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The Role of Glycoconjugates in Metastatic Melanoma Blood-Borne Arrest and Cell Surface Properties

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The role of glycoconjugates in cell surface and blood-borne implantation properties of murine metastatic melanoma sublines of low (B16-F1) or high (B16-F10) potential to colonize lungs was investigated by treating melanoma cells with the antibiotic tunicamycin. This drug prevents glycosylation of glycoproteins by inhibiting the formation of lipid-linked oligosaccharide precursors. The degree of tunicamycin-mediated modifications in glycoproteins was assessed by monitoring the decrease in cell surface sialogalactoproteins by binding of ^{125}I-labeled Ricinus communis agglutinin I. Scanning electron microscopy of tunicamycin-treated B16-F1 and B16-F10 cells showed morphologic changes such as cell rounding and formation of numerous surface blebs. Tunicamycin-treated B16-F1 and B16-F10 cells lost their lung colonization abilities when injected intravenously into C57BL/6 mice, concomitant with lowered rates of adhesion to endothelial cell monolayers, endothelial extracellular matrix (basal lamina), and polyvinyl-immobilized fibronectin in vitro, suggesting that this drug inhibits experimental metastasis by modifying the surface glycoproteins involved in determining the adhesive properties of malignant cells.

Key words: adhesion, blood-borne implantation, extracellular matrix, glycoprotein, melanoma metastasis, tunicamycin, lectin

The process of tumor metastasis involves a complex series of sequential steps from malignant cell invasion of surrounding tissues at the primary tumor site to colonization of distant secondary sites [1-3]. The tumor cell properties related to each step of the metastatic process have been investigated using animal tumor models [3, 4]. Of particular interest are cell surface properties, because the cell surface has been implicated in metastatic events such as blood-borne arrest and organ colonization [5, 6]. Three different experimental approaches have been used to demonstrate the role of cell surface components in metastasis: incorporation of exogenous molecules from cells of differing metastatic potential into the plasma membranes of malignant cells [5, 6]; modification of blood-borne arrest properties by cell surface enzymatic alterations [7, 8]; and correlation of malignant cell surface glycoproteins [9-14], enzymes [15-17], or antigens [13, 18] with experimental metastasis.

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We have found that glycoconjugates on B16 melanoma sublines are involved in the blood-borne arrest properties of these cells. Using drugs such as tunicamycin, an antibiotic produced by Streptomyces lysosuperificus that inhibits formation of asparagine-linked oligosaccharide chains on glycoproteins [19–23], we found that experimental metastasis of B16 melanoma cells can be inhibited significantly [24]. Several studies have utilized tunicamycin to evaluate the role of glycoconjugates in glycoprotein activities [25–37]. In this report we found that tunicamycin-mediated modifications in B16 melanoma cell surface sialogalactoproteins could be monitored by the loss in binding of ^{125}I-labeled Ricinus communis agglutinin I to neuraminidase-treated B16 cells. Tunicamycin treatment inhibited B16 melanoma experimental metastasis and adhesion of B16 cells to endothelial cell monolayers, extracellular matrix (basal lamina) made by endothelial cells, and polyvinyl-immobilized fibronection.

MATERIALS AND METHODS

Cells and Cell Cultures

Murine B16 melanoma sublines selected once (B16-F1) or ten times (B16-F10) for blood-borne lung implantation, survival, and growth [38] were supplied by Dr. I.J. Fidler (NCI-Frederick Cancer Research Center, Frederick, Maryland) and were grown on plastic in Dulbecco modified Eagle's minimum essential medium (DMEM) (Gibco, Grand Island, New York) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Inc., Inglewood, California) and 1% nonessential amino acids (GIBCO). Low passage cell cultures (< 8 passages) were grown under humidified atmosphere in an incubator containing 5% CO_2 and 95% air at 37°C. Subconfluent cell cultures were harvested by treatment for 10–15 min with 2 mM EDTA in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS). After suspension into single cells and washing by brief centrifugation and resuspension in DMEM without serum, cell viabilities were determined by trypan blue exclusion. Cell lines were tested routinely for mycoplasma by use of Hoechst 33258 stain [39]. Bovine aortic endothelial cells were obtained from Dr. D. Gospodarowicz (University of California, San Francisco) [40] and cultured in alpha-modified minimum essential medium (AMEM, GIBCO) supplemented with 10% calf serum (GIBCO). Fibroblast growth factor was purified as described [41] and added to endothelial cells every other day at a concentration of 100–500 ng/ml. For adhesion assays endothelial cells were grown to confluency in 24-well Costar tissue culture dishes [42]. Other conditions were the same as for B16 melanoma sublines [8–10, 14].

Treatment of B16 Melanoma Cells With Tunicamycin

Tunicamycin (lot 177382) was obtained from the U.S. National Cancer institute (contract of Dr. G. Tamura, University of Tokyo) and was solubilized in 10 mM sodium hydroxide at a concentration of 2 mg/ml and kept at −20°C for up to 3 weeks. Immediately before each experiment the tunicamycin solution was diluted into culture medium and sterilized by passing through Acrodisc (0.2 μm; Gelman, Ann Arbor, Michigan). From the results of dose-response experiments described elsewhere [24] a concentration of 0.5 μg/ml was used. The incubation was carried

out for 24 or 36 hours in order to effect maximally cell surface glycoproteins while maintaining cell viability at 90–95% [24]. Because of B16 melanoma growth inhibitory activity of this drug, the initial cell densities were chosen so that both treated and untreated cultures reached equivalent subconfluent densities (2–4 × 10^6/T-75 tissue culture flask) at the end of the pretreatment period.

Experimental Blood-Borne Metastasis Assays

Female C57BL/6 mice, 4–6 weeks old, were obtained from Charles River, Inc. (Kingston, Maryland) and quarantined for 2 weeks. Disease-free animals were fed normal rodent chow and unchlorinated spring water, and their weights were recorded twice weekly. Washed B16 cell suspensions 2 × 10^5 viable cells/ml in DMEM) were kept on ice prior to injection into animals. Fifteen mice per experimental group were inoculated IV with 4 × 10^4 tumor cells in 0.2 ml. Mice were killed 20 days later and autopsied. The numbers of pulmonary tumor nodules were counted after the lung was perfused via the trachea with 4% formalin in DPBS. Extrapulmonary tumor formation was assessed in each animal and recorded.

Binding of Ricinus communis Agglutinin I to Cell Surface

Carrier-free Na(^{125}I) was obtained from New England Nuclear (Boston). Ricinus communis agglutinin I (RCA_I) was prepared as previously described [43] and iodinated according to the procedures of Burridge [44]. The specific radioactivity of ^{125}I-labeled RCA_I was 3.0 × 10^4 cpm/μg of protein. Binding experiments were carried out according to Kawaguchi et al [45] as follows. Briefly, serially diluted ^{125}I-RCA was incubated with 1.0 × 10^5 B16-F1 or B16-F10 cells previously treated or untreated with tunicamycin for 24 hours, detached with 2 mM EDTA in Ca^{2+}, Mg^{2+}-free DPBS, and suspended in DMEM containing 1% bovine serum albumin (BSA, fraction V, Sigma Chemical, St. Louis). The reaction was performed in a final volume 0.4 ml of DMEM containing 1% BSA (fraction V, Sigma) at room temperature for 60 min with occasional gentle mixing. To remove sialic acid from the cell surface prior to lectin labeling, the cell suspension was incubated with 10 m unit/ml of neuraminidase (Arthrobactor ureafaciene; Calbiochem-Behring, La Jolla, California) in DPBS containing 1% BSA at 37°C for 60 min and washed with DMEM. After 60 min binding reaction with ^{125}I-RCA_I, the cells were washed twice with 3 ml of DPBS by centrifugation. Bound radioactive ^{125}I-lectin was counted in Beckman Model 8000 automatic gamma counter. Data were plotted according to Steck and Wallach [46]. The number and association constants of major receptor sites were calculated according to Kawaguchi and Osawa [47].

Scanning Electron Microscopy

B16 melanoma cells were grown on sterilized glass coverslips. Tunicamycin (0.5 μg/ml) was added in DMEM containing 5% FBS and 1% nonessential amino acids, and the cells were incubated for 12–24 h at 37°C. The excess medium was removed, and the cells were rinsed in complete medium plus serum and incubated for 24 additional h, or the excess medium was removed and replaced by a drop of fixative. Tunicamycin-treated and untreated B16 cells were fixed in 0.1% formaldehyde, 0.72% glutaraldehyde, 0.114 M sodium cacodylate buffer, pH 7.3 (390 mOsm), for 15 min at room temperature for this and all subsequent steps. After rinsing three

times in 0.16 M sodium cacodylate buffer, pH 7.3 (340 mOsm), for 1 min each, the samples were postfixed in 2% OsO_4 in 0.125 M sodium cacodylate buffer, pH 7.3, for 15 min, rinsed in the same buffer three times for 3 min each, rinsed in distilled water once for 5 sec, and stained with a filtered, saturated solution of thiocarbohydrazide for 5 min. The samples were again rinsed in distilled water five times for 1 min each, fixed in 2% OsO_4 in distilled water for 15 min, and rinsed five additional times for 1 min. each. The fixed samples were dehydrated in ethanol, critical-point dried using liquid CO_2, and coated with AuPd prior to examination in an AMR-1000 scanning electron microscope.

Adhesion Assays

Tumicamycin-treated and untreated B16 melanoma cells were radiolabeled with ^{51}Cr. Labeling was performed with 0.25 mCi Na $(^{51}Cr)O_4$ (carrier-free in sterile saline solution, New England Nuclear) per T-75 tissue culture flask for 3 h in DMEM plus 5% FBS and 1% nonessential amino acids. B16 cells (2–4 $\times$ 10^6/flask) were harvested with 2mM EDTA in Ca^{2+}, Mg^{2+}-free DPBS, washed three times with DMEM containing 1% BSA, pH 7.4, and suspended in the same solution at a concentration of 5 $\times$ 10^4 cells/ml. Endothelial cells were carefully washed with DMEM containing 1% BSA, pH 7.4, 30 min prior to the assays. Endothelial extracellular matrix was prepared as described previously [42]. Bovine plasma fibronectin purified from calf serum (Irvine, Scientific, Irvine, California) according to Engvall and Ruoslahti [48] was used for coating polyvinyl dishes (Linbro, Hamden, Connecticut) by the method previously described [42]. Tumor cell suspensions were placed on endothelial monolayers, endothelial extracellular matrices, or immobilized fibronectin, and incubated with agitation at 37°C. At given time intervals the samples were washed carefully three times with 0.5 ml DMEM containing 1% BSA, pH 7.4, at 37°C. Adhering ^{51}Cr-labeled cells were counted in a Beckman Model 8000 automatic gamma counter.

RESULTS

Tunicamycin Treatment of B16 Melanoma Cells

As described elsewhere [24], the effects of tunicamycin on macromolecular synthesis in B16-F1 and B16-F10 melanoma sublines were not specific for glycoconjugate biosynthesis. In order to block incorporation of (^3H)mannose into acid, insoluble macromolecules within 24 h, 0.5 μg/ml or higher concentration of this drug was required [24]. Incubation of B16 melanoma cells for more than 12 h in 0.5 μg/ml tunicamycin resulted in inhibition of protein synthesis as shown by incorporation of (^3H)leucine into acid-insoluble macromolecules. At this dose protein synthesis was approximately 50% of control level [24]. DNA synthesis and growth rates of both B16-F1 and B16-F10 variant sublines decreased such that the cell doubling times were approximately twice those of control cells. However, even after 36 h of tunicamycin treatment, viabilities of these cells shown by trypan blue dye exclusion were more than 90%. Furthermore, the rates of increase in cell numbers, as well as the rates of DNA synthesis, recovered to control levels within 24 h after removal of tunicamycin from the culture media [24].

Table I. Lung and Extrapulmonary Tumors Induced by Injections of B16 Melanoma Sublines

Cell line	Tunicamycin treatment[a]	No. of lung tumor colonies/animal	Median (range)	No. of animals with extrapulmonary tumor	Location and no. of extrapulmonary tumor
B16-F1	–	0, 0, 0, 0, 0, 1, 2, 3, 4, 5, 10, 13, 28, 32, 35, 40	4 (0-40)	2	subcutaneous 2
	+	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 3, 5	0 (0-5)	1	ovary 1
B16-F10	–	19, 27, 28, 32, 35, 44, 48, 57, 62, 66, 83, 91, 127	48 (19-127)	6	ovary 5 subcutaneous 1 mesentry 1
	+	0, 0, 0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 1, 2, 2	0 (0-1)	2 2	ovary 2 mesentry 1

[a]0.5 μg/ml for 36 h.

Experimental Metastasis of B16 Melanoma Sublines After Tunicamycin Treatment

Both B16-F1 and B16-F10 melanoma variant cells, which were selected for lung colonization in vivo once or ten times, respectively, yielded tumor foci in the lungs. However, subline B16-F10 formed approximately ten times more pulmonary tumors than subline B16-F1 as expected [3, 5, 8, 14]. After 24 h treatment of B16 cells with 0.5 μg/ml tunicamycin, the incidence of lung colonization was reduced significantly (Table I). In terms of inhibiting lung colonization, B16-F1 and B16-F10 sublines responded to this drug to similar extents. Tumor colonization did not appear to increase at extrapulmonary sites, suggesting that tunicamycin does not shift the distribution of experimental metastases from lungs to other organs.

Effect of Tunicamycin on Adhesion Characteristics of B16 Melanoma Cells

Since growth rates in vitro of B16-F1 and B16-F10 cells recovered within 24 h after tunicamycin removal, the differences in tumor colonization in vivo appeared to be due to specific initial arrest of tumor cells in the lung microcirculation. We therefore carried out an adhesion experiment in vitro that was designed to mimic the initial steps of tumor cell colonization. Using a non-shear assay for attachment of tumor cells to confluent endothelial cell monolayers, the rates of adhesion of (^{51}Cr)-labeled B16-F1 or B16-F10 cells were lowered by previous treatment of tumor cells with 0.5 μg/ml tunicamycin (Fig. 1a). Comparison of B16 melanoma adhesion to endothelial monolayers (Fig. 1a) or to endothelial extracellular matrix or basal lamina (Fig. 1b) indicated that the melanoma cells attach more rapidly to extracellular matrix produced by endothelial cells than to endothelial cells themselves, as found previously [42]. Tunicamycin treatment also reduced the rate of adhesion of melanoma cells to isolated endothelial matrix (Fig. 1b). The adhesion kinetics of melanoma cells to extracellular matrix and to polyvinyl-immobilized fibronectin were similar, suggesting that matrix fibronectin is important in the enhanced tumor cell adhesion to extracellular matrix [42]; tunicamycin also inhibited attachment of B16 cells to the immobilized fibronectin (Fig. 1c). This observation, in combination with the fact that fibronectin is the predominant glycoprotein component of endothelial extracellular matrix, supported our previous proposal that matrix fibronec-

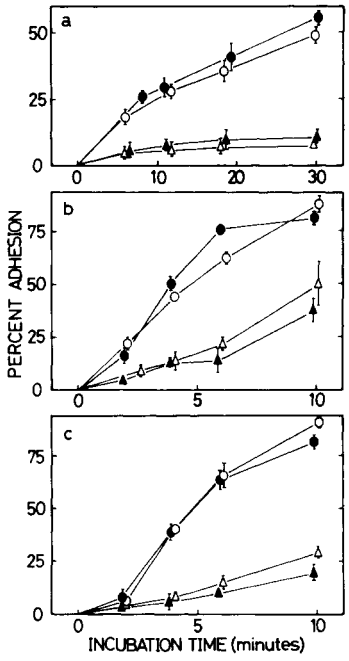

Fig. 1. Kinetics of adhesion of untreated or tunicamycin-treated (0.5 μg/ml, for 24 or 36 h) B16 melanoma cells to: a) confluent monolayer of endothelial cells, b) isolated endothelial cell extracellular matrix, c) polyvinyl-immobilized fibronectin. ● – ●, Untreated B16-F1 cells; ○ – ○, untreated B16-F10 cells; ▲ – ▲, tunicamycin-treated B16-F1 cells; △ – △, tunicamycin-treated B16-F10 cells.

tin is responsible, at least in part, for tumor cell attachment to vascular endothelium [42]. Because the adhesion process is mediated by tumor cell surface components, tunicamycin-sensitive cell surface molecules are likely to play an important role.

Ricinus communis Agglutinin I Binding Sites on B16 Melanoma Sublines

Since tunicamycin is known to impair the synthesis of asparagine-linked sugar chains in glycoproteins by affecting the formation of N-acetylglucosamine-containing lipid intermediates, complex-type sugar chains on B16 melanoma cells were analyzed by use of ^{125}I-RCA$_1$. This lectin is known to bind preferentially to β-galactosyl-N-acetylglucosaminyl terminal sequences in complex-type sugar chains [49]. Without prior treatment of cells with neuraminidase, virtually no binding of RCA$_1$ was detected on either B16-F1 or B16-F10 cell surfaces (Fig. 3) indicating that nonreducing termini of oligosaccharide chains are heavily sialylated, which blocks binding of RCA$_1$ [43]. After neuraminidase treatment ^{125}I-RCA$_1$ bound to B16-F1 and B16-F10 cells with biphasic kinetics, indicating the existence of major lectin receptors with an association constant of about 2×10^7 M^{-1}. The data also suggested that there are other lectin receptors with lower association constants on B16-F1 and B16-F10 cells. The numbers of high affinity receptor sites were approximately 5.0×10^7 and 2.8×10^7 for B16-F1 and B16-F10, respectively. After a 24-h treatment with tunicamycin, the high affinity receptor sites, which are probably composed of complex-type sugar chains, disappeared, whereas the lower affinity receptor sites re-

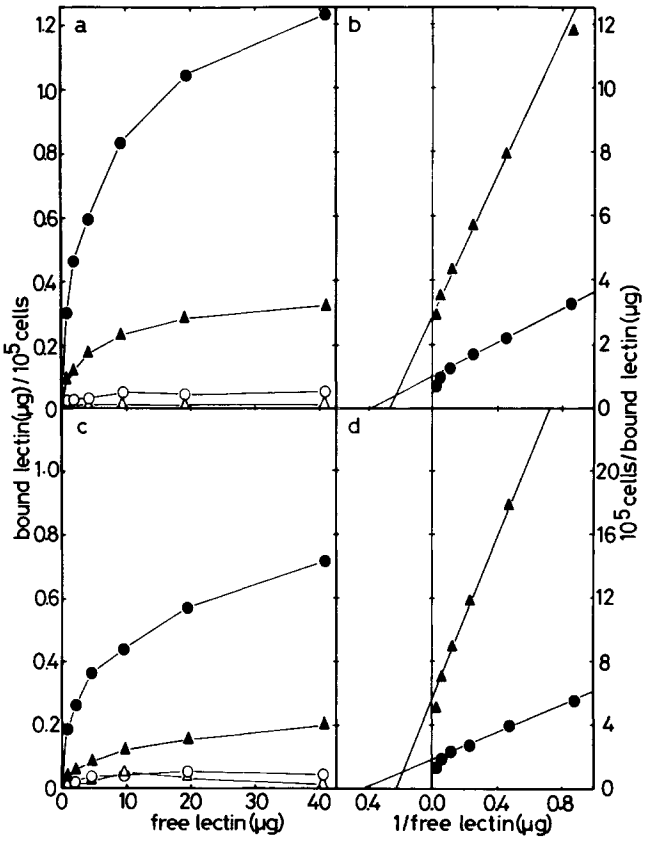

Fig. 2. Quantitative binding of ^{125}I-labeled Ricinus communis agglutinin I to untreated and tunicamycin-treated (0.5 μg/ml for 24 h) B16-F1 (a, b) or B16-F10 (c, d) cells. ● — ●, Neuraminidase-treated control cells; ○ — ○, control cells; ▲ — ▲, neuraminidase-treated, tunicamycin-treated cells; △ — △, tunicamycin-treated cells. Panels b and d were plotted according to Steck and Wallach [46].

TABLE II. Binding of Ricinus communis Agglutinin$_I$ to Neuraminidase-Treated B16 Melanoma Cells

	Untreated		Cultured in the presence of tunicamycin[a]	
Cell line	K_O[b]	n[c]	K_O[b]	n[c]
B16-F1	2.0×10^7	5.0×10^7	1.3×10^7	1.6×10^7
B16-F10	2.1×10^7	2.8×10^7	1.1×10^7	0.8×10^7

[a]0.5 μg/ml for 24 h.
[b]Apparent association constant (M^{-1}) for major receptor sites.
[c]Number of major receptor sites on single cell.

mained (Fig. 2 and Table II). These results indicated that the nonreducing ends of asparagine-linked sugar chains whose nonreducing ends are composed of sialyl-galactosyl residues disappear or decrease greatly on cells treated with tunicamycin.

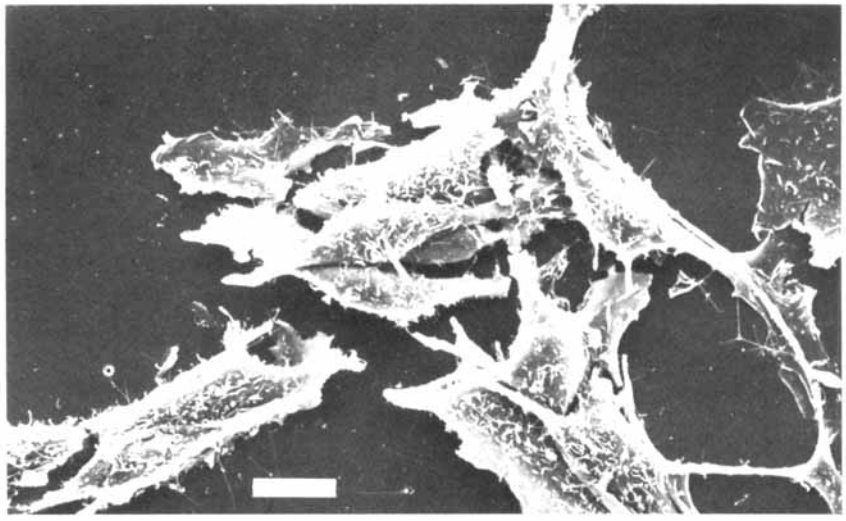

Fig. 3. Scanning electron micrograph of untreated B16-F1 cells. Bar equals 10 μm.

Morphological Changes on B16 Melanoma Cells Detected by Scanning Electron Microscopy

Surface morphologies of tunicamycin-treated and untreated B16-F1 and B16-F10 cells were studied by scanning electron microscopy. Normally B16 cells are flat in appearance with numerous microvilli and cellular processes (Fig. 3). After a 12-h treatment with 0.5 μg/ml tunicamycin, a fraction of cells became rounded in appearance with no apparent difference between B16-F1 and B16-F10 cells (data not shown). By 24 h more than 70% of the melanoma cells became rounded in shape (Fig. 4). Although all cells at this time remained adherent to the culture substrate, they could be detached easily by a brief treatment with an EDTA solution. Tunicamycin treatment caused the surfaces of cells to lose microvili and be covered with small bleblike projections (Fig. 4). Within 24 h after removal of this drug cells regained their flattened morphologies, showing that the reversibility of the morphological changes correlated with tunicamycin presence (Fig. 5). These changes might be attributed to a modification of surface molecules such as glycoproteins, which are known to be important in plasma membrane organization and cell adhesion to substratum.

DISCUSSION

Tunicamycin has been used as a specific inhibitor of protein glycosylation because it affects formation of lipid-linked precursors required for the formation of asparagine-linked oligosaccharide moieties. However, the effects of tunicamycin on the synthesis, transport, localization, and function of glycoproteins on different cell types have been quite variable [25-37]. Moreover, in some cases unglycosylated molecules were found to be more susceptible to proteolytic digestion such that these molecules were turned over or degraded at higher rates [30, 37]. The effects of

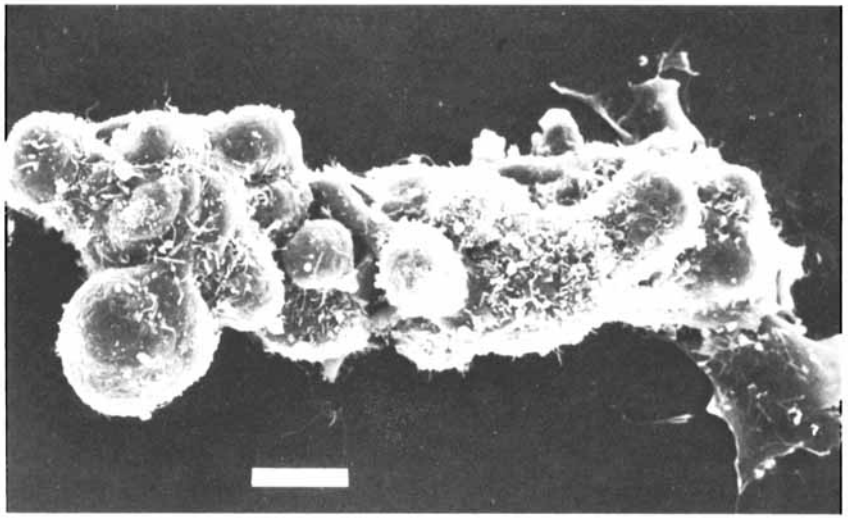

Fig. 4. Scanning electron micrograph of B16-F1 cells treated 24 h with 0.5 μg/ml tunicamycin. Bar equals 10 μm.

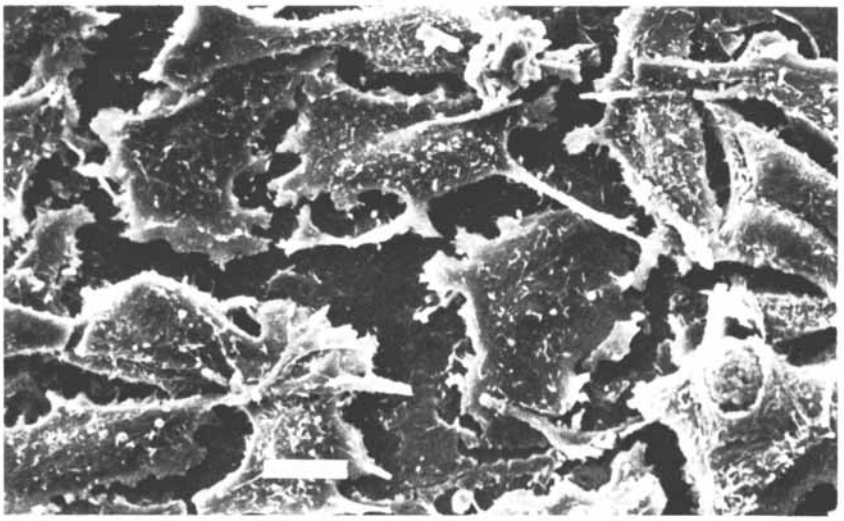

Fig. 5. Scanning electron micrograph of B16-F1 cells treated 24 h with 0.5 μg/ml tunicamycin and then incubated an additional 24 h in the absence of tunicamycin. Bar equals 10 μm.

tunicamycin on cellular physiology appear to be more complex. Duskin and Bornstein [50] found that tunicamycin was toxic to virally transformed mouse 3T3 and human WI-38 cells but not to the corresponding untransformed cell lines. Olden et al [51] confirmed this observation on a variety of fibroblast cell lines and also found that permanent transformed cell lines were resistant to tunicamycin treatment. However, even cells in which tunicamycin was not toxic showed reductions in their

growth rates and modifications in their morphologies [50]. Morphological alterations in chick embryo fibroblasts during tunicamycin treatment was shown by Olden et al [30] and Pratt et al [52] to occur concomitant with decreases in fibronectin and sulfated proteoglycans. Similar observations have been made using 3T3 fibroblasts [53]. Cellular differentiation was found to be modified by tunicamycin. Surani [54] documented that a particular stage of mouse embryonic development was blocked by tunicamycin, and Nakayasu et al [55] reported that this drug induced differentiation of human and moue myeloid leukemic cells in vitro. Although these effects, as well as effects on chick embryo chondrocyte differentiation [56], were likely a consequence of cell surface modification by tunicamycin, the molecular mechanisms involved in cellular interaction and differentiation remain to be elucidated.

Tumor metasis is one of the more important unsolved biological phenomena in which the cell surface plays an important role [3, 6]. The significance of plasma membrane carbohydrates in the metastasis of malignant B16 melanoma cells is unknown. Cell surface modifications have been detected in B16 sublines selected for enhanced blood-borne arrest, invasion, and survival at particular organ sites. Lung-selected sublines such as B16-F10 have modifications in cell surface glycoproteins detected by galactose oxidase-borohydride labeling procedures [14], whereas ovary-selected [10] and brain-selected [9] B16 sublines possess cell surface protein alterations detectable by lactoperoxidase-catalyzed iodination that correlate with preferred organ colonization.

In this study we have utilized tunicamycin to modify cell surface carbohydrates of lung-selected B16 melanoma cells in order to assess their role in blood-borne arrest and experimental lung metastasis. It is clear from our data that tunicamycin-mediated modification of the B16 cell surface results in a marked reduction in the potential of blood-borne melanoma cells to arrest and survive to form pulmonary tumor colonies, indicating the importance of carbohydrates and glycoproteins in this process. However, the exact roles of carbohydrates and glycoproteins are not clear. Although tunicamycin reduced the growth rates of B16 melanoma cells, cell viability after 36 h treatment was greater than 90%; furthermore, treated cells returned to normal growth rates after removal of this drug. Therefore, the effects of tunicamycin were apparently not due to a general depression of activities important for cell survival. We have shown that metastatic tumor cells are very adept at binding to and invasion of endothelial cell monolayers [42, 57], so we examined the kinetics of attachment of untreated and tunicamycin-treated B16-F1 and B16-F10 cells to endothelial cell monolayers in vitro. B16 cell–endothelial cell adhesion was dramatically inhibited by tunicamycin, and experimental metastatic implantation was reduced. As described previously [42], malignant cell adhesion to endothelial cells occurs at lower rates than to extracellular matrix produced by endothelial cells, as well as to an immobilized-fibronectin surface. In all of these processes tunicamycin lowered the kinetics to approximately the same extents. The similarity of adhesive characteristics after tunicamycin treatment supports our previous finding that fibronectin, a major component of extracellular matrix, plays, at least in part, a significant role in tumor cell adhesion to vascular endothelium. We expect that the tunicamycin-sensitive cell surface molecules on melanoma cells responsible for adhesion and tumor implantation bind to fibronectin. In fact, we found a difference between the binding of ^{125}I-labeled serum fibronectin (CIg) to tunicamycin-treated and untreated B16 melanoma cells. However, the amounts

bound and the association constants of this binding reaction were extremely low, and it was therefore difficult to interpret these data.

Cell surface biochemical changes due to tunicamycin were detected on B16 melanoma cells by binding of Ricinus communis agglutinin I, which clearly demonstrated cell surface carbohydrate modifications. The binding of ^{125}I-RCA_I to B16 cells was observed only after neuramindase pretreatment of B16-F1 and B16-F10 melanoma cells; therefore, complex-type sugar chains on B16 and B16-F10 cells are fully sialilated at their nonreducing termini. After incubating cells with tunicamycin, RCA_I receptor sites of high affinity were lost, but sites of low affinity remained. These latter RCA_I binding sites were composed presumably of glycolipids or mucin-type sugar chains [47, 49], the synthesis of which did not seem to be particularly sensitive to tunicamycin. These results also suggested that the net surface charge of melanoma cells could be modified by tunicamycin treatment, which affects cellular adhesion and blood-borne arrest processes. Although asparagine-linked sugar chains on glycoproteins were the most probable target of tunicamycin, the possibility that this drug modified directly or indirectly other classes of cell surface molecules has not been eliminated. Cell surface glycosaminoglycans are the other candidates for tunicamycin modification, because Satoh et al [58] demonstrated that B16 melanoma cells produce hyalurounic acid, chondroitin sulfate, and heparan sulfate. In addition, proteoglycan synthesis in chick embryo fibroblasts and embryonic chick cornea were inhibited by tunicamycin [51, 59], and proteoglycans appear to be involved in cell adhesion to substratum [60, 61].

The results and findings in this report suggesting that cell surface complex carbohydrates are important in the B16 melanoma metastatic process are consistent with previous reports [11, 14, 62]. Elsewhere we have described the effects of tunicamycin on B16-F1 and B16-F10 cellular metabolism and glycoproteins synthesis in relation to the blood-borne arrest and lung colonization potentials [24]. It remains to be demonstrated if the sialo-oligosaccharide that binds to RCA_I after removal of terminal sialic acid will be a useful marker of surface molecules that mediate endothelial arrest and blood-borne metastasis of B16 melanoma cells.

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