References and Notes

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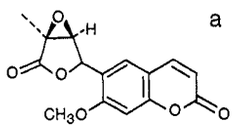

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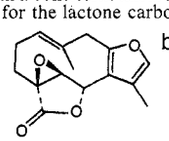

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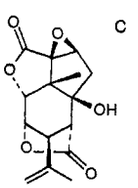

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Metastatic Potential Is Positively Correlated with Cell Surface Sialylation of Cultured Murine Tumor Cell Lines

Abstract. *The ability of murine tumor cells to metastasize spontaneously from subcutaneous sites is positively correlated with the total sialic acid content of the cells in culture, the degree to which the sialic acid is exposed on the tumor cell surface, and, most strongly, with the degree of sialylation of galactosyl and N-acetylgalactosaminyl residues in cell surface glycoconjugates. These findings suggest that sialic acid on the cell surface may play a role in tumor cell metastasis.*

Much attention has been devoted to studying alterations in cell surface carbohydrates in neoplastic transformation (*1*). Particular interest has been focused on changes in the composition and metabolism of sialic acid (*N*-acetylneuraminic acid) in transformed cells (*1–8*). No consistent patterns have so far emerged (*1*); sialic acid levels (*2–4*), sialylated membrane components (*5*, *6*), and sialyl transferase enzymes (*4*, *5*, *7*, *8*) in transformed cells have been found to be increased in some studies (*2*, *5–8*) and decreased in others (*3*, *4*).

Several studies have suggested, however, that cell surface sialic acid might play a role in determining the metastatic properties of neoplastic cells. Sialic acid has been implicated in determining cellular adhesiveness (*9*), the ability of intravenously injected cells to implant in various organs (*10*), cellular motility and invasiveness in vitro (*11*), and immunogenicity (*12*). Each of these properties has been linked to the metastatic behavior of cells (*13*). Previous studies with variants of the B16 mouse melanoma that differ in lung implantation properties after intravenous injection showed higher levels of neuraminidase-releasable cell surface sialic acid on cells from the cell line with enhanced ability to implant in lungs (*14*, *15*). Prompted by these findings, we examined the sialic acid content and cell surface sialylation of a variety of murine tumor cell lines of diverse types which vary in their spontaneous metastatic properties (*16–19*).

The cultured cell lines we used include spontaneously transformed, DNA and RNA virus–transformed, and chemically transformed cells derived from (or producing in vivo) carcinomas, sarcomas, and melanomas (Fig. 1, legend). The metastatic properties of the cell lines were defined by determining the percentage of animals developing spontaneous metastases to any site after the excision of a primary subcutaneous tumor (*18*, *20*). Tumors were excised at sizes ranging from 2 to 6 g for rat tumors and 1 to 3 g for mouse tumors; animals were killed either when obvious metastases developed or after prolonged observation (5 to 17 months after excision in rats, 4 months in mice). One to five experiments were performed for each cell line, with 5 to 65 animals tested per line and with reproducible results from experiment to experiment (*18*, *20*). The cell lines exhibited a wide range of metastatic behavior, with the overall incidence of metastasis ranging from 0 to 100 percent (Fig. 1). Three nontumorigenic cell lines were also studied.

Using cells that were within several tissue culture passages of those used in the in vivo experiments, we examined three aspects of the sialylation of cultured cells: (i) the total sialic acid content of the cells (Fig. 1A), (ii) the degree to which metabolically labeled sialic acid was exposed to neuraminidase on the cell surface (Fig. 1B), and (iii) the extent to which galactosyl (Gal) and *N*-acetylgalactosaminyl (GalNAc) residues on cell surface glycoconjugates were substituted with sialic acid (Fig. 1C). All three of these parameters correlated positively with the spontaneous metastatic potential of the cells. The best correlate of metastatic potential was the degree of sialylation of cell surface Gal and GalNAc.

The sialic acid contents of the ganglioside and glycoprotein fractions of cultured cells from 23 of the cell lines were determined separately by resorcinol and thiobarbituric acid assays (*17*), and the values were combined to give the total cellular content of sialic acid. The total sialic acid content of the cultured cells correlated significantly with their metastatic potential (Fig. 1A). The average sialic acid content of high metastatic cells (> 50 percent of incidence of metastasis) was 1.4-fold greater than the average sialic acid content of low metastatic cells (< 50 percent incidence of metastasis).

The exposure of sialic acid on the surface of cultured cells from 29 of the cell lines was determined by metabolically labeling cells for 48 hours in medium containing *N*-acetyl-D-[6-^{3}H]mannosamine (1 μCi/ml) (29 Ci/mmole; New England Nuclear), which specifically labels sialic acid residues (*21*), and then incu-

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bating triplicate cultures (at or near confluence) for 1 hour in calcium- and magnesium-containing phosphate-buffered saline (*p*H 6.0) either with or without *Vibrio cholera* neuraminidase (25 U/ml) (Calbiochem-Behring) (*15*). The average percent of label released into the supernatant medium in control cultures (*C*) was subtracted from the average percent of label released in the neuraminidase-treated cultures (*T*) to give the amount of sialic acid specifically released by the enzyme, expressed as a percent of the total incorporated label (*T*−*C*). The percentage of the total incorporated label released by neuraminidase correlated significantly with the metastatic potential of the cells (Fig. 1B). The average percentage of releasable sialic acid was 2.0-fold greater in high metastatic than in low metastatic cells.

The extent to which potential sialic acid acceptor sites on cell surface glycoconjugates were substituted with sialic acid was assessed for all 35 cell lines by comparing galactose oxidase–sodium borotritide labeling of neuraminidase-treated and untreated cells (*22*). The percent sialylation of cell surface Gal and GalNAc residues determined by this method was highly correlated with the metastatic potential of the cells (Fig. 1C). The average value of percent sialylation for high metastatic cells was 3.6-fold higher than that for low metastatic cells.

The values obtained for the least metastatic cell lines corresponded closely with those of the nontumorigenic cell lines in each study (Fig. 1). This finding suggests that altered sialic acid metabolism may be observed primarily in cells that are both tumorigenic and metastatic. It may thus be important to consider metastatic properties when comparing the sialylation of transformed cells with that of untransformed cells (*1*–*8*).

The mechanisms responsible for the differences in sialylation and cell surface sialic acid exposure of the metastatic variant cells have not been determined. Although, in other systems, the sialic acid content of whole cells or of cell surface glycoproteins correlated with the activity of sialyl transferase enzymes (*4*, *5*), in our studies, sialyl transferase levels did not show a correlation with the metastatic potential of the cells (*23*). Other factors, such as cell surface sialidase activity (*8*, *24*), the rate of membrane turnover, and the microarchitecture of the cell surface, have not been explored.

In agreement with our results, a decrease in the sialylation of surface components has been found in nonmetastatic variant cells selected from a metastatic B16 melanoma line by resistance to wheat germ agglutinin (*25*, *26*). In addition, cell surface glycoproteins (*27*, *28*) and lectin-exposed glycoconjugates (*29*) have been found to be highly sialylated in human chronic myelogenous leukemia cells (*29*), human leukemic cell lines (*27*), and human melanoma cell lines (*28*). In contrast, low metastatic B16 cells resistant to *Ricinus communis* agglutinin do not exhibit decreased cell surface sialylation (*26*); no differences in the sialylation of cell surface glycoproteins were detected in three variants of the B16 melanoma cell line that differ in their lung implantation properties when injected intravenously (*30*); and no difference in total and plasma membrane–bound sialic acid was found in cells obtained from seven metastatic variant rat mammary adenocarcinomas grown in vivo (*31*). These divergent findings may result from differences in the metastatic assays (*26*, *30*) or from differences in the preparation of cells for biochemical assays (*31*), or they may reflect the existence of factors other than cell surface sialylation that influence metastasis in these cell systems.

The increased sialylation of cell surface glycoconjugates that we observed may contribute to the metastatic behavior of tumor cells through (i) an increased adhesiveness (*9*, *32*) leading to larger tumor cell emboli (*33*) or to an increased capacity to adhere to vascular endothelium at secondary sites of implantation (*34*), (ii) an increased capacity to aggregate blood platelets (*18*), or (iii) a decrease in the susceptibility of the cells to destruction by host immune defense mechanisms (*12*). Alternatively, the degree of cell surface sialylation may not contribute to the metastatic properties of cells, but may vary incidentally as a consequence of other factors responsible for producing the differences in metastatic behavior. Delineating the mechanisms

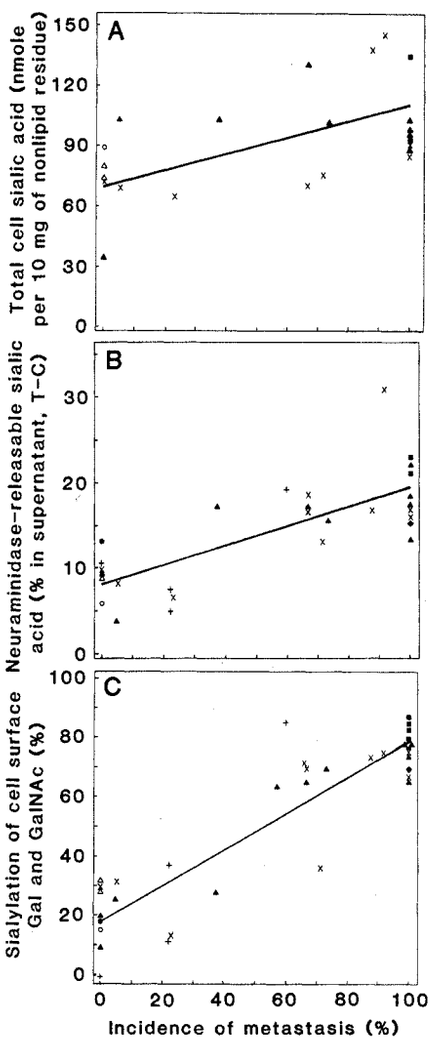

Fig. 1. Correlation between the metastatic potential of cultured tumor cells and (A) their total sialic acid content ($r = .54$, $P < .02$); (B) the percentage of metabolically labeled sialic acid that is releasable by neuraminidase ($r = .74$, $P < 10^{-5}$); and (C) the percent sialylation of cell surface galactosyl (Gal) and *N*-acetylgalactosaminyl (GalNAc) residues ($r = .90$, $P < 10^{-12}$). Symbols: (×) ten derivatives of the polyoma virus–induced PW20 Wistar-Furth rat renal sarcoma cell line (*18*); (+) four rat mammary adenocarcinoma cell lines (two derived from the methylcholanthrene-induced Wistar-Furth MT/W9A tumor line, one from the dimethylbenzanthracene-induced Fischer DMBA 8 tumor line, and one from the spontaneous Fischer R3230AC tumor line; (●) SV40-transformed Balb/3T3 mouse cell line (SV-A31) (*35*); (▲) 11 Kirsten and Moloney sarcoma virus–transformed Balb/3T3 cell lines (*17*, *20*); (■) five B16 C57B1/6 mouse melanoma–derived cell lines [F1 and F10 (*15*) and three further derivatives (*36*)]; (◆) cell line derived from the spontaneous C57B1/6 Lewis lung carcinoma; (○) untransformed Balb/3T3 A31 cells [nontumorigenic (*37*)]; and (△) R20 and R24 flat revertants of the K234 Kirsten sarcoma virus–transformed Balb/3T3 cell line (*17*) [nontumorigenic (*36*)]. Total sialic acid values in (A), representing the means of two or three independent determinations, had standard errors ranging from 1 to 9 nmole of sialic acid per 10 mg of nonlipid residue. In (B), standard errors of the values for the neuraminidase-releasable sialic acid ranged from 1 to 7 percent of the total incorporated label. Values of percent sialylation in (C) represent the mean of two to seven independent determinations, with standard errors ranging from 1 to 13 percent sialylation (in four cases duplicate values from one experiment were averaged). Regression lines were calculated by linear regression and correlation analysis (omitting values for the nontumorigenic cell lines). The correlation coefficients obtained for (B) and (C) were unchanged when only the 20 tumorigenic cell lines examined in (A) were considered.

that underlie the observed correlations should contribute to our understanding of the metastatic process.

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The in vitro Classical Conditioning of the Gill Withdrawal Reflex of *Aplysia californica*

Abstract. *Associative learning has been demonstrated in a reduced siphon, mantle, gill, and abdominal ganglion preparation of* Aplysia. *The preparations learned to respond to a previously neutral stimulus as a consequence of training in a classical conditioning paradigm. Backward conditioning, presentation of the conditioned stimulus alone, or presentation of the unconditioned stimulus at some random interval after presentation of the conditioned stimulus failed to produce conditioning. This model system can be used to study the neural mechanisms underlying associative learning.*

If simple model systems such as the well-studied *Aplysia* preparation (*1*) exhibit associative learning (*2*), the neuronal mechanisms underlying learned behavior may be analyzed. Already much of our understanding of the neuronal mechanisms underlying two types of nonassociative learning—habituation and sensitization—have been obtained from the *Aplysia* model system (*3*). Although it has been difficult in the past to demonstrate associative learning in *Aplysia* (*4*), associative learning has recently been conclusively demonstrated

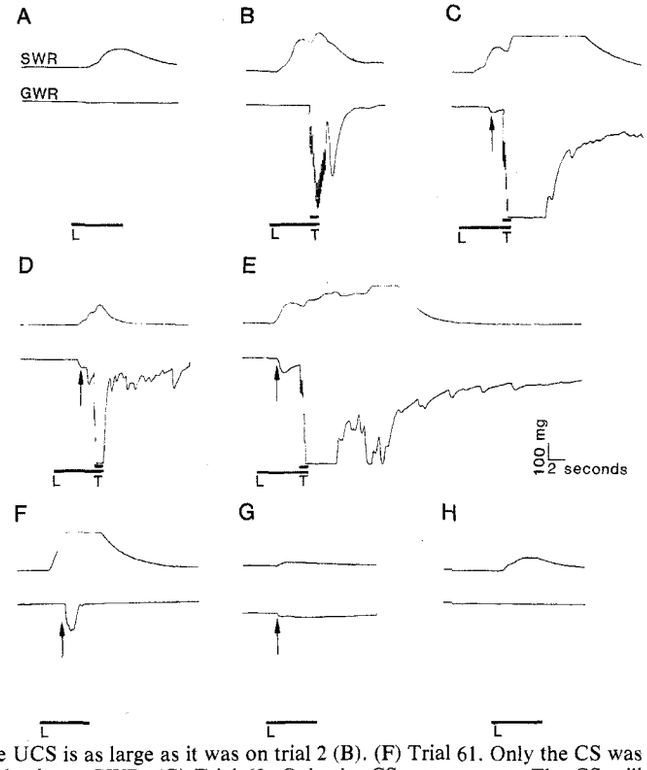

Fig. 1. Data from a preparation in the experimental forward conditioning group. (A) Trial 1. The CS [light (*L*)] was presented to the preparation on trial 1; it evoked a siphon withdrawal response (*SWR*), but did not evoke a gill withdrawal reflex (*GWR*). The CS is indicated by a bar under the tracings. (B) Trial 2. The first trial on which the CS was paired with the UCS [tactile stimulus (*T*)]. The CS evoked only an SWR. The UCS, however, evoked a large GWR as well as an SWR. The GWR evoked by the UCS did not readily habituate. (C) Trial 25. The CS evoked not only an SWR but also a small GWR (arrow). (D) Trial 40. (E) Trial 60. The GWR evoked by the UCS is as large as it was on trial 2 (B). (F) Trial 61. Only the CS was presented, and it evoked a large GWR. (G) Trial 63. Only the CS was present. The CS still evoked the GWR but its amplitude was smaller than it was on trial 61. (H) Trial 65. CS alone. It no longer evoked a GWR. Presentation of the UCS also led to sensitization of the SWR.

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