Effects of Tunicamycin on B16 Metastatic Melanoma Cell Surface Glycoproteins and Blood-borne Arrest and Survival Properties

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ABSTRACT

The role of cell surface glycoproteins in determining in vivo blood-borne arrest and survival characteristics of murine melanoma sublines of low (B16-F1) or high (B16-F10) potential to form experimental lung metastases after injection i.v. was assessed after inhibiting tumor cell protein glycosylation with tunicamycin. Incubation of B16-F1 or B16-F10 cells with 0.5 µg (or above) tunicamycin per ml for 12 to 36 hr inhibited significantly lung tumor colony formation. Examination of B16 cells in the presence of 0.5 µg drug per ml indicated that complex oligosaccharide synthesis was inhibited >90%, while protein synthesis remained at about 50% of the control levels. Tunicamycin induced morphological changes in B16-F1 and B16-F10 cells such as cellular rounding. Cell growth was also inhibited by tunicamycin. These effects were reversible, and B16 cells recovered their normal morphologies and growth rates within 24 hr after removal of the drug. Exposed cell surface protein analyzed by lactoperoxidase-catalyzed 125I-iodination-sodium dodecyl sulfate-polyacrylamide gel electrophoresis-autoradiography showed few changes after tunicamycin treatment; however, sialogalactoproteins (detected by the binding of 125I-labeled R. communis agglutinin I to polyacrylamide gels containing desialized B16 cell surface components) were reduced dramatically by the drug. The adhesive properties of untreated and tunicamycin-treated B16 cells were assessed by the binding of 31Cr-labeled B16 cells to endothelial cell monolayers. Tunicamycin-treated B16-F1 and B16-F10 cells adhered at lower rates to endothelial cells such that after 24 to 36 hr of drug (0.5 µg/ml) treatment adhesion was almost completely blocked, suggesting that tunicamycin-induced cell surface glycoprotein changes in B16 melanoma cells may interfere with tumor cell-host cell interactions that lead to arrest and survival of blood-borne malignant cells.

INTRODUCTION

The formation of distant metastases involves several discrete, selective steps that depend on a complex array of cellular interactions between tumor and host cells (15, 18, 44, 45, 50, 55). It is thought that many, if not all, of these interactions are mediated by cell surface molecules. Evidence for this has come from experiments where the surface membranes of cells of low metastatic potential have been modified by fusion and incorporation of plasma membrane vesicles obtained from cells of high metastatic potential. The vesicle-modified tumor cells transiently and specifically displayed enhanced arrest and experimental metastasis formation compared to the unmodified tumor cells or cells modified with vesicles from cells of low metastatic potential (51). In addition, there are numerous examples where cell surface differences in the display, dynamics, amounts, or structures of cell surface proteins-glycoproteins exist between tumor cell sublines selected in vivo or in vitro for altered metastatic properties (4, 5, 18, 26, 43, 45, 54-57). Since most cell surface proteins appear to be glycosylated (3, 10) and oriented with their oligosaccharides facing the exterior side of the cell membrane (47), it is possible that certain metastatic properties, such as blood-borne tumor cell arrest, may depend on the correct display of specific plasma membrane glycoproteins.

In the studies reported here, we asked whether the oligosaccharides found on tumor cell surface glycoproteins are involved in determining blood-borne arrest properties by interfering with protein glycosylation using the antibiotic tunicamycin (61-63) which specifically blocks synthesis of the saccharide donor N-acetylglucosaminyl pyrophosphoryl polyisoprenol (39, 64). Using the murine B16 melanoma sublines selected once (B16-F1) and 10 times (B16-F10) for blood-borne lung implantation, survival, and growth (14), we found that treating B16 cells in vitro with low doses of tunicamycin inhibits experimental lung tumor colony formation without increasing extrapulmonary tumor formation after injection i.v. of the B16 tumor cells.

MATERIALS AND METHODS

Cells and Cell Culture. Murine B16 melanoma sublines of low (B16-F1) and high (B16-F10) lung tumor colony formation or experimental lung metastasis characteristics were supplied by Dr. I. J. Fisher (National Cancer Institute-Frederick Cancer Research Center, Frederick, Md.) and were grown on plastic in DMEM (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% heat inactivated fetal bovine serum (Flow Laboratories, Inc., Inglewood, Calif.) and 1% nonessential amino acids (GIBCO). Cell lines were tested routinely for Mycoplasma by use of Hoechst 33258 stain (9). Bovine aortic endothelial cells were obtained from Dr. D. Gospodarowicz (University of California, San Francisco, Calif.) (24) and cultured in α-modified minimum essential medium (GIBCO) supplemented with 10% calf serum (GIBCO). Fibroblast growth factor was purified as described (23) and added to endothelial cells every other day at a concentration of 100 to 500 ng/ml. Endothelial cells were grown to confluency in 24-well Costar tissue culture plates. Other conditions were the same as for B16 melanoma sublines.

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Experimental Blood-borne Metastasis Assays. Female C57BL/6 mice, 4 to 6 weeks old, were obtained from Charles River, Inc. (Kingston, Md.) and quarantined for 2 weeks. Animals were fed normal rodent chow and unchlorinated spring water, and their weights were recorded twice weekly. Washed B16 cell suspensions (2 x 10^6 viable cells/ml in DMEM) were kept on ice prior to injection into animals. Fifteen mice/experimental group were inoculated i.v. with 4 x 10^6 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.

**Treatment of B16 Melanoma Cells with Tunicamycin.** Tunicamycin (Lot 177382) was obtained from the United States 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. To examine reversibility of the antibiotic effect, the culture medium containing tunicamycin was removed, and the cells were rinsed twice with the same volume of fresh culture medium and incubated without tunicamycin for various times at 37°C.

**Synthesis of Carbohydrate, Protein, and DNA.** B16 melanoma cells (4 x 10^6 in 2 ml culture medium) were cultured in 6-well plastic cluster dishes (Costar, Cambridge, Mass.) for 2 days. Various concentrations of tunicamycin and 5 μCi of [2-3H]mannose (16 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) per ml were added at the same time. After incubation for 24 hr, the medium containing radioactive precursor was removed by aspiration. Cells were washed twice with 3 ml of ice-cold DPBS, and 2 ml of ice-cold 10% TCA were added to each well. TCA-soluble material was removed by washing twice with 2 ml of 10% TCA and twice with 3 ml of 99% ethanol. Cells were dried in air and solubilized with 1 ml of 1 M sodium hydroxide. One aliquot (0.5 ml) was neutralized with 0.3 ml 2 M acetic acid and added to 10 ml of Aquasol-2 for liquid scintillation radioisotope determination in a Beckman Model LS7500. Another aliquot (0.2 ml) was subjected to protein determination according to the method of Lowry et al. (40) using bovine serum albumin fraction V as a standard. For time course experiments, [2-3H]mannose, [6-3H]glucosamine (19.0 Ci/mmol; New England Nuclear, Boston, Mass.), [3H]lucine (56.5 mCi/mmol; New England Nuclear), or [methyl-3H]thymidine (54.2 mCi/mmol; New England Nuclear) was added at a final concentration of 5 μCi/ml (or 0.5 μCi/ml for [methyl-3H]thymidine) for 6 hr. The radioactivity incorporated into macromolecules was measured by the method described above.

**Analysis of Cell Surface Proteins and Glycoproteins of B16 Melanoma Cells.** Lactoperoxidase (purified grade) was purchased from Calbiochem-Behring Co. (La Jolla, Calif.). Glucose oxidase from Aspergillus nigeria (type V) was a product of Sigma Chemical Co. (St. Louis, Mo.). A mixture of ovalbumin, bovine serum albumin, phosphatydylase b, b-galactosidase, and myosin was purchased from Bio-Rad Laboratories (Richmond, Calif.) and used for molecular weight standards. Carrier-free Na251 was from New England Nuclear. RCA was prepared as described previously (46). Exterior proteins of B16 melanoma cells were labeled (33). Labeling was performed with 0.25 mCi Na251CrO4 (carrier-free sterile saline solution, New England Nuclear) per T-75 tissue culture flask for 3 hr in DMEM plus 5% fetal bovine serum. B16 cells (3 to 5 x 10^6/flask) were harvested with 2 ml EDTA in Ca2+, Mg2+-free DPBS, washed 3 times with DMEM containing 1% bovine serum albumin, and suspended in the same solution at a concentration of 4 x 10^6/ml. The kinetics of adhesion of B16 melanoma cells onto endothelial cell monolayers were measured as described previously (33).

**RESULTS**

**Effects of Tunicamycin on B16 Cell Macromolecular Synthesis.** Tunicamycin inhibits incorporation of [3H]mannose into TCA-insoluble material during a 24-hr incubation. Dose-response curves indicate that tunicamycin, when present at concentrations of 0.5 μg/ml or greater, almost completely blocks incorporation of mannose into protein in both B16-F1 and B16-F10 cells (Chart 1). The low background incorporation of [3H]mannose in the presence of 0.5 μg (or above) tunicamycin per ml is probably due to a lag effect or less likely to mannose incorporation into complex carbohydrates that do not depend on polyisoprenol precursors; therefore, 0.5 μg tunicamycin per ml was used for subsequent experiments.

We assessed the time course of tunicamycin-mediated inhibition of glycoprotein, total protein, and DNA synthesis at various time periods after drug addition. Incorporation of mannose and glucosamine decreases rapidly and by 12 hr is less than 10 to 20% of control cultures in the absence of tunicamycin (Chart 2). The decrease in rate of glucosamine incorporation is slightly slower than that of mannose, probably because the former can be incorporated into mucin-type sugar chains as well as glucosaminoglycans. Synthesis of protein is decreased slightly but reaches a plateau at approximately 50% of initial incorporation at 12 hr and washed in 3% acetic acid. Destaining was performed in 3% acetic acid at room temperature. Low concentrations of acetic acid were used to minimize cleavage of sialic acid. To remove sialic acid from glycoproteins, the gels were heated in 50 mM sulfuric acid at 80°C for 1 hr (59) or 1.7 M acetic acid at 100°C for 30 min. Elution of lectin and direct visualization of sugar chains by use of labeled lectin were carried out according to Burridge (6). 125I-RCA (15,000 cpm/μg protein) was used at a final concentration of 0.3 mg in 10 ml, and staining was performed for 2 hr with 2 mg bovine serum albumin (Fraction V, Sigma) per ml used to reduce nonspecific binding. After washing, gels were dried on filter paper (Whatman 3 MM) and autoradiographed with intensifying screens (60) on Kodak X-Omat R-5 X-ray film.

**Adhesion of B16 Melanoma Cells onto Endothelial Monolayers.** Tunicamycin-treated and untreated B16 melanoma cells were radiolabeled (33). Adhesion of B16 melanoma cells onto endothelial cell monolayers were measured as described previously (33).

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**Chart 1.** Dose-response curve of the effect of tunicamycin on the incorporation of [3H]mannose into the TCA-insoluble fraction of whole cells. •, B16-F1; O, B16-F10. Values at each point are the average of triplicate samples. Standard deviations were less than 10% of data value.
Effects of Tunicamycin on Metastatic Colonization

Chart 2. Time course of the effects of tunicamycin on the biosynthesis of carbohydrate, protein, and DNA. Values are shown by percentage of control incorporation of radioactive precursors into TCA-insoluble fractions of whole cells. Each point represents an average of triplicate samples; bars, S.D.; a, B16-F1; b, B16-F10. O, incorporation of $\alpha$-[2-$^{3}$H]mannose; $\Delta$, incorporation of $\alpha$-[6-$^{3}$H]glucosamine; $\triangle$, incorporation of $[^{3}$H]leucine; $\blacktriangle$, incorporation of [methyl-$^{3}$H]thymidine.

of control levels after 12 hr. Tunicamycin also inhibits incorporation of thymidine into DNA, and although the rate of inhibition of DNA synthesis is less than that of protein synthesis, by 36 hr it decreases to 10% of the control levels (Chart 2). The inhibition of DNA synthesis may be attributed to secondary changes subsequent to inhibition of glycosylation. No difference is apparent between the effects of tunicamycin on B16-F1 compared to B16-F10 cells (Chart 2).

B16 melanoma cells recover from tunicamycin treatment. Although growth rates of B16-F1 and B16-F10 cells are decreased in the continuous presence of 0.5 $\mu$g tunicamycin per ml, they recover within 24 hr to normal rates when the drug is removed (Chart 3). The decrease in initial cell numbers in Chart 3 is probably related to lower plating efficiencies and/or adhesion of tunicamycin-treated cells to tissue culture plastic. Similar results are found when [methyl-$^{3}$H]thymidine incorporation into TCA-insoluble material is measured. Within 12 hr of tunicamycin treatment, $[^{3}$H]thymidine incorporation into DNA is reduced but recovers to control levels within 24 hr after drug removal in both B16-F1 or B16-F10 cells (Chart 4). The delay in recovery is due possibly to the effect of drug previously incorporated into cells or the time required for turnover of modified glycoproteins essential to events in DNA replication.

Effects of Tunicamycin on B16 Cell Morphology. After 12 hr of tunicamycin (0.5 $\mu$g/ml) treatment, a fraction of the B16 melanoma cells become rounded in appearance (Fig. 3). By 24 hr of drug treatment, approximately 70% of the cells possess modified, rounded morphologies, adhere weakly onto the cell culture substrate, and are easily detached by a short treatment of EDTA. Removal of tunicamycin from the culture media results in reversal of these effects so that, by 24 hr without drug, the cells regain their flattened morphologies. Tunicamycin induced similar morphological changes in both B16-F1 and B16-F10 cells.

Effects of Tunicamycin on Experimental Metastasis following Injection i.v. of B16 Cells. In 3 separate experiments, tunicamycin treatment (0.5 $\mu$g/ml for 12 to 36 hr) reduced significantly lung tumor colony formation assessed 20 days after injection i.v. of B16 melanoma cells (Table 1). The relative degree of tunicamycin-mediated reduction of lung tumor colonies appears to be similar for both B16-F1 and B16-F10 cells and is dependent on the length of drug treatment such that
incubation in tunicamycin (0.5 μg/ml) for 36 hr is more effective than 12 hr. In addition, the incidence of B16 extrapulmonary tumor colony formation is not increased by tunicamycin treatment, indicating that tumor colonization is not shifted to other organ sites. Since the growth rates in vitro of B16-F1 and B16-F10 cells are not affected 1 day after tunicamycin removal, the differences in tumor colonization in vivo may be due to specific initial arrest of B16 cells at pulmonary sites.

**Effects of Tunicamycin on Adhesion of B16 Cells to Endothelial Cell Monolayers.** Since the adhesion of circulating tumor cells to the vascular endothelium is thought to be an important step in the metastatic process (18, 33–35, 44, 50, 51), we tested the effects of tunicamycin on B16 cell adhesion to endothelial cell monolayers. Using a nonshear assay for adhesion of 51Cr-labeled B16 cells onto cell monolayers (34), tunicamycin (0.5 μg/ml) treatment lowers the kinetics of endothelial cell adhesion of B16-F1 or B16-F10 cells (Chart 5). Drug treatment for 36 hr is more effective in inhibiting endothelial cell adhesion than 12 hr, and these results are reversible after drug removal (data not shown).

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### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>B16 subline</th>
<th>Treatment time (hr)</th>
<th>Median no. of lung tumor colonies</th>
<th>Location and no.</th>
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<td>F10</td>
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<td>3/15</td>
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<tr>
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<td>24</td>
<td>11 (2–80)</td>
<td>Ovary, 3; brain, 1</td>
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<td>0 (0–15)</td>
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<tr>
<td></td>
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<td>6/15</td>
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<td>36</td>
<td>0 (0–2)</td>
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<td>1/15</td>
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<tr>
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<tr>
<td></td>
<td>F10</td>
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<td>4/15</td>
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<td>F10</td>
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<td>3 (0–58)</td>
<td>Ovary, 4</td>
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<td></td>
<td>F10</td>
<td>36</td>
<td>0 (0–2)</td>
<td>Ovary, 4</td>
</tr>
</tbody>
</table>

* a Tunicamycin at a final concentration of 0.5 μg/ml.
* b Groups of C57BL/6 mice were injected i.v. with 4 × 10⁶ B16 cells in 0.2 ml, and lung tumor colonies were determined after 20 days.
* c Numbers in parentheses, range.
* d Significant difference between control and tunicamycin-treated group (p < 0.01).
* e Significant difference between control and tunicamycin-treated group (p < 0.001).
Analysis of Cell Surface Proteins and Glycoproteins on Tunicamycin-treated and Control B16 Cells. Cell surface proteins of B16 cells accessible to lactoperoxidase-catalyzed $^{125}$I iodination have been determined by SDS-polyacrylamide gel electrophoresis-autoradiography. Twelve hr of tunicamycin (0.5 $\mu$g/ml) treatment does not appear to modify greatly the gel electrophoresis-autoradiography. Twelve hr of tunicamycin $^{125}$I iodination have been determined by SDS-polyacrylamide gel electrophoresis-autoradiography. Twelve hr of tunicamycin treatment, reductions of bands of approximate m.w. 84,000 and 70,000 are seen in autoradiograms along with a concomitant increase of radioactivity at the top of the gel representing material which fails to penetrate into the gel (Fig. 1). No differences are seen in the lactoperoxidase-catalyzed iodination surface labeling profiles between B16-F1 and B16-F10 cells as seen previously (45, 55), and there is no apparent difference between the effects of tunicamycin on these B16 sublines.

In order to visualize cellular glycoproteins, SDS-polyacrylamide gels can be labeled with radiolaabeled lectins. Bands on autoradiograms made after removal of sialic acids in oligosaccharides by treatment with mild acid (59) and labeling with $^{125}$I-RCA, do not correspond to major surface proteins labeled by lactoperoxidase, and no differences are seen between sublines B16-F1 and B16-F10 (cf. Fig. 1 with Fig. 2). However, the major galactoproteins visualized by $^{125}$I-RCA labeling (approximate m.w. 63,000, 67,000, 80,000, 93,000, 96,000, and 115,000) are thought to be cell surface constituents, because gels do not bind significant amounts of $^{125}$I-RCA, if the terminal sialic acid residues are not removed from oligosaccharide chains prior to labeling (data not shown). Sialic acid is believed to exist almost exclusively on the termini of glycoprotein oligosaccharides at the outer surfaces of cells (3, 10).

Galactoproteins on B16 cell surfaces visualized by $^{125}$I-RCA, labeling on SDS gels are decreased by tunicamycin treatment and reappear after tunicamycin removal. Incubation of B16 cells in 0.5 $\mu$g tunicamycin per ml for 36 hr results in almost complete loss of $^{125}$I-RCA labeling to SDS gels (Fig. 2, Lane 7). Treatment of B16 cells with the drug for 12 or 36 hr and then subsequent incubation in drug-free media for 12 to 24 hr results in recovery of $^{125}$I-RCA labeling (Fig. 2, Lanes 3 to 6 and 8 to 11). The rate of recovery for each lectin-labeled galactoprotein appears to be different, but most bands reappear by 24 hr.

DISCUSSION

The antibiotic tunicamycin was discovered by Takatsuki et al. (61) and Takatsuki and Tamura (63) to possess potent antiviral activities. Subsequently, tunicamycin was found to act by preventing glycosylation of glycoproteins via formation of N-acetylgalcosaminyl pyrophosphoryl polysoprenol (39, 64); thus, it has proven useful in elucidating the role of carbohydrate in mediating glycoprotein functional activities. For example, in the presence of tunicamycin, interferon was synthesized at normal rates and retained its biological activities but did not contain its carbohydrate chain (19, 41). In other systems, tunicamycin caused alterations in glycoprotein-biosynthetic secretory pathways. Tunicamycin causes procollagen (29), immunoglobulin (27, 28), and other glycoproteins (37, 38, 48) to be synthesized but not normally secreted. Some glycoproteins are turned over or degraded abnormally in tunicamycin-treated cells. Fibronectin is a glycoprotein found in both cell surface and secreted forms in fibroblasts, and Olden et al. (49) found that tunicamycin causes fibronectin to be synthesized normally but degraded more rapidly than normal due to a greater susceptibility to protease digestion. A similar finding has been made by Prives and Olden (53) for the acetylcholine receptor of embryonic muscle cells. Not all glycoprotein subunits are uniformly affected by tunicamycin. Nishikawa et al. (48) reported that tunicamycin treatment reduced the relative amount of glycosylated HLA-DR heavy chains in membranes of lymphoma cells without affecting glycosylation of HLA-DR light chains.

Carbohydrate appears to be important in viral maturation. When baby hamster kidney cells are treated with tunicamycin and infected with vesicular stomatitis or Sindbis viruses, G1 protein migration is impaired, and it does not appear on the outer cell surface (37, 38); however, the few nonglycosylated virions that are produced possess normal infectivities but altered stabilities (20).

The role of cell surface glycoproteins 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. Although lung-selected sublines such as B16-F10 do not show changes in lactoperoxidase-catalyzed iodination patterns compared to parental or a once-selected B16-F1 subline (45, 55), they do have modifications in glycoproteins detected by galactose oxidase-borohydride labeling procedures (55). On the other hand, ovary-selected (5) and brain-selected (4) B16 sublines possess different cell surface alterations detectable by lactoperoxidase-catalyzed iodination that correlate with preferred organ colonization.

In the present study, we have utilized tunicamycin to modify cell surface glycoprotein of 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 modifications of the B16 cells result in a marked reduction in the potential of blood-borne melanoma cells to arrest and survive to form pulmonary tumor colonies and adhere to monolayers of endothelial cells, indicating the importance of carbohydrates of glycoproteins in this process. However, it is difficult to know which specific cell surface carbohydrates or glycoproteins are involved. Moreover, glycoproteins function in a wide variety of biological activities at the cell surface and elsewhere, and blocking membrane glycoprotein glycosylation could result in complex cellular effects as seen by others (12, 52). The effects of tunicamycin on B16 melanoma cell experimental metastatic properties could be secondary and not the simple result of a specific modification in cell surface membrane glycoproteins. Since, in the in vivo assay used here, blood-borne tumor colonization ability required 14 to 20 days to score and the cellular effects of tunicamycin appeared to be reversible within 1 day of drug removal, tunicamycin probably acts only on the initial stages of the assay, similar to the results of Hart et al. (26) for the effects of cytotoxic drugs on blood-borne experimental lung metastasis of B16-F10 cells. Elsewhere, we have shown that metastatic tumor cells are very
adept at binding to and invasion of endothelial cell monolayers (33, 34), 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 under conditions where tumor cell surface sialoglycoproteins were modified, and this drug appeared to act differently from the cytoskeletal disrupting drugs that Hart et al. (26) used to modify arrest and endothelial cell adhesion by modifying internal cell components. From our data, it is not clear whether tunicamycin blocks appearance of these sialoglycoproteins at the cell surface or whether these molecules are present but unglycosylated. Since lactoperoxidase-iodination techniques did not detect major tunicamycin-induced changes in cell surface proteins, these components could be unglycosylated or glycoproteins with mucin-like sugar chains (1) which should be unaffected by the drug. Labeling with 125I-RCA on SDS gels indicated that sialogalactoproteins were modified. Migration distances of these glycoproteins on SDS gels did not coincide with any of the major bands detected by lactoperoxidase-catalyzed iodination. This indicates that the sialoglycoproteins are minor components on the cell surface or are resistant to iodination. The RCA-binding galactoproteins were reduced markedly after tunicamycin treatment or were present as unglycosylated molecules and not detectable by this procedure. Differences were not found between the sialogalactoprotein patterns of sublines B16-F1 and B16-F10. This finding is consistent with previous data on cell surface components of these sublines (45, 55), indicating that cell surface sialoglycoproteins are not related to the components determining organ preference of colonization. On the other hand, differences in the dynamics of cell surface anionic components such as sialoglycoproteins have been found in these same sublines (54), and this could affect the display of molecules important in metastatic colonization.

A high-molecular-weight glycoprotein present on fibroblasts, fibronectin, appears to be involved in cell substratum adhesion (11, 13, 49, 65, 66). There are several reports that claim a relationship between cell surface fibronectin and metastatic potential (8, 58). However, this relationship has been disputed in other systems (42), and various sublines of B16 melanoma do not display significant amounts of fibronectin on their surfaces as shown with a radioimmune assay. Although RCA binds to the sugar chain of fibronectin (7), we did not detect a B16 galactoprotein of approximate m.w. 200,000 to 220,000 on gels. Although low-molecular-weight forms of fibronectin-like molecules have been detected on spermatozoa (32) and in serum (65), it is unlikely from our results that one of the RCA-binding galactoproteins is fibronectin.

In conclusion, we found that: (a) tunicamycin treatment of B16 melanoma cells reduced lung colonization potential in vivo; (b) at the same time, cell surface adhesive properties were modified as detected by the adhesion assays; and (c) the most plausible cell surface molecules susceptible to tunicamycin are glycoproteins, and we found changes in the carbohydrate chains on sialogalactoproteins. However, we cannot conclude that B16 melanoma sialoglycoproteins are the only molecules affected by tunicamycin treatment. The importance of cell surface components in the metastatic process has been documented previously (15, 18, 51). It has been known for some time that the blood-borne arrest and survival of B16 melanoma cells is not random (16–18, 26, 44), and treatment of B16 melanoma cells with enzymes such as trypsin (15) modifies blood-borne arrest properties. Although detailed purification and reconstituting experiments will be necessary in order to prove the involvement of specific cell surface glycoproteins in B16 tumor cell blood-borne arrest, there exists sufficient information suggesting that cell surface components mediate this important process (15, 18, 33, 45, 50, 51, 55) to warrant further work on their isolation and characterization.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 2. Autoradiography of SDS-polyacrylamide slab gel stained with \(^{3}S\)-labeled RCA, after electrophoresis removal of sulphate acid. a, B16-F1; b, B16-F10. Lane 1, untreated cells; Lane 2, pretreated with tunicamycin for 12 hr; Lane 3, pretreated for 12 hr, washed, and incubated 3 hr without tunicamycin; Lane 4, pretreated for 12 hr, washed, and incubated for 6 hr without tunicamycin; Lane 5, pretreated for 12 hr washed, and incubated for 12 hr without tunicamycin; Lane 6, pretreated for 12 hr, washed, and incubated for 24 hr without tunicamycin; Lanes 7 to 11, same conditions as Lanes 2 to 6 except pretreated for 36 hr. Molecular weight markers are the same as in Fig. 1.

Fig. 3. Phase-contrast microscopic photographs of B16 melanoma cells after treatment with 0.5 \(\mu \text{g}\) tunicamycin per ml a to e, B16-F1; f to i, B16-F10. a and f, untreated; b and g, pretreated for 12 hr with tunicamycin; c and h, pretreated for 36 hr with tunicamycin; d and i, pretreated for 12 hr with tunicamycin, washed, and incubated for 24 hr without tunicamycin; e and j, pretreated for 36 hr with tunicamycin, washed, and incubated for 24 hr without tunicamycin. x 215.
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