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Metastatic potential of transformed rat 3Y1 cell lines is inversely correlated with lysosomal-type sialidase activity

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Abstract

We have investigated sialidase activities in transformed rat 3Y1 cells of different metastatic potential. Only lysosome-type sialidase was apparent in the particulate fractions of 3Y1 cells and their transformants. As compared with control 3Y1 cells, *src*-transformed cells exhibited decreased sialidase activity, and v-*fos* transfer to these latter induced even more severe decrease in the sialidase activity with acquisition of high lung metastatic ability. Various lysosomal enzymes other than sialidase were hardly affected by the transformation. Sialic acid transfer to *N*-linked glycoproteins was slightly elevated in the transformants, but not in parallel with their metastatic potential.

Key words: Sialidase; Metastasis; Oncogenic transformation; Lysosome; Rat 3Y1 cell

1. Introduction

Cell surface carbohydrates are known to undergo various alterations during neoplasia. The changes generally involve glycoproteins expressed on tumor cell surface with increased branching of complex-type *N*-glycans, increased polylactosaminoglycan chains and increased sialylation [1–5], which are correlated not only with tumorigenicity but also with elevated metastatic potential [6,7]. In particular, the alterations in sialylation have been proposed to be intimately associated with invasiveness and metastatic ability [4,6,8,9]. To elucidate how such aberrant sialylation occurs in cancer cells, we have been studying sialidase and sialyltransferase in rat hepatoma [10–12] and other tumor models [13]. We are now focusing on the enzyme studies in relation to invasiveness and metastasis.

Our previous studies on rat sialidases demonstrated four enzyme types differing in subcellular distribution and in catalytic and immunological properties. Located in the lysosomal matrix [14], cytosol [15] lysosomal membrane and plasma membrane [16], they are altered quantitatively in hepatocarcinogenesis [10] and in phorbol ester-induced malignant transformation of mouse epidermal JB6 cells [13]. We also found that Galβ1–4GlcNAcα 2–6 sialyltransferase activity was selectively increased in hepatomas [11,12]. Based on these results, we have determined the activity levels of sialidase and sialyltransferase of transformed rat fibroblast 3Y1 cell lines of different metastatic potential. The transformed cells were found to demonstrate much more prominent alteration of sialidase than sialyltransferase activities.

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Abbreviations: NeuAc, *N*-acetylneuraminic acid; 4MU-NeuAc, 4-methylumbelliferyl-α-D-*N*-acetyl-neuraminic acid; Lac-cer, lactosylceramide.

2. Materials and methods

2.1. Materials

4MU-NeuAc was purchased from Nakarai (Kyoto, Japan). GM3 labeled in the NeuAc moiety was synthesized enzymatically from lactosylceramide and CMP-NeuAc as described previously [17]. CMP-[^{14}C]NeuAc was obtained from New England Nuclear (Boston, MA) and diluted with cold CMP-NeuAc from Sigma (St. Louis, MO) to give a final specific activity of 2.5 Ci/mol. The sources of gangliosides and glycoproteins used as substrates for sialidase and sialyltransferase assays were described elsewhere [11,15].

2.2. Cells

Cells were routinely cultivated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 10% CO_2 and 90% air. SR-3Y1–2 is tumorgenic but low metastatic cell line established by infection of a normal rat fibroblast cell line, 3Y1-B clone 1–6 with the Schmidt-Ruppin D strain Rous sarcoma virus [18]. The fos-SR-3Y1–202, 203 and 205 are highly metastatic cell lines established by transfer of pFBJ-2 plasmid containing proviral v-*fos* DNA and pSV2-neo into SR-3Y1–2 [19]. v-*fos* transfer enhances lung metastasis in relation to an increase in invasiveness [20]. Cells harvested prior to confluency were rinsed in culture flasks with phosphate-buffered saline, scraped out with a rubber policeman, and collected at 600 × *g* for 5 min. The packed cells were washed with phosphate-buffered saline and sonicated on ice in 9 vols. of ice-cold 0.25 M sucrose containing 1 mM EDTA 2 times each for 15 min at the maximum speed. The mixture was then centrifuged at 600 × *g* for 10 min and the supernatant at 105,000 × *g* for 60 min at 4°C. The resultant supernatant was used as the cytosolic fraction, and the pellet suspended in 9 vols. of the sucrose solution was used as the particulate fraction. The fractions were either used immediately for the assays or kept frozen at −70°C. Protein was determined by the method of Bradford [21].

2.3. Determination of sialic acid

Cells (1×10^6) were incubated with 0.02 units of *Arthrobacter ureafaciens* sialidase in 1 ml of 0.01 M sodium phosphate buffer (pH 6.5)

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containing 0.15 M NaCl at 37°C for 1–2 h. After centrifugation, the supernatant was assayed for sialic acid by the thiobarbituric acid method of Aminoff [22]. Viability remained over 90%. For total sialic acid determination, cells were treated in 0.05 M H_2SO_4 for 60 min at 80°C and centrifuged. The resulting supernatant was assayed for sialic acid as above.

2.4. Sialidase assay

4MU-NeuAc and ^{14}C-labeled GM3 were routinely used as substrate, since 4MU-NeuAc was previously found to be an appropriate substrate for intralysosomal and cytosolic sialidases and GM3 for membrane-associated sialidases. The assays were performed under the optimal conditions as follows. When 4MU-NeuAc was the substrate, the assay mixture contained 60 nmol of the substrate, 5 μmol of sodium acetate buffer (pH 4.7), 100 μg of bovine serum albumin and particulate fractions (20–60 μg protein) in a final volume of 0.1 ml. For the assay of cytosolic sialidase, sodium acetate buffer was replaced by sodium cacodylate buffer (pH 6.0). The incubation was conducted at 37°C for 1–2 h and terminated by the addition of 0.25 M glycine–NaOH (pH 10.4). 4-Methyl-umbelliferone released was determined fluorometrically as described previously [15]. With [^{14}C]GM3 as substrate the assay mixture was composed of 10 nmol of the substrate, 5 μmol of sodium acetate (pH 4.7), 100 μg of bovine serum albumin, 50 μg of sodium deoxycholate and enzyme in 0.1 ml. After incubation at 37°C for 60 min, the radioactivity in the trichloroacetic acid-soluble fraction was determined [17]. When other substrates were employed, sialic acid released was determined after AG1X-2 column chromatography.

2.5. Sialyltransferase assay

Sialyltransferase activities were determined under the optimal conditions described below. When asialo- (AS-) glycoproteins were acceptors, the assay mixture contained 0.04 μmol of CMP-[^{14}C]NeuAc, 50–100 μmol (expressed in terms of acceptor site) of acceptor, 5 μmol of sodium cacodylate (pH 6.3), 100 μg of Triton X-100 and enzyme (20–60 μg) in 0.1 ml. After incubation at 37°C for 1–2 h, protein-bound radioactivity was determined as described previously [11]. With glycolipid as acceptor, the assay mixture was the same as for glycoprotein acceptors except 20–50 μmol of acceptor was used. The reaction product was measured by counting the radioactivity incorporated into the corresponding area co-migrating with a standard in a thin layer plate [12].

2.6. Other assays

β-Galactosidase, β-*N*-acetylglucosaminidase and α-fucosidase were assayed with the respective 4MU-glucosides as described [17]. Assays of acid phosphatase and cathepsin D were also conducted as described previously [14].

3. Results

Table 1 shows that total sialic acid and sialidase-releasable sialic acid contents were increased by transformation with v-*src* and v-*fos*. A transformed rat fibroblast clone (SR-3Y1–2), induced by infection with Rous sarcoma virus, possessed twice the amount in the control rat fibroblast cell line (3Y1 clone). However, augmentation of spontanous lung metastasis by v-*fos* transfer into SR-3Y1–2 did not further increase the sialic acid contents as evidenced by the results for fos-SR-3Y1–202 and -203. When the 3Y1 cells and their transformants were assayed for sialidase activity using 4MU-NeuAc or GM3 as substrates, they exhibited sialidase activity toward 4MU-NeuAc but little toward GM3 at pH 4.7 in the particulate fractions. The cytosolic fraction could not hydrolyze any of these substrates at either pH 4.7 or 6.0. Sialidase in the particulate fractions hydrolyzed sialyllactose and fetuin glycopeptides as well as 4MU-NeuAc at pH 4.7; the relative rates of hydrolysis were 45%, 40% and 100%, respectively. Glycoproteins were hardly hydrolyzed and gangliosides practically failed as substrates even in the presence of detergents such as Triton X-100 or sodium cholate. When the cell homogenates were fractionated according to de Duve et al. [23] and the resulting fractions were assayed with 4-MU-NeuAc as substrate, more than 60% of this acidic sialidase activity was found in the lysosomal/mitochondrial fraction, in which as much activity of acid phosphatase, a marker enzyme for lysosomes, was present. These results indicate that 3Y1 cells and their transformants possess only a lysosomal matrix-type sialidase, which was previously characterized as one of the four types of rat liver sialidase able to hydrolyze only low molecular weight substrates. As compared with the sialidase activity of control 3Y1 cells, the activity levels were markedly reduced in the transformants (Table 2). This was more pronounced in highly metastatic cells, fos-SR-3Y1, than in those with low metastatic potential, SR-3Y1–2. To determine if the low levels of sialidase activity in the transformants were due to the presence of any inhibitor, the particulate fractions of transformed cells were added to that of control 3Y1 cells, and the combined fractions measured for sialidase activity. The combined fractions showed essentially equivalent values to the sums of the two data obtained individ-

Table 1
Sialic acid contents of 3Y1 cells and their transformants

Cell lines	Total sialic acid (μg/mg protein)	Sialidase-accessible sialic acid (μg/mg protein)
3Y1	13.41 ± 1.50	3.42 ± 0.22
SR-3Y1-2	24.32 ± 2.31*	6.25 ± 0.16**
fos-SR-3Y1-202	24.31 ± 3.33*	6.28 ± 0.45**
fos-SR-3Y1-203	26.20 ± 2.15*	5.67 ± 0.12**

Values are means ± S.D. of three batches of cells.
Significant difference from 3Y1: *$P < 0.01$; **$P < 0.001$.

Table 2
Sialidase activities of 3Y1 cells and their transformants

Cell lines	Substrate	
	4MU-NeuAc (mmol/h/μg protein)	[^{14}C]-GM3 (mmol/h/μg protein)
3Y1	92.5 ± 12.5	0.21 ± 0.05
SR-3Y1-2	31.2 ± 2.4*	N.D.
fos-SR-3Y1-202	11.4 ± 3.6**,***	0.16 ± 0.10
fos-SR-3Y1-203	10.5 ± 6.1**,***	N.D.
fos-SR-3Y1-205	18.7 ± 2.7**,***	N.D.

Values are means ± S.D. of four different batches of cells. Significant difference from 3Y1 (*$P < 0.025$; **$P < 0.01$) and from SR-3Y1-2 (***$P < 0.01$); N.D. = not detected.

Table 3
Low levels of sialidase activity in transformants are not due to the presence of an inhibitor

Particulate fraction	Sialidase activity (nmol/h)
3Y1	7.4
SR-3Y1-2 (2)	1.7
fos-SR-3Y1-202 (3)	0.93
(1) + (2)	8.4
(1) + (3)	7.8

Sialidase activity was assayed at pH 4.7 in the particulate fraction(s) of 3Y1 cells and/or their transformants. Values are means of two experiments.

ually as shown in Table 3, indicating that a special inhibitor may not be involved in decrease of sialidase activity in the transformants. As the sialidases of the 3Y1 cells and their transformants were suggested to be of lysosome-type, we examined whether several lysosomal enzymes other than sialidase might also be altered by the oncogenic transformation (Table 4). Unlike the sialidase, the activities of four glycosidases and cathepsin D were hardly affected. Although decrease in some of the enzyme activities in v-*fos* transferred cells was appreciable, in all cases it occurred to a much lesser extent than for sialidase. We also compared the activity levels of sialyltransferases between control 3Y1 cells and their transformants using glycoprotein and glycolipid acceptors as shown in Fig. 1. The activity toward asialo-orosomucoid was somewhat increased in the transformants while little changes were observed in the activities with two asialo-submaxillary mucins and asialo-transferrin as acceptors. The sialyltransferases responsible for ganglioside formation were rather reduced in activity by the transformation.

4. Discussion

The present study revealed that oncogenic transformation of rat 3Y1 cells causes alterations of sialidase and sialyltransferase activities accompanied by an increase of sialic acid contents. The most striking findings were that transformed cells demonstrate severely reduced lysosome-type sialidase activity and that acquisition of highly metastatic potential with v-*fos* transfer was associated with further remarkable decrease in sialidase activity. Interestingly, this seems to be specific to sialidase among lysosomal enzymes. Total sialic acid as well as surface sialic acid contents were higher in the transformed cells than in control cells, but did not correlate with metastatic potential. This may imply that only the sialic acids of particular sialoglycoconjugates are involved in metastasis. The transformed cells exhibited to some degree increased sialyltransfer to asialo-orosomucoid containing tri- and tetra-antennary complex-type chains. On the other hand, sialyltransfer to glycolipid acceptor was rather decreased. These data suggest that increase in sialic acid bound to *N*-linked glycoprotein would occur with a concomitant decrease in that bound to glycolipid. Such a tendency was also shown previously for phorbol ester-induced transformants of JB6 mouse epidermal cells, characterized decreased lysosomal sialidase activity and increased sialyltransfer to *N*-linked glycoproteins [13].

High metastatic potential of fos-SR-3Y1 cells was earlier described to be closely related to high invasiveness, in association with augmentation of cell motility [20]. Consequently, their metabolic shift to an increased sialic acid density, at least of some sialoglycoproteins responsible, may bring about the enhanced metastasis, especially invasiveness. This is also consistent with other work demonstrating good correlations between sialic acid contents and invasiveness [4,6,8,9]. We have further found that BL6 cells, a high invasive variant of B16 melanoma cells, have higher levels of sialyltransferase activity than cells of low invasiveness, although in that case sialidase activity was hardly affected (unpublished data).

src gene products most likely act at a post-translational level such as phosphorylation, whereas fos gene products are involved in many cellular events due to their trans-acting transcription function [24]. It is at present far from understood how expression of *src* and *fos* genes is related to alterations in sialidase and sialyltransferase and how these oncogenes modify sialidase activity selectively among various lysosomal enzymes. Investigations of whether transfection of a recently cloned rat sialidase gene [25] into fos-SR-SY1 cell might reduce their metastatic potential are now under way.

Table 4
Glycosidase and cathepsin D activities of 3Y1 cells and their transformants

	3Y1	SR-3Y1-2	fos-SR-3Y1-202
		(nmol/h/mg protein)	
Sialidase	84.4	29.1	12.6
		(nmol/min/mg protein	
β-Galactosidase	15.1	18.6	11.4
β-*N*-Acetyl-glucosaminidase	24.9	28.3	22.5
Fucosidase	10.9	9.7	8.3
Acid phosphatase	149.6	138.8	120.4
		(μg/min/mg protein)	
Cathepsin	25.7	26.7	19.5

Values are means of two experiments.

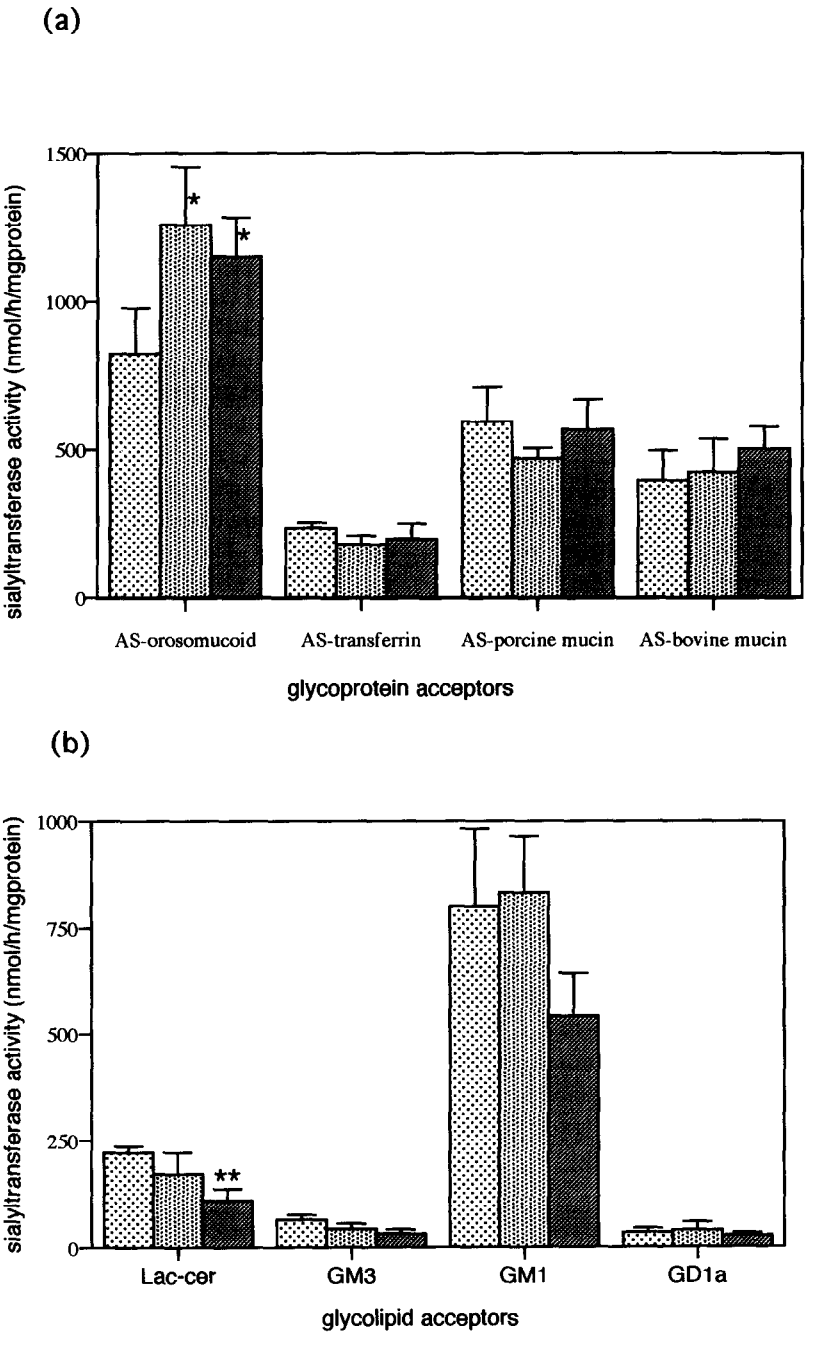

Fig. 1. Sialyltransferase activities toward glycoprotein (a) and glycolipid (b) acceptors in 3Y1 cells and their transformants. Asialo- (AS-) orosomucoid, -transferrin, -bovine submaxillary mucin were prepared by mild acid hydrolysis (0.1 N H_2SO_4 at 80°C for 1 h) and AS-porcine submaxillary mucin by treating with 1 N H_2SO_4 at 70°C for 3 h to remove sialic acid and fucose. Activities are means±S.D. of three batches of cells. Significant differnce from 3Y1 (*$P < 0.05$, **$P < 0.01$). ▦ = 3Y1; ▨ = SR-3Y1-2; ▩ = fos-SR-3Y1.

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