Carbohydrate Chain Analysis by Lectin Binding to Electrophoretically Separated Glycoproteins from Murine B16 Melanoma Sublines of Various Metastatic Properties

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ABSTRACT

Cellular glycoprotein carbohydrate chains of B16 melanoma sublines of various metastatic colonization capacities were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and direct lectin staining, combined with chemical modification of carbohydrate chains in situ. For these studies, we utilized B16 sublines selected for low (B16-F1) or high lung (B16-F10), high brain (B16-B15b), or high ovary (B16-O13) colonization properties, or high tissue invasiveness in vitro (B16-BL6). The major B16 cell surface sialoglycoproteins were of Mₐ ~ 115,000, ~90,000, ~82,000, and 60,000 to 65,000, and were detectable by periodate NaB₃H₄ labeling and binding of ¹²⁵I-wheat germ agglutinin (WGA). Terminal sialic acid residues in the carbohydrate chains were responsible for WGA binding, since chemical removal of sialic acid prevented WGA labeling of the glycoproteins. However, removal of sialic acid residues followed by Smith degradation resulted in reappearance of WGA-binding sites on these sialglycoproteins, indicating that the carbohydrate chains possessed at least one branching point at an outer a-mannosyl residue. This structural feature was further indicated by the failure of ¹²⁵I-Lens culinaris hemagglutinin to bind to these sialylglycoproteins. The fact that the carbohydrate residues of the Mₐ ~ 115,000, ~90,000, and ~82,000 sialoglycoproteins were of the complex type was confirmed by their reactivity with ¹²⁵I-Ricinus communis agglutinin I, which preferentially binds to Gal → GlcNAc sequences after removal of sialic acid in situ. In contrast, ¹²⁵I-peanut (Arachis hypogaea) agglutinin, specific for Gal → GaINAc sequences, failed to bind to the major WGA-reactive sialoglycoproteins, but strongly interacted after removal of sialic acid with Mₐ ~ 51,000 and ~56,000 glycoproteins from sublines B16-F1, -F10, and -BL6 and with a Mₐ ~63,000 glycoprotein from sublines B16-F10, -BL6, -O13, and -B15b. Thus, the small, mucin-type carbohydrate chains were expressed almost exclusively on these lower Mₐ sialoglycoproteins, and very little on the Mₐ ~82,000, ~90,000, and ~115,000 sialoglycoproteins. Differences in lectin binding to glycoproteins were observed with different sublines. These glycoproteins included: (a) a WGA-binding Mₐ 60,000 to 75,000 sialoglycoprotein prominent on B16-B15b cells. (b) the peanut (A. hypogaea) agglutinin-binding Mₐ ~51,000, ~56,000, and ~63,000 sialoglycoproteins on lung-colonizing sublines, and (c) a L. culinaris hemagglutinin-binding Mₐ ~50,000 nonsialylated glycoprotein on B16-O13 cells.

INTRODUCTION

It is now accepted that neoplastic transformation of cells is associated with changes in the amount and/or display of cell surface glycoconjugates (22, 25, 39–41, 49, 54). Although alterations in glycoproteins, glycolipids, and glycosaminoglycans have been observed after neoplastic transformation, the relationships between these changes and certain important characteristics of cancer involving interactions between tumor cell surface molecules and host cells and tissues, such as characteristics that determine metastatic colonization of distant organ sites, are unknown (41).

Previously, we suggested that the surface sialoglycoproteins of B16 melanoma cells were responsible for the blood-borne arrest and lung colonization properties of these cells. In experiments using tunicamycin, an inhibitor of glycoprotein biosynthesis (26, 28), we were able to inhibit B16 melanoma cell lung colonization, and these modifications of in vivo properties correlated with the presence of certain sialoglycoproteins at the B16 cell surface (26). Another approach for assessing the role of cellular sialoglycoproteins in metastatic phenomena been to examine the structural variations of sialoglycoproteins among the tumor cell sublines with different metastatic colonization capacities. Although such comparisons may be meaningful only if all glycoproteins could be examined and compared individually, the presence of numerous unique glycoproteins on each cell renders such an analysis complicated. For example, Bhavanandan et al. (8) have biochemically characterized one of the major antigenic glycoproteins from a B16 melanoma subline, but it is extremely difficult to perform such analyses simultaneously on several different glycoproteins from various cell sublines. Therefore, we have separated tumor glycoproteins on the basis of their size by polyacrylamide gel electrophoresis in the presence of NaDodSO₄, and have estimated the carbohydrate structures of individual, separated glycoproteins by the binding of specific ¹²⁵I-labeled lectins in combination with chemical modifications of the carbohydrate chains in situ (29).

MATERIALS AND METHODS

Lectins. WGA was purified according to the method of Bloch and Burger (10). RCA, and PNA were purified as described previously (42, 51), except that acid-treated Sepharose 4B was used instead of Sepharose 4B or Sepharose 6B (2). LCH was purified according to the method of Sage and Green (48), and Con A was purified according to the method of Agrawal and Goldstein (1). The currently accepted carbohydrate-binding specificities of these lectins are summarized in Chart 1 (4, 5, 7, 29, 37, 36, 57). All lectins were iodinated with Na¹²⁵I (New England Nuclear, Boston, Mass.), using chloramine-T according to the method of Burridge (13), and they were then purified on affinity columns. Specific radioactivity

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essential medium (Grand Island Biological Co., Grand Island, N. Y.) and tissue culture dishes in a mixture (1:1) of Dulbecco-modified minimum (B1 6-O1 3) or 15 times for brain colonization (B1 6-B1 5b) were established (14), or 6 times for invasion of mouse bladder wall (B16-BL6) (23, 44) by T. Irimura and G. L. Nicolson.

Cells and Cell Lysates. Murine B16 melanoma sublines selected once (B16-F1) or 10 times for blood-borne lung colonization B16-F10 (14), or 6 times for invasion of mouse bladder wall (B16-BL6, 23, 44) were provided by T. J. Fidler (Frederick Cancer Research Facility, Frederick, Md.). Sublines selected 13 times for blood-borne ovary colonization (B16-O13) or 15 times for brain colonization (B16-B15b) were established previously in our laboratory (11, 12, 35). All cells were grown on plastic tissue culture dishes in a mixture (1:1) of Dulbecco-modified minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) and Ham's F-12 medium (Grand Island Biological Co., supplemented with 5% heat-inactivated fetal bovine serum (Reheis Division, Armour Pharmaceutical Co., Kankakee, Ill.). Cell cultures were grown to subconfluency in a humidified atmosphere of 5% CO2 and 95% air at 37°. Surface labeling of cells with lactoperoxidase and 125I was carried out by a slight modification of the Hubbard and Cohn method (24), as described previously (38). The labeling of cell surface sialic acid was performed according to the method of Gahmberg and Andersson (18). Unlabeled or surface-labeled cells were rinsed with Dulbecco's phosphate-buffered saline and maintained for 30 min. The labeling of cell surface sialic acid was performed according to the method of Gahmberg and Andersson (18). Unlabeled or surface-labeled cells were rinsed with Dulbecco's phosphate-buffered saline and maintained for 30 min.

RESULTS

B16 Sialoglycoproteins Revealed by Cell Surface Labeling. For comparisons, the results of polyacrylamide gel electrophoretic analyses of murine B16-O13 melanoma sialoglycoproteins, surface-exposed proteins, and RCA-binding glycoproteins are shown in Fig. 1a. The major surface proteins labeled by lactoperoxidase-catalyzed iodination did not coincide with the major sialoglycoproteins, as found previously with B16-F1 and B16-F10 cells (26). Polyacrylamide gel electrophoresis of B16-O13 melanoma cell glycoproteins after labeling by the periodate:NaB3H4 method indicated that sialic acid-containing components migrate to the same relative positions as the glycoproteins revealed by the binding of 125I-RCAI, after removal of sialic acid from the glycoproteins (Fig. 1a). It is likely that these RCA-

Chart 1. Carbohydrate-binding specificities of plant lectins based on previous reports (4, 5, 7, 27, 36, 57).

Chart 2. Positions of B16 melanoma glycoproteins and standard glycoproteins after electrophoresis in 7% polyacrylamide slab gels in the presence of 1% NaDodSO4. Standard glycoproteins are: PTG, porcine thyroglobulin; BPN, bovine serum albumin; HTF, human serum transferrin; BFE, bovine fetuin; OVG, hen ovalbumin with galactosyl, hybrid-type carbohydrate chain; OWM, hen ovalbumin with high mannosetype carbohydrate chain. The representative structures of carbohydrate chains in the standard glycoproteins are indicated in the dotted enclosures. Monosaccharides are symbolized as follows: S, α-N-acetylgalactosaminyl; G, β-galactosyl; N, β-N-acetylgalactosaminyl; M, α- or β-mannosyl; F, α-L-fucosyl residues. Linkage positions are indicated at the bottom right of the chart.

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Carbohydrate Chain Analysis of Melanoma Glycoproteins

Carbohydrate Chains of B16 Glycoproteins Revealed by WGA Binding. Detergent-extracted glycoproteins from various B16 melanoma sublines that had been separated by polyacrylamide gel electrophoresis in NaDodSO₄ and stained with ¹²⁵I-WGA showed similar glycoprotein-staining patterns (Fig. 1b). The major WGA-binding components of B16 cells had apparent Mₕ -115,000, -90,000, -82,000, and 60,000 to 65,000. The diffuse band at an approximate Mₕ 60,000 to 65,000 stained variably with ¹²⁵I-WGA in different preparations of the same cell subline, and it was likely that the expression of this glycoprotein(s) was unrelated to the biological properties of B16 cells. The diffuse band at molecular weights of between 60,000 and 75,000 was found exclusively in subline B16-B15b (Fig. 1b; Chart 3a). There were several minor WGA-binding components with an apparent molecular weights ranging from 45,000 to 55,000 in sublines B16-BL6 and B16-O13. Slight differences among B16 sublines were also found in the high-molecular-weight region (~300,000) (Chart 3a). When gels were treated with mild acid to hydrolyze sialic acid residues prior to ¹²⁵I-WGA staining, all of the B16 glycoprotein components lost their capacity to bind WGA (Fig. 1c). Only the ovalbumin standards with a hybrid-type carbohydrate chain were WGA reactive after this treatment, indicating that sialic acid at the terminal carbohydrate moieties of B16 glycoproteins was responsible for the interaction with WGA.

In order to obtain further information on the structural features of carbohydrate chains from separated B16 glycoproteins, the glycoproteins were desialized in the polyacrylamide gels, subjected to Smith degradation, and then examined for their interactions with ¹²⁵I-WGA. The major sialoglycoproteins, after desialization and Smith degradation, were restored to their WGA-binding capacities (Fig. 1d). Slight WGA reactivity was detectable with the standard glycoprotein with porcine thyroglobulin, and strong reactivity with human transferrin and bovine fetuin, which contain triantennary complex-type carbohydrate chains branching at position 4 on the α-mannosyl residue (37, 43, 56). After Smith degradation, this carbohydrate chain is converted to GlcNAcβ1 → 4(GlcNAcβ1 → 2)Galβ1 → 3GalNAcβ1 → 4GlcNAc → Asn (33), resulting in strong interactions with WGA (33). The results suggest that the Mₕ ~115,000, ~90,000, ~82,000, and 60,000 to 65,000 glycoproteins, as well as the Mₕ 60,000 to 75,000 glycoprotein on B16-B15b cells, contain complex-type carbohydrate chains with at least one branching chain at the outer mannose residue.

Carbohydrate Chains of B16 Glycoproteins Revealed by RCA₁ and PNA Binding. As demonstrated previously with B16-F1 and -F10 cells, the binding of ¹²⁵I-RCA₁ to glycoproteins of all melanoma sublines was detectable only after removal of sialic acid. The major glycoprotein components detected by RCA₁ were almost identical to those that bound WGA (Fig. 2a). Among the standard glycoproteins, RCA₁ bound strongly to glycoproteins with complex-type sugar chains, such as porcine thyroglobulin, bovine fibronectin, human transferrin, and bovine fetuin, but only after removal of sialic acid. Since RCA₁ preferentially binds to the Gal→ GlcNAc sequence (27), this result is strong evidence for the presence of complex-type carbohydrate chains in the major B16 sialoglycoproteins.

The binding of ¹²⁵I-PNA to B16 glycoproteins was detected only after removal of sialic acid. In sublines B16-F1, -F10, and -BL6, the glycoproteins revealed by ¹²⁵I-PNA binding consisted mainly of Mₕ ~63,000, ~56,000, and ~51,000 components, whereas the glycoproteins at Mₕ ~82,000 were stained only slightly. Therefore, the Mₕ ~115,000, ~90,000, and ~82,000 components apparently did not contain significant amounts of the small, mucin-type carbohydrate chains (Fig. 2b). In B16-O13 and -B15b cells, the Mₕ ~63,000 component was rather prominent, and this band apparently overlapped with one of the major WGA-binding glycoproteins found in B16-B15b cells (Chart 3). Among the standard glycoproteins, only PNA exclusively interacted with bovine fetuin (Fig. 2b), which was the only glycoprotein containing small, mucin-type carbohydrate chains having a Gal→ GalNAc sequence (29).

Carbohydrate Chains of B16 Glycoproteins Revealed by LCH and Con A binding. The B16 melanoma glycoprotein component that was the most intensely stained with ¹²⁵I-LCH had an apparent Mₕ ~50,000. However, this glycoprotein appeared either to be a minor component of B16 cells or to have low ¹²⁵I-LCH-binding affinity, judging from its low intensity in autoradiograms, as compared to ¹²⁵I-LCH binding to porcine...
thyroglobulin, the only LCH-stainable standard glycoprotein used (Fig. 2c). That LCH bound only to porcine thyroglobulin among the standard glycoproteins was consistent with the results of Kornfeld et al. (32), who showed that LCH bound to glycopeptides with 2 α-mannosyl residues that contained free C-3 and C-4 hydroxyls and a α-fucosyl residue at the innermost GlcNAc. This type of oligosaccharide is usually seen as a biantennary complex-type sugar chain containing fucose (56). The highest intensity of 129I-LCH labeling to the M, ~50,000 glycoprotein was found in B16-O13 cells, while on the other B16 sublines the intensity of labeling diminished in the order: O13 > F1 > F10 > BL6 > B15b (Chart 3d). 129I-LCH failed to bind to glycoproteins from B16-B15b cells. As described above, the major sialoglycoproteins did not bind LCH, probably because they contained highly branched carbohydrate chains.

Con A is known to possess a specificity similar to, but broader than that of LCH, and it binds to high mannose-type, hybrid-type, and biantennary complex-type carbohydrate chains (4). When B16 melanoma glycoproteins were separated on polyacrylamide gels and stained with 129I-Con A, more than 15 components were revealed (Fig. 2d). Relatively small differences among B16 sublines were found with 129I-Con A. Since LCH failed to bind to most of the Con A-reactive components, and since no hybrid-type carbohydrate chains in these cells were indicated by the WGA-binding experiments after removal of sialic acid, most of the Con A-reactive components were probably high mannose-type carbohydrate chains.

DISCUSSION

Melanoma cell sublines of the murine B16 system with a variety of invasive and organ colonization capacities have been sequentially selected in vivo or in vitro (11, 12, 14, 23, 35, 41, 44). Since blood-borne metastatic colonization results from a series of complex cellular interactions, the significance of tumor cell surface-labeling of sialic acid residues were also revealed by the binding of WGA and RCA, after the removal of sialic acid. These sialoglycoproteins (M, ~115,000, ~90,000, ~82,000, and 60,000 to 65,000) have been found to contain sialylated, triantennary, or more branched complex-type, carbohydrate chains.

The small, mucin-type carbohydrate chains that bind PNA after removal of sialic acid were found on M, ~36,000, ~56,000, and ~51,000 sialoglycoproteins. The relative amounts of 129I-PNA binding to these 3 glycoproteins varied among the different sublines, as indicated from the densitometric scans of the autoradiograms (Chart 3). The presence of mucine-type carbohydrate chains in B16 melanoma cells was demonstrated by Bhavanandan and Davidson (6), and a mucin-type glycoprotein has been isolated and partially purified from human melanoma cells by Umemoto et al. (53). Our results indicate that the B16 glycoproteins that carry small, mucine-type carbohydrate chains are distinct from the major B16 sialoglycoproteins, except for the M, 60,000 to 65,000 components. That the different glycoproteins have different types of carbohydrate chains was suggested by similar techniques using human skin fibroblast glycoproteins (20).

The estimated structures of the carbohydrate chains found on various major B16 sialoglycoproteins have been summarized in Chart 4. Elsewhere, Bhavanandan et al. (8) have purified and characterized a major B16 antigenic glycoprotein that migrates between phosphorylase b and fetuin upon polyacrylamide gel electrophoresis in NaDodSO4. This antigen may be a portion of the M, ~82,000 glycoprotein that contains sialylated complex-type, high mannose-type, and possibly a small amount of mucin-type sugar chains.

We noted apparently weak interactions between LCH and B16 glycoproteins compared to porcine thyroglobulin. Presumably, this can be explained by the highly branched nature of the complex-type carbohydrate chains of the B16 sialoglycoproteins, an explanation consistent with their WGA-binding properties after Smith degradation. Kornfeld et al. (32) reported that LCH has high affinity to biantennary complex-type carbohydrate chains with Fuc on the innermost GlcNAc. The strongest LCH binding was detected on a M, ~50,000 glycoprotein, and the degree of LCH binding to this component among variant B16 cells followed this order: O13 > F1 > F10 > BL6 > B15b. The complex-type carbohydrate chain may be incomplete, similar to one of the small, LCH-binding carbohydrate chains of human erythrocyte membrane band-3 glycoprotein (52). In contrast to major B16 sialoglycoproteins, the M, ~50,000 glycoprotein was not strongly stained with RCA, before and after mild acid hydrolysis. It was interesting that Con A binding was equivalent among the various B16 sublines to approximately 15 separated glycoproteins, and all of these glycoproteins did not appear to be sialoglycoproteins, but rather were glycoproteins with high mannose-type carbohydrate chains, using conventional biochemical techniques. Glycopeptides have been analyzed from whole cells or whole membrane fractions (9, 54, 55), but information is not available from these studies on the nature and the localization of the glycoprotein molecules carrying these carbohydrate chains. We have recently developed a simple method that uses lectin binding and in situ chemical modifications to estimate the carbohydrate structures of individual glycoproteins separated on polyacrylamide gels. Only minute quantities of glycoproteins are necessary for this method, and side-by-side comparisons of glycoproteins from various cell lines were possible.
marker proteins, nor did binding occur in the presence of 0.1 M of the appropriate inhibitory monosaccharides. (b) Comigration of 2 different glycoproteins could not be distinguished from the presence of a single glycoprotein with multiple, heterogeneous sugar chains. (c) Saccharide chains not detected by the panel of lectins used here could be important components of B16 glycoproteins. For example, we reported that pokeweed mitogen is specific for branched, poly-N-acetyllactosamine-type carbohydrate chains (30), but B16 melanoma glycoproteins did not appear to have this type of carbohydrate chain structure. It should be noted that the molecular weight of the glycoproteins separated by polyacrylamide gel electrophoresis could only be estimated, because of their anomalous migration behaviors in such gels (21). Finally, we found only a small portion of insoluble sialoglycoproteins using the procedures described here; therefore, the analyses presented pertain to virtually all of the major cell-associated sialoglycoproteins of B16 cells.

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* T. Hiruma and G. L. Nicolson, unpublished data.

**Chart 4. Proposed basic structures of carbohydrate chains present in the individual glycoproteins common in murine B16 melanoma sublines. Electrophoretic pattern is from a fluorogram of periodate/NaBH₄-labeled B16-O13 melanoma cell lysate. The complex-type carbohydrate chains are primarily localized on Mᵰ ~115,000, ~90,000, ~82,000, and 60,000 to 65,000 component. They are highly sialylated and possess at least one branching at outer α-mannosyl residue. Although the presence and location of α-fucosyl residues are unknown, they are shown in the structure, since incorporation of radioactive fucose into B16 melanoma glycopeptides was previously reported (54). The mucin-type carbohydrate chains are located on distinct sialoglycoproteins other than sialoglycoproteins, except the Mᵰ 60,000 to 65,000 component. The proposed mucin-type carbohydrate chain is a plausible structure based on PNA binding and information provided elsewhere (6, 9).

**Figure 1.** Differences found in the glycoproteins of various B16 melanoma sublines such as the LCH-binding, Mᵰ ~50,000 glycoprotein and the low-molecular-weight sialoglycoproteins bearing small, mucine-type carbohydrate chains were not qualitative, consistent with the results obtained in other metastatic systems (40, 46, 47, 50). Since metastasis is a complex, multistep process, glycoprotein alterations could affect the biological behavior of malignant cells at any of several discrete steps requiring cellular interactions. This may explain the diversity in cell surface changes found in experiments in which B16 cells were sequentially selected in vivo for organ colonization (data here), selected in vitro for surface carbohydrate alterations (16, 17), or modified biosynthetically (26, 28). Therefore, it was difficult to determine whether the observed changes were strictly related to differences in organ colonization properties among the various B16 sublines.

**Figure 2.** The methods described here appeared to be quite useful in estimating the types and relative amounts of carbohydrate chains on individual glycoprotein components in polyacrylamide gels. However, certain precautions must be heeded. (a) Weakly stained bands could have resulted from low amounts of the component, low lectin affinities of the saccharide chains, or both. Assuming that the association constants of known lectins with glycopeptide receptors are >5 × 10⁶ M⁻¹ (4, 31), we used lectin concentrations as low as 1 to 2 × 10⁻⁷ M. Under these conditions, ¹²⁵I-lectin does not bind to the unglycosylated molecular-weight
Fig. 1. Electrophoretic patterns of B16 melanoma glycoproteins on 7% polyacrylamide slab gels. a, electrophoretic separation of B16-O13 melanoma surface glycoproteins and proteins on 7% polyacrylamide gels: Lane 1, fluorograph of the glycoproteins labeled by the periodate-NaH₂PO₄ method; lane 2, autoradiogram of cell surface proteins labeled by lactoperoxidase catalyzed iodination; lane 3, autoradiogram of glycoproteins detected by 125I-RCA binding after removal of sialic acid from the glycoproteins. In situ. Arrows, positions of molecular weight markers: MW, myosin (Mₐ ~200,000); GA, β-galactosidase (Mₐ ~116,250); PH, phosphorylase b (Mₐ ~92,500); B, bovine serum albumin (Mₐ ~66,200); OV, ovalbumin (Mₐ ~45,000). b, autoradiogram of NaDodSO₄-polyacrylamide gels stained with 125I-WGA. Before removing sialic acid, binding was not detected on any components of the B16 melanoma and normal tissue extracts. Lanes 7 to 8 and molecular weight markers are the same as in Fig. 1i. a, autoradiogram of NaDodSO₄-polyacrylamide gels stained with 125I-RCA. Prior removal of sialic acid did not cause significant effects; Lanes 7 to 8 and molecular weight markers are the same as in Fig. 1b. c, autoradiogram of NaDodSO₄-polyacrylamide gels stained with 125I-Con A. Prior removal of sialic acid did not cause significant effects; Lanes 7 to 8 and molecular weight markers are the same as in Fig. 1b.
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