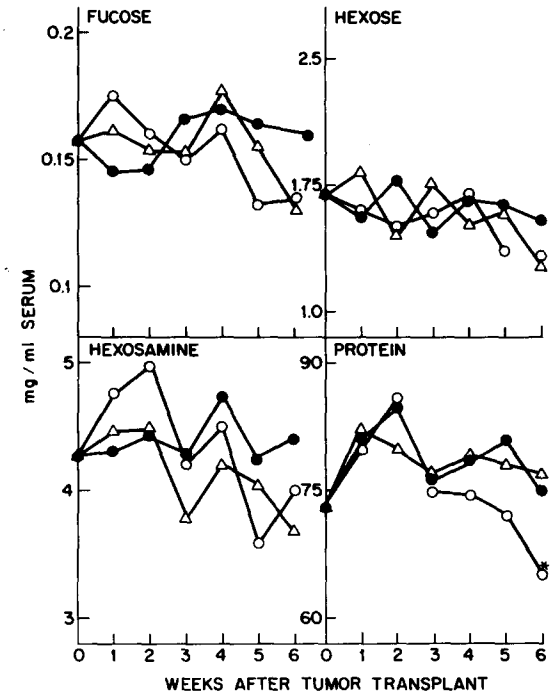

Fig. 2. Serial determination of protein-bound sugar and sugar-amine in the sera of rats bearing metastasizing and non-metastasizing mammary tumors and control rats. For details of the procedures see Materials and Methods and legend to Fig. 1 for the meaning of symbols.

and Kim [10] observed that the specific activity of sialyltransferase in the microsomes of SMT-2A were higher than that of MT-W9B. In these studies, enzyme assayed in homogenates prepared from SMT-2A tumors obtained on 5 different occasions showed specific activity values ranging from 1820 to 3365 (pmole/hr/mg protein) with a mean of 2445, while those for MT-W9B ranged from 3097 to 4034 with a mean value of 3578 ($n=5$). Thus, the specific activity of sialyltransferase in the MT-W9B is significantly higher than SMT-2A ($P<0.05$), although it has the lowest value among the non-metastasizing strains, overlapping with those of the metastasizing tumors (Table 1).

The differences in the levels of glycosyltransferases in the metastasizing and non-metastasizing tumors suggest that the spectrum of glycoproteins synthesized by the two types of tumors are probably different. The non-metastasizing tumors synthesize more with sialic acid as the terminal monosaccharide, while the metastasizing tumors synthesize the ones ending in fucose.

Although the activity of sialyltransferase is lower in the metastasizing tumor, it is interesting that the serum levels of this enzyme were significantly higher beginning with the 4th week of tumor development, in the animals bearing SMT-2A compared to those with MT-W9B or control group of rats. On the 4th week, the size of the primary SMT-2A tumor was only slightly higher than MT-W9B, but at this point it has already started metastasis to the distant sites. On the 6th week, the total tumor mass was 2–3 fold higher for SMT-2A than MT-W9B. Since the specific activity of galactosyltransferase in the metastasizing tumor is 3 times as high as in the non-metastasizing tumor [4], one would expect that the increase of this enzyme activity in the serum of SMT-2A rats over the control will be 9 fold as high as that of MT-W9B over the control. This indeed was found for galactosyltransferase, but for sialyltransferase one finds 4 times increase in the serum level against the expected value of 2 fold. Fucosyltransferase B had a specific activity 6 times as high in SMT-2A than MT-W9B [5], but increase of this enzyme in the serum over the control was similar for both types of tumors. Therefore, the elevation of the serum levels of the glycosyltransferases is probably not controlled by the total content of this enzyme in the tumor only. Subcellular localization of these enzymes, their stability and the rate of clearance from the circulation, and the nature of the tumor itself probably play significant roles in the process.

Liver is the major site of glycoprotein biosynthesis and a potential source of glycosyltransferase in the serum. No significant difference in the total content of any of these enzymes in the liver of animals bearing either of the two types of tumor could be found. Therefore, the source of the additional sialyl- and galactosyltransferase in the serum of the tumor hosts is most likely the tumor tissues. The elevation of these glycosyltransferases are thus good indicators for the presence of tumor. The shed enzymes may also provide the tumor with an immune escape mechanism by interfering with the immune response of the host to the tumor [4, 5].

Winzler [7] found that the amounts of neutral sugars and sugar amines in the serum increase under various pathological conditions including advanced cancer. Bernacki and Kim [10] observed that the amounts of sialoconjugates in the sera of rats bearing these metastasizing tumors increased concomitantly with the elevation of sialyltransferase. No such correlation between the levels of glycosyltransferase and the transferred glycose were found for any other glycosyltransferase.

In the sera of rats bearing transplantable mammary tumors, Bosmann and Hilf [11]

also found elevated levels of sialyltransferase. Elevations of various glycosyltransferases in the sera of cancer patients have been reported from a number of laboratories [12–17]. Recently, a cancer-associated isoenzyme of galactosyltransferase has been found in the sera of cancer patients [18]. These spontaneously metastasizing tumor models are very useful for study of the mechanism of release of the glycosyltransferase from the tumor and the use of these enzymes as tumor markers.

Acknowledgements—I extend my thanks to Dr. U. Kim, in whose laboratory this work was initiated.

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