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Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells

(processing/metastasis/tumorigenicity)

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ABSTRACT **Oligosaccharide moieties of cell-surface glycoconjugates are thought to be involved in recognition events associated with tumor metastasis and invasion. Using swainsonine (SW), an inhibitor of Golgi α-mannosidase II that results in the formation of hybrid-type oligosaccharides on *N*-linked glycoproteins, we have tested the hypothesis that specific glycan structures are required for pulmonary colonization by tumor cells. B16-F10 murine melanoma cells were treated with SW in growth medium and then injected intravenously into syngeneic C57BL/6 mice. This treatment resulted in dramatic inhibition of colonization, but it had no effect on B16-F10 viability or on cellular tumorigenicity after subcutaneous implantation. SW-treated radiolabeled B16-F10 cells were cleared from the lungs at a greater rate than control cells, suggesting that one effect of treatment is to alter tumor cell retention in the target organ. Our results implicate specific glycan structures in pulmonary colonization and offer a potential approach for identification of specific macromolecules involved in tumor cell–organ recognition during metastasis.**

Growing evidence suggests that carbohydrate residues on cell-surface glycoconjugates play an important role in the metastatic spread of tumor cells. Malignant and transformed cells commonly possess more highly branched and sialylated *N*-linked oligosaccharides than corresponding normal cells (reviewed in refs. 1 and 2). This difference apparently results in the altered lectin-binding characteristics of tumor cell sublines of varying metastatic potential (reviewed in refs. 3 and 4). Recently, a direct correlation between sialylation of galactose and *N*-acetylgalactosamine residues and metastatic capacity has been observed in a series of tumor cell lines (5, 6), suggesting that the extent of completion of membrane glycoprotein processing may affect metastatic activity. Furthermore, cell lines selected for both lectin resistance and galactose deficiency that possess lesions in oligosaccharide synthesis and contain undersialylated complements of glycoproteins, are often less metastatic than their parental lines (reviewed in ref. 7).

In assays for blood-borne metastasis, treatment of B16 murine melanoma cells with tunicamycin, an inhibitor of the biosynthesis of N-linked glycoproteins, appeared to abrogate pulmonary colonization in syngeneic mice (8–10). This effect has been correlated with the presence of certain surface sialoglycoproteins (8, 9, 11). However, since tunicamycin is known to be toxic at least for most tumor cells (12, 13), the significance of this finding remains unclear.

Swainsonine (SW) is one of several recently discovered drugs that specifically inhibit different stages of glycoprotein processing (reviewed in refs. 14 and 15). The inhibition of Golgi α-mannosidase II by SW results in formation of complex–high mannose hybrid-type oligosaccharides where ordinarily complex chains would be synthesized (16, 17). We report here that treatment of B16-F10 murine melanoma cells with SW under nontoxic conditions dramatically inhibits their ability to colonize the lungs of C57BL/6 mice after intravenous injection. This inhibition correlates with alterations in the oligosaccharide structure of B16-F10 membrane glycoproteins and suggests that not only the presence but also the specific structure of cell-surface carbohydrates is required for successful completion of the colonization phase of metastasis.

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MATERIALS AND METHODS

Cell Culture. B16-F10 murine melanoma cells, a line selected *in vivo* for high pulmonary colonization (ref. 18; a gift from I. J. Fidler, M. D. Anderson Hospital, University of Texas, Houston, TX), were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum, MEM vitamins, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine (all from GIBCO), and gentamycin sulfate at 50 μg/ml (M. A. Bioproducts, Walkersville, MD), and passaged twice weekly by short exposure to 0.25% trypsin/0.02% EDTA (GIBCO). Only cells <60 days old were used for these studies. Cultures were routinely tested for microbial infection and verified to be free of mycoplasma.

Pulmonary Colonization. Six-week-old pathogen-free C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) were quarantined for 1 week and used over an age range of 8–10 weeks. SW was obtained either as a gift from H. P. Broquist (Vanderbilt University, Nashville, TN) or from Boehringer Mannheim and was dissolved in water. B16-F10 cells were plated at 10^6 or 5×10^5 cells per 75-cm^2 flask and 2 or 3 days later, respectively, were treated with SW in growth medium for 18–22 hr. The just confluent cultures were then washed with divalent cation-free Dulbecco's phosphate-buffered saline (PBS^-; GIBCO), detached with 0.02% EDTA for 2 min, and resuspended gently to 2.5×10^5 cells per ml in Dulbecco's MEM containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (GIBCO). Prior to injection, animals were warmed for 10 min to elicit vasodilation, and 0.2-ml aliquots of each cell suspension (containing 5×10^4 cells) were then injected slowly into the lateral tail vein as described by Fidler (19). Fourteen days later, the animals were sacrificed with ether and their lungs were excised and fixed in 10% formaldehyde. Surface melanoma colonies were counted visually or with the aid of a dissecting microscope. Extrapulmonary tumor formation was checked for each group.

Pulmonary Retention. B16-F10 cells were plated at 2×10^6 cells per 75-cm^2 flask, and the next day they were labeled with [5-^{125}I]iododeoxyuridine (0.4 μCi/ml, 200 mCi/mmol; 1 Ci = 37 GBq; New England Nuclear) in the presence of SW

Abbreviations: endo H, endoglycosidase H; SW, swainsonine.

at 3 μg/ml for 18 hr in growth medium (20). Both sets of cells were then detached and aliquots of 5×10^4 cells were injected into the lateral tail vein of C57BL/6 mice. At various times, mice were sacrificed by cervical dislocation and the lungs were excised and exsanguinated in 70% ethanol (20) before assaying for radioactivity.

Growth in Soft Agar. The ability of B16-F10 cells to grow suspended in soft agar was tested by the procedure of Nagata and Ichikawa (21). Base layers (2 ml) of 0.5% agar were prepared in 35-mm tissue culture wells by boiling sterile 3.3% agar (Difco), cooling to 45°C, mixing with Dulbecco's MEM and fetal bovine serum (10% final concentration), and allowing gelation at room temperature for 30 min. Sterile 1.8% agar was then boiled, cooled to 41°C, and mixed with fetal bovine serum (10% final concentration) and either control or SW-treated (5 μg/ml for 18 hr) cells suspended in Dulbecco's MEM. Aliquots of these mixtures containing 2×10^4 cells and 0.33% agar in 0.5 ml were quickly seeded onto base layers and gelation was allowed as described above. After 8 days incubation at 37°C, the number of original cells forming colonies was determined visually by counting 30 random microscope fields.

Incorporation of [^{14}C]Leucine and [^{3}H]Mannose into Trichloroacetic Acid-Insoluble Material. B16-F10 cells were seeded in 1 ml of growth medium at 6×10^4 cells per 23-mm tissue culture well, and 2 days later they were treated with SW for 18 hr at 37°C. L-[1-^{14}C]leucine (55 mCi/mmol) and D-[2-^{3}H(N)]mannose (15–27 Ci/mmol; both from New England Nuclear) were then added at 0.2 μCi/ml and 5 μCi/ml, respectively, and the incubation was continued for 20 min at 37°C. Cell monolayers were washed two times with 1 ml of cold PBS$^-$ and trichloroacetic acid-soluble label extracted with 1 ml of cold 5% trichloroacetic acid for 1 hr at 4°C. The trichloroacetic acid-insoluble material was washed once with 1 ml of ethanol, solubilized in 0.9 ml of 0.1 M NaOH for 1 hr at room temperature with agitation and 0.5-ml aliquots were counted after mixing with 3 ml of Aquasol (New England Nuclear).

[^{3}H]Mannose Labeling and Endoglycosidase H (endo H) Digestion of Membrane Proteins. B16-F10 cells were seeded at 7×10^5 cells per 100-mm dish in growth medium and 2 days later were pretreated with SW at 5 μg/ml for 3 hr at 37°C. [^{3}H]Mannose (50 μCi/ml) was added and the incubation continued for 18 hr. Cell monolayers were washed four times with cold PBS$^-$ and scraped into 50 mM Tris·HCl, pH 7.0/soybean trypsin inhibitor (50 μg/ml)/50 μM leupeptin (both from Sigma)/1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$; Calbiochem). The cells were then homogenized with 10 strokes of an all-glass Dounce and pelleted by centrifugation at 40,000 × *g* for 30 min at 4°C. Membranes were solubilized in cold 50 mM Tris·HCl, pH 7.2/150 mM NaCl/0.1% $NaDodSO_4$/1% Triton X-100/soybean trypsin inhibitor (50 μg/ml)/50 μM leupeptin/1 mM $PhMeSO_2F$ for 2 hr, diluted into 50 mM sodium acetate, pH 5.5/100 mM NaCl/soybean trypsin inhibitor (50 μg/ml)/50 μM leupeptin/1 mM $PhMeSO_2F$, and centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant was made 0.01% in $NaDodSO_4$ and either used immediately or stored at −80°C.

To 40-μl aliquots of control and SW-treated cell membrane proteins was added 6 μl of 1% $NaDodSO_4$/6% 2-mercaptoethanol, and the mixture was boiled for 3 min. After cooling, 10.7 μl of 265 mM sodium acetate, pH 5.5/250 mM NaCl/50 μM leupeptin/1 mM $PhMeSO_2F$ and 10 μl of endo H (2 units/ml)/50 mM sodium acetate, pH 5.5 (Miles, Naperville, IL) was added and incubated for 40 hr at 37°C. The reaction was arrested by addition of 9.5 μl of 0.4 M Tris·HCl, pH 6.7/24% $NaDodSO_4$, 7.6 μl of glycerol, 3.9 μl of 2-mercaptoethanol, and by boiling for 5 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed under reducing conditions in slab polyacrylamide gels containing 0.1% $NaDodSO_4$. A discontinuous buffer system with a 5% polyacrylamide stacking gel (pH 6.8) and a 7–15% gradient polyacrylamide resolving gel (pH 8.8) was used (22). Gels were fixed in 10% trichloroacetic acid, incubated for 30 min in Enlightning (New England Nuclear), and dried. Fluorography was performed at −80°C with Kodak X-Omat AR XAR-5 film for 7 days.

RESULTS

Inhibition of Pulmonary Colonization by SW Treatment. To modify the *N*-linked oligosaccharide subunits of B16-F10 cell glycoproteins, subconfluent cultures were incubated with SW for 18–22 hr. This time is equivalent to ≈2 generation times of B16-F10 (20) and allows substantial turnover of cellular glycoproteins. As shown in Figs. 1 and 2, SW treatment resulted in a dramatic dose-dependent inhibition of pulmonary colonization 14 days after injection of cells into the lateral tail vein of C57BL/6 mice. Greater than 80% inhibition of colony formation has been consistently observed with SW at 3 μg/ml in multiple experiments. Similar results have been obtained with several different batches of SW including lots obtained commercially or as a gift from H. P. Broquist (Vanderbilt University, Nashville, TN).

The appearance of melanotic colonies in organs other than the lung is seldom observed in this model system, but the possibility that SW treatment caused an increase in the frequency of extrapulmonary metastases by redistribution of cells to other sites was routinely checked. No evidence to support this explanation was obtained.

As a consequence of its inhibition of Golgi α-mannosidase II, SW results in aberrant processing of *N*-linked glycan precursors to form endo H-sensitive hybrid oligosaccharides containing both complex-type and high mannose-type branches (16, 17). Glycoproteins that ordinarily contain endo H-resistant complex sugar chains are therefore rendered sensitive to enzymatic digestion. To demonstrate that SW did indeed function in this way in B16-F10 cells, the endo H sensitivity of membrane glycoproteins isolated with or without prior SW treatment was examined. As shown in Fig. 3 (lanes 1 and 2), endo H treatment of membrane glycoproteins

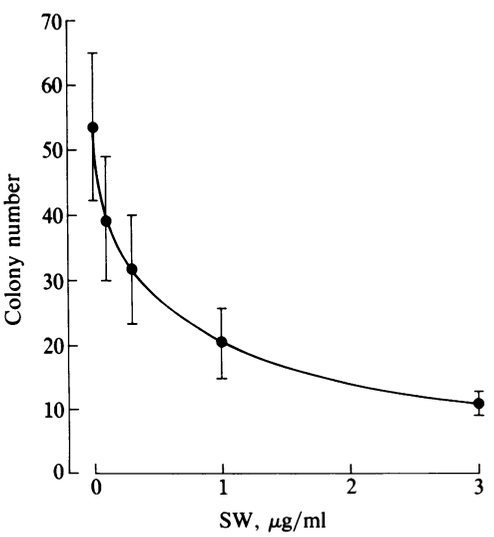

FIG. 1. Inhibition of pulmonary colonization by SW treatment. Cultures of B16-F10 cells were treated with the indicated concentrations of SW in growth medium for 18–22 hr and then aliquots of 5×10^4 cells were tested for their lung colonizing capacity. The results are the average of two separate experiments. Bars represent SEM ($n = 8$ for each experiment). $P < 0.005$ at 3 μg/ml by Student's *t* test.

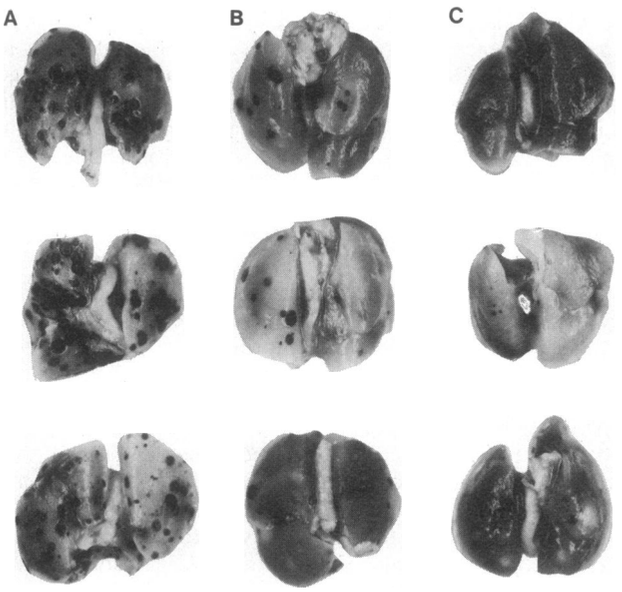

FIG. 2. Representative lungs to show inhibition of melanotic colony formation by 10^5 B16-F10 cells after no treatment (*A*) or incubation with SW at 0.3 μg/ml (*B*) or 3 μg/ml (*C*).

isolated from control cells labeled metabolically with [^{3}H]mannose resulted in the disappearance of several labeled bands. These glycoproteins appear to possess high mannose-type glycans. Some proteins were unaffected by endo H and presumably contain processed complex chains, whereas others, identifiable by both a lowered molecular weight and a decrease in labeling intensity, appear to have a mixture of high mannose and complex oligosaccharides. The pattern of [^{3}H]mannose-labeled membrane glycoproteins from cells treated with SW was similar to the control (lane 3). When this mixture was treated with endo H, an almost complete loss of labeled bands was observed, indicating that almost all complex sugar chains had been converted to structures with high mannose character as a result of SW treatment (lane 4). To exclude the presence of nonspecific proteolytic activity in preparations of endo H, the gel shown in Fig. 3 was stained

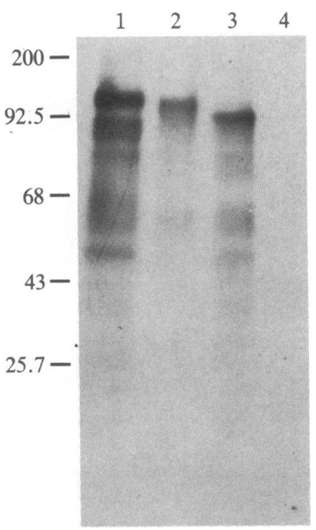

FIG. 3. Endo H sensitivity of SW-treated membrane glycoproteins. Cultures of B16-F10 cells were labeled with [^{3}H]mannose in the absence or presence of SW at 5 μg/ml and membrane fractions were isolated as described. Samples of control (lanes 1 and 2) and SW-treated (lanes 3 and 4) membranes were analyzed electrophoretically under reducing conditions with (lanes 2 and 4) or without (lanes 1 and 3) prior endo H digestion. Numbers on left represent $M_r \times 10^{-3}$.

with Coomassie blue prior to fluorography. The banding pattern in all lanes appeared indistinguishable, indicating that endo H treatment removed only sugar residues and did not degrade the polypeptide portion of the glycoproteins (data not shown). Therefore, at the concentrations used in Figs. 1 and 2, SW affects glycoprotein processing and the consequences of drug treatment are likely to be due to alterations in carbohydrate structure.

Controls for Toxicity. The effect of SW treatment on incorporation of the radiolabeled precursors [^{14}C]leucine and [^{3}H]mannose into trichloroacetic acid-insoluble material was examined to check for a potential inhibition of B16-F10 growth. B16-F10 cultures were incubated with SW for 18 hr before measuring amino acid and sugar incorporation over a short 20-min period. The use of this brief pulse was designed to detect any toxic effect that long-term exposure to the drug might have. As shown in Fig. 4, SW at concentrations up to 10 μg/ml did not impair either leucine or mannose incorporation into trichloroacetic acid-insoluble material, in agreement with previous findings (23). Indeed, at no time has SW ever exerted a toxic effect at these concentrations, even for incubation periods of up to 3 days (data not shown). In studies analogous to those in Fig. 4, but using continuous labeling with leucine and mannose, no difference in leucine incorporation was apparent over an 18-hr period (data not shown). A slight stimulation (10–20%) of mannose incorporation over the control was observed. This phenomenon, reported previously by Elbein *et al.* (23), may reflect SW-mediated inhibition of mannose removal from glycan precursors.

The results of microscopic examination of B16-F10 morphology (Fig. 4 *Inset*) and trypan blue dye exclusion revealed no differences between control and SW-treated cells. We therefore conclude that the drug is completely nontoxic, at least up to 10 μg/ml, and that the inhibition of pulmonary colonization mediated by SW cannot be explained by cytotoxicity.

Controls for Suppression of Tumorigenicity. Two approaches were taken to rule out the suppression of B16-F10 tumorigenicity by SW. First, in the most rigorous test, control and SW-treated (3 μg/ml for 18 hr) cells were injected

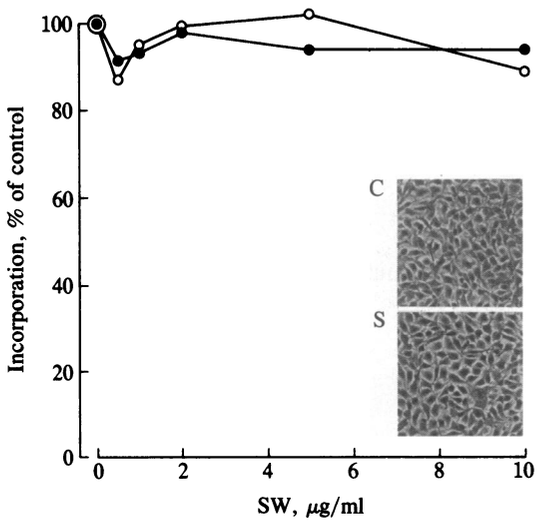

FIG. 4. Effect of SW treatment on B16-F10 leucine and mannose incorporation. Cultures of B16-F10 cells were pretreated with SW at the indicated concentrations for 18 hr and then the incorporation of [^{14}C]leucine (●) and [^{3}H]mannose (○) into trichloroacetic acid-insoluble material was measured over a 20-min period. The results are expressed as percentage of control incorporation, which was 2800 cpm and 53,500 cpm for leucine and mannose, respectively. (*Inset*) Phase-contrast micrographs of control (C) and SW-treated (3 μg/ml; S) B16-F10 cells.

subcutaneously into C57BL/6 mice and the rate of appearance of palpable tumors was noted. In all animals, tumors appeared at similar times (visible at days 11–12) and progressed to kill the host at comparable rates (mean killing times of 19.5 ± 3.3 and 19.9 ± 2.6 days for control and SW-treated cells, respectively). Incubation of B16-F10 cells with SW does not, therefore, affect their ability to form tumors in syngeneic animals. Second, the growth of control and SW-treated cells was tested in a standard soft agar assay (21). B16-F10 cells grew well suspended in 0.33% agar in the presence of 10% serum (86.3 ± 6.1% of inoculated cells forming colonies), and SW treatment did not impair this ability (83.2 ± 5.6% formation). The size of the colonies was similar in both cases, indicating that the anchorage independence of B16-F10 growth was not affected by drug treatment. We conclude as a result of these studies that SW does not affect the tumorigenic properties of B16-F10 cells.

Pulmonary Retention of Radiolabeled B16-F10 Cells. As an initial step toward investigating the mechanisms involved in SW-mediated inhibition of pulmonary colonization, the retention of [^{125}I]iododeoxyuridine-labeled B16-F10 cells in the lungs of C57BL/6 mice was monitored as a function of time after lateral tail vein injection. As shown in Fig. 5, almost complete binding of both control and SW-treated cells to the lungs was observed 2 min after injection. Both sets of cells were subsequently cleared at a negative exponential rate as is characteristic for this assay (20), but interestingly SW-treated cells were lost more rapidly (50% loss occurring in 65 min compared to 110 min for the control; Fig. 5). After only 6 hr of incubation, less than half as many SW-treated cells remained in the lungs compared to the control (Fig. 5). This result suggests that one mechanism by which SW blocks metastatic colonization is interference with tumor cell retention in the target organ during the early post-injection time period.

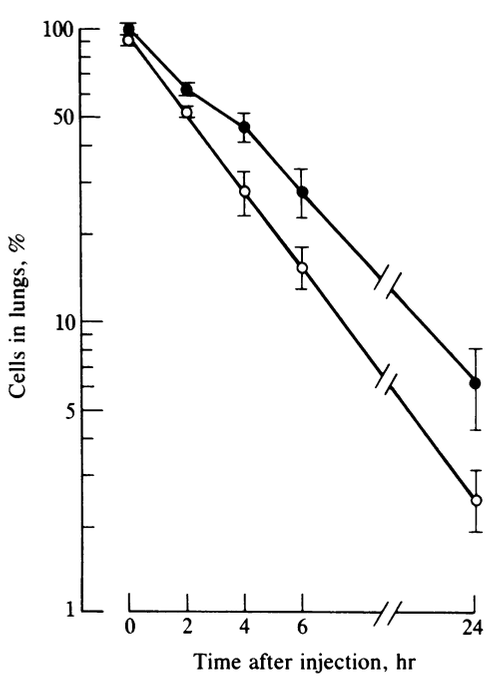

FIG. 5. Pulmonary retention of radiolabeled B16-F10 cells. Control (●) and SW-treated (3 μg/ml; ○) cells were labeled with [^{125}I]iododeoxyuridine and injected intravenously into C57BL/6 mice. The pulmonary radioactivity was determined at the indicated times after sacrifice and exsanguination. Bars represent SEM ($n = 4$). The level of radioactivity in other organs was also monitored, but no significant difference between control and SW-treated cells was observed.

DISCUSSION

The process of tumor metastasis can be subdivided into a complex series of steps, many of which are still poorly understood (3, 24–26). Several of these steps are thought to be mediated by cell-surface macromolecules of the malignant cells (reviewed in refs. 27–29), and a full understanding of the biochemical mechanisms involved offers the potential for selectively interfering with crucial metastatic events.

By using SW to specifically alter carbohydrate structures, we have investigated the role of the oligosaccharide subunits of B16-F10 cellular glycoproteins in metastatic blood-borne arrest and colonization. The colonization capacity of these cells was substantially impaired by SW treatment, suggesting that the particular orientation and/or sequence of glycoprotein sugar residues is critical for the cells to be able to successfully colonize target organs.

Several precedents exist for carbohydrate-mediated cellular functions that may be involved at different stages of the metastatic cascade and that may be inhibited by SW. In general, alteration of surface carbohydrates appears to interfere with the adhesiveness of tumor cells together with their recognition of target organs. Lectin-resistant mutant cell lines often possess altered adhesive properties (6, 30, 31), and some specificity in the ability of cellular glycopeptides to inhibit adhesion to extracellular matrix molecules has been demonstrated (32). Treatment of cells with either glycosidases (33), lectins (33), tunicamycin (34, 35), or neuraminidase (31) modulates their attachment and spreading *in vitro*. Recently, cell-surface lectins on normal hepatocytes that aggregate liver-metastasizing tumor cells and that may be involved in the organ selectivity of these cells have been identified (36–38). These findings support the conclusions of earlier correlative studies that linked the cell-surface protein profiles of different tumor cell sublines with their preferred organ colonization (39, 40).

The novel requirement for strict carbohydrate specificity that is apparent from our studies appears to strongly implicate an oligosaccharide–lectin-type of recognition event in metastatic colonization by B16-F10 cells. We suggest a model in which early interference in either B16-F10 cell adhesion or recognition is one mechanism for SW-mediated inhibition of pulmonary colonization. A contribution from other more complex cellular effects of SW should not, however, be ruled out. Indeed, alteration of exposed oligosaccharide residues has been shown to affect glycoprotein immunogenicity and to elicit activation of the immune system (41, 42). A detailed study of the changes in cellular glycoproteins induced by SW should yield important results concerning the surface macromolecules involved in metastatic colonization.

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