[Chem. Pharm. Bull. 31(6)2039—2044(1983)]

Cell Surface Radiolabeling of the Carbohydrate Moieties of the Plasma Membrane Major Glycoprotein of AH-66 Hepatoma Ascites Cells

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(Received October 30, 1982)

The major plasma membrane glycoproteins of AH-66 cells were radiolabeled by three methods which are known to label cell surface carbohydrates. The labeled components were separated by polyacrylamide gel electrophoresis and detected by fluorography. The AH-66 cells were found to be unusual because a single major glycoprotein with an apparent molecular weight of 165000 was almost exclusively labeled by both neuraminidase–galactose oxidase–NaB^3H_4 and dilute periodate–NaB^3H_4 treatments. The major glycoprotein was not labeled by galactose oxidase–NaB^3H_4 treatment. When the major glycoprotein labeled by the neuraminidase–galactose oxidase–NaB^3H_4 procedure was solubilized with Triton X-100 and then subjected to affinity chromatography on Sepharose-conjugated *Ricinus communis* agglutinin II, the ^3H-labeled major glycoprotein bound to Sepharose-conjugated *Ricinus communis* agglutinin II lectin and was eluted with lactose. These results indicated that the major glycoprotein contained sialyl-galactosyl or sialyl-*N*-acetylgalactosaminyl terminal groups, which are exposed on the external surface of the plasma membranes of AH-66 cells.

Keywords——glycoprotein; cell-surface labeling; carbohydrate structure; AH-66 cell; hepatoma cell

Introduction

Since the surface structures of cells are most probably involved in cell-cell interaction, growth regulation, and the establishment or modulation or antigenic properties of the cells, it would be useful to investigate the surface component profile of tumor cells and also to isolate and characterize their surface components. In a series of papers we have examined cell surface proteins of the plasma membranes of AH-66 hepatoma ascites cells both by chemical modification of plasma membrane proteins[1] and by urea treatment of the intact cells.[2] We also characterized the major glycoprotein of the plasma membranes of AH-66 cells; this glycoprotein was first purified by Funakoshi and Yamashina[3] and later by Nakajo, Nakaya and Nakamura.[4]

In order to further elucidate the surface structure of AH-66 cells, we examined the structure of cell surface carbohydrate moieties of the major glycoprotein of the plasma membranes of AH-66 cells. For this purpose, the following three labeling procedures, known to label cell surface carbohydrate residues, were used; 1) sequential treatment of cells with neuraminidase and galactose oxidase prior to reduction with NaB^3H_4;[5] 2) treatment of cells with galactose oxidase prior to reduction with NaB^3H_4;[5] and 3) treatment of cells with dilute periodate followed by reduction with NaB^3H_4.[6] Labeled components were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis and analyzed by fluorography. To ascertain the structure of the cell surface carbohydrate analyzed by the methods described above, the major glycoprotein labeled by neuraminidase–galactose oxidase–NaB^3H_4 was further subjected to affinity chromatography on Sepharose-conjugated *Ricinus communis* agglutinin II (RCA II).

Materials and Methods

Materials——NaB^3H_4 (661 mCi/mmol) was purchased from the Radiochemical Centre, Amersham.

Galactose oxidase (Type IV) of *Dactylium dendoides* obtained from Sigma Chemical Company, with a stated activity of 180 units per mg of protein, was dissolved in phosphate-buffered saline (PBS), pH 7.0, and treated at 50°C for 30 min before use as described by Gahmberg and Hakomori.[5] Neuraminidase of *Arthrobacter ureafaciens* was obtained from Nakarai Chemicals, Ltd. Sepharose-conjugated RCA II was purchased from E.Y. Laboratories, Inc. Standard proteins for the estimation of apparent molecular weight on polyacrylamide gel electrophoresis were purchased from Boehringer Mannheim Corp.

Growth of Hepatoma Cells——AH-66 hepatoma cells were grown in the cavity of male Donryu rats weighing approx. 120—160 g and obtained by drainage of the ascites fluid 7 d after the implantation of the tumors.[1,2,4]

Cell Surface Labeling Procedures——The surface labeling of AH-66 cells by sequential treatment with neuraminidase, galactose oxidase, and NaB^3H_4 was done essentially according to the method of Gahmberg and Hakomori.[5] In some experiments, cells were treated directly with galactose oxidase without neuraminidase treatment. To 1 ml of packed cells (approx. 3×10^8 cells), 4 ml of 0.1 M sodium phosphate buffer (pH 6.0) was added, and the cell suspension was incubated for 1 h at 37°C in the presence of 0.5 unit of neuraminidase. After incubation, the cells were collected by centrifugation at $100\times g$ for 5 min and washed twice with PBS (pH 7.0). The washed cells were suspended in 5 ml of PBS (pH 7.0) containing 20 units of galactose oxidase, and reaction was carried out for 1 h at 37°C. The cell suspension was centrifuged at $100\times g$ for 5 min and the pellet was washed 3 times with PBS (pH 7.4). Next, 4 ml of PBS (pH 7.4) was added to the washed cells, and they were reduced with 0.5 mCi of NaB^3H_4 for 30 min at room temperature. In order to complete the reduction, 1 mg of unlabeled $NaBH_4$ was added and the labeled cells were washed 4 times with PBS (pH 7.4) by centrifugation at $100\times g$ for 5 min.

Labeling of cell surface sialoglycoproteins by periodate-NaB^3H_4 was carried out essentially by the procedure of Liao, Gallop and Blumenfeld[6b] as modified by Gahmberg and Anderson.[6a] Packed cells (1 ml) were suspended in 4 ml of PBS (pH 7.4) and oxidized with 5 μmol of sodium periodate. The oxidation was performed for 30 min at 0°C, and then 2.5 μmol of glucose was added to the cell suspension. AH-66 cells were collected by centrifugation at $100\times g$ for 5 min and washed 3 times with PBS (pH 7.4). The reduction was performed using 0.5 mCi of NaB^3H_4 as described above. After ^3H-labeling of the cells surface, plasma membranes of AH-66 cells were prepared as described previously.[4]

Affinity Chromatography——The plasma membranes of AH-66 cells modified by the neuraminidase–galactose oxidase–NaB^3H_4 procedure were applied to a Sepharose-conjugated RCA-II affinity column. Two mg of isolated plasma membranes was solubilized by sonication for 3 min in PBS (pH 7.4) containing 2% Triton X-100. The solubilized membrane component was removed by centrifugation at $16000\times g$ for 30 min. The supernatant was diluted to 1% Triton X-100 with PBS (pH 7.4) then applied to an immobilized RCA II column (0.5×5 cm). The column was washed with PBS (pH 7.4) containing 1% Triton X-100, and bound glycoprotein was eluted with PBS containing 1% Triton X-100 and 0.1 M lactose.

Radioactivity Measurement——Isolated plasma membranes were dissolved in 0.5 ml of 1 N NaOH, and then 10 ml of scintillation mixture was added. The mixture was neutralized by the addition of 0.5 ml of 1 N HCl. Scintillation mixture consisted of 6 g of 2,5-diphenyl-oxazole, 0.15 g of 1,4-bis(5-phenyloxazolyl)-benzene, 1000 ml of toluene and 500 ml of Triton X-100. Radioactivity was counted in an Aloka LSC-671 liquid scintillation counter.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis——Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli[7] using 10% gel. After electrophoresis, Coomassie blue staining for proteins and periodate-Schiff staining for carbohydrate were carried out by the procedures of Fairbanks, Steck and Wallach.[8] Fluorography of gels was conducted by the method of Bonner and Laskey.[9] Protein determination was carried out by the method of Lowry *et al.*[10] with bovine serum albumin as a standard.

TABLE I. Cell-surface Labeling of AH-66 Cells

Treatment	[^3H]-Incorporated (cpm/mg protein)
A) None	8161
B) Galactose oxidase	8140
C) Neuraminidase-galactose oxidase	30806
D) Sodium periodate	44220

AH-66 cells (approx. 1.5×10^8) were labeled with NaB^3H_4, without enzyme treatment (A), after treatment with galactose oxidase prior to reduction with NaB^3H_4 (B), after treatment with neuraminidase prior to treament with galactose oxidase (C), and after oxidation with sodium periodate prior to reduction with NaB^3H_4 (D). The experimental conditions are described in detail in "Materials and Methods".

Results

External Radiolabeling of AH-66 Cells

Table I shows the results of radiolabeling of intact AH-66 cells by different cell surface labeling procedures. The tritium label was significantly incorporated into the plasma membranes of AH-66 cells by the treatment of the cells with NaB^3H_4 alone. The same phenomenon has been noted by several investigators and referred to as nonspecific labeling. Gahmberg and Hakomori[11] reported that nonspecific labeling occurs in proteins and in lipids of NIL and 3T3 cells. Treatment of AH-66 cells with galactose oxidase prior to the reduction with NaB^3H_4, the procedure that labels galactosyl and *N*-acetylgalactosaminyl residues on external surfaces of cells with ^3H, did not cause any increase in the incorporation of ^3H. By contrast, when AH-66 cells were initially treated with neuraminidase prior to treatment with galactose oxidase and reduction with NaB^3H_4, the incorporation of ^3H was increased approx. 4-fold as compared to that obtained by the galactose oxidase–NaB^3H_4 method. The concentrations of neuraminidase and galactose oxidase used in these experiments correspond to a great excess. Therefore, no increase in the incorporation of ^3H was observed after the treatment of AH-66 cells with higher concentrations of these enzymes. Mild oxidation by sodium periodate followed by reduction with NaB^3H_4, a technique known to label cell surface neuraminic acid residues,[5] was the most effective method for the incorporation of ^3H.

Analyses of [^3H]-Labeled Proteins

As reported previously,[1,2,4] the plasma membranes of AH-66 cells contained about 30 bands stainable with Coomassie blue (lane A in Fig. 1) and one major periodate-Schiff-positive band (lane B in tha same figure) as analyzed by sodium dodecyl sulfate gel electrophoresis. The major periodate-Schiff-positive band is a glycoprotein with an apparent molecular weight of 165000 in 10% gels and is composed of 54% carbohydrate and 46% protein.[4] The major glycoprotein was markedly radiolabeled by treatment of the intact AH-66 cells with neuraminidase prior to treatment with galactose oxidase and reduction with NaB^3H_4 (lane C in Fig. 1) but was not labeled by the galactose oxidase–NaB^3H_4 method (lane D in the same figure). These results suggest that the major glycoprotein has no galactosyl or *N*-acetylgalactosaminyl residues at the nonreducing terminus and that these sugars are probably substituted with sialic acid residues. The possibility that nonreducing terminal galactosyl or *N*-acetylgalactosaminyl residues are sterically unavailable to galactose oxidase prior to neuraminidase treatment has also to be considered. Nonspecific labeling of AH-66 cells by reduction only with NaB^3H_4 occurred on bands corresponding to molecular weights between 60000 and 65000 (lane E in Fig. 1). Although the nonspecific labeling was reported to be enhanced in cells transformed by viruses,[11] the nature of the nonspecific labeling was not studied further in the present work. Radiolabeling of the major glycoprotein was also observed by oxidation of AH-66 cells with sodium periodate followed by reduction with NaB^3H_4 (lane F in Fig. 1), indicating that the sialic acid residues of the major glycoprotein are located at the outer surface of AH-66 cells. It should be noted here that the mobility of the radiolabeled band is faster than that obtained by reduction with NaB^3H_4 (compare lane C with lane F in Fig. 1). The slower mobility of glycoproteins after neuraminidase plus galactose oxidase treatment than after periodate treatment was also observed for AS-30D hepatocellular carcinoma cells by Glenney *et al.*[12] This molecular weight shift is considered to be due to variations resulting from the removal of sialic acid residues from glycoproteins.

Affinity Chromatography of Radiolabeled Cell Surface Glycoproteins

AH-66 cells were initially treated with neuraminidase prior to treatment with galactose oxidase and reduction with NaB^3H_4, and then plasma membranes were prepared and solubilized with Triton X-100. The solubilized components were subjected to affinity chromatography

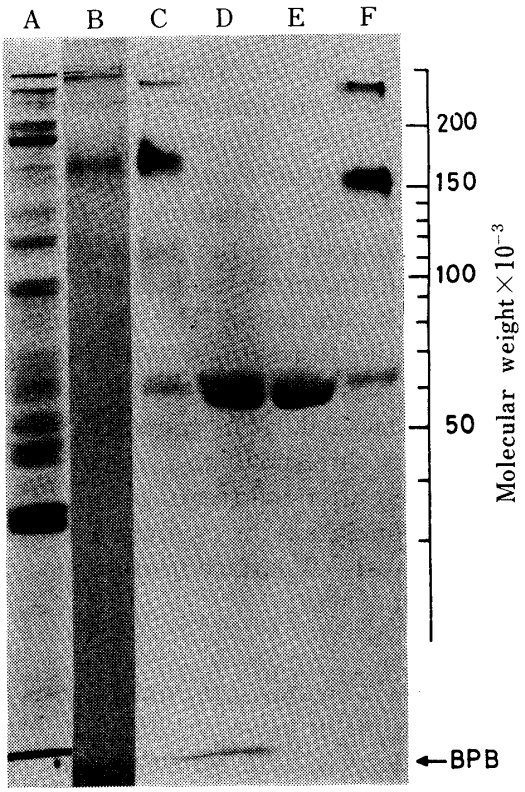

Fig. 1. Electrophoretic Profiles and Fluorographs of Plasma Membranes of AH-66 Cells Labeled by Three Different Methods

The labeled plasma membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure of Laemmli[7] and stained with Coomassie blue(A) or with periodate-Schiff reagent (B). After staining and destaining, the gel was processed for fluorography. Fluorography patterns (C–E) of AH-66 cells which were labeled by NaB^3H_4 after treatment with neuraminidase plus galactose oxidase (C), after treatment with galactose oxidase prior to reduction with NaB^3H_4 (D), without enzyme treatment (E), and after oxidation by sodium periodate (F).

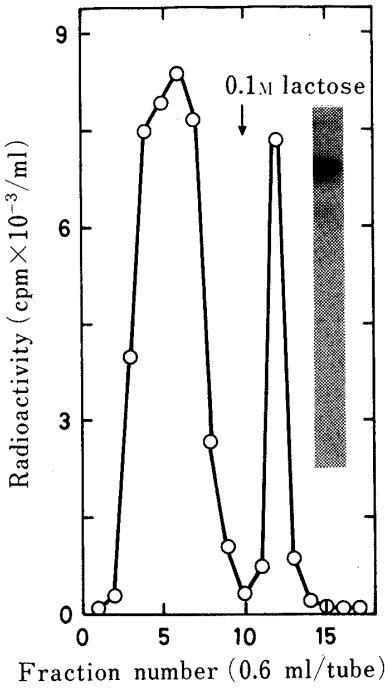

Fig. 2. Affinity Chromatography of Radiolabeled Cell Surface Glycoproteins on a Sepharose-conjugated RCA II Column

AH-66 cells were labeled by NaB^3H_4 after treatment with neuraminidase plus galactose oxidase, and then plasma membranes of the radiolabeled cells were prepared. The plasma membranes thus prepared were solubilized with Triton X-100, the insoluble material was removed by centrifugation, and the supernatant was applied to a column of Sepharose-conjugated RCA II. The column was washed with PBS (pH 7.4) containing 1% Triton X-100, and eluted with PBS containing Triton X-100 and 0.1 M lactose. Fractions (0.6 ml/tube) were collected and 0.1 ml of each fraction was analyzed for radioactivity. The inset shows the fluorography patterns of glycoproteins eluted with PBS containing Triton X-100 and lactose. The conditions of electrophoresis and fluorography were the same as in Fig. 1.

on Sepharose-conjugated RCA II (Fig. 2). Approx. 30% of the total radioactivity was adsorbed on the column, and the adsorbed radioactivity was eluted with 0.1 M lactose. The peak fraction thus eluted was analyzed by sodium dodecyl sulfate-gel electrophoresis and fluorography. As shown in the inset of Fig. 2, the peak contained two glycoproteins. The major band corresponds to the major glycoprotein with an apparent molecular weight of 165000 and the minor band corresponds to the band only faintly labeled by neuraminidase plus galactose oxidase followed by reduction with NaB^3H_4. It should be noted that the flow-through fraction contained both non-specifically labeled protein and aggregates of the major glycoprotein labeled by neuraminidase–galactose oxidase–NaB^3H_4. When the galactose oxidase treatment was omitted, no labeled proteins were adsorbed on the column (result not shown). Since RCA II has a high binding specificity for galactosyl and *N*-acetylgalactosaminyl residues, the above result also supports the conclusion that the nonreducing termini of carbohydrate moieties of the major plasma membrane glycoproteins of AH-66 cells is consist of sialyl-galactosyl or sialyl-*N*-acetylgalactosaminyl residues.

Discussion

Recently, the cell surface carbohydrate moieties of various tumor cells have been examined by means of various external radiolabeling methods.[12,13] In these studies, several glycoproteins in a tumor cell have been radiolabeled by the cell surface labeling methods. For example, at least 10 and 6 major glycoproteins were labeled by the periodate-NaB^3H_4 method in Novikoff[13c] and AS-30D carcinoma[12] cells, respectively. In human erythrocytes, three major glycoproteins (PAS 1, PAS 2 and PAS 3) were labeled by periodate-NaB^3H_4 and Band 3 protein in addition to these glycoproteins was labeled by the neuraminidase–galactose oxidase–NaB^3H_4 method.[5] In contrast to these studies, the present study showed that a single major glycoprotein of the plasma membranes of AH-66 cells was almost exclusively labeled by both the periodate-NaB^3H_4 and neuraminidase–galactose oxidase–NaB^3H_4 methods, although two other bands were faintly labeled. Therefore, it seems that AH-66 cells afford a simple experimental system for studying the role of a cell surface glycoprotein.

As demonstrated in the present study, the major glycoprotein of the plasma membranes of AH-66 cells was labeled by galactose oxidase–NaB^3H_4 only after neuraminidase treatment. Moreover, the major glycoprotein after labeling with neuraminidase–galactose oxidase–NaB^3H_4 was bound to Sepharose-conjugated RCA II. Based on these results, the nonreducing termini of carbohydrate moieties of the major glycoprotein are deduced to be siallyl-galactosyl or sialyl-*N*-acetylgalactosaminyl. The fact that treatment with dilute periodate labeled the same glycoprotein supports this interpretation. However, an alternative possibility that galactosyl or *N*-acetylgalactosaminyl residues are sterically unavailable to galactose oxidase prior to neuraminidase treatment is not excluded.

Increases in total sialic acid content have been observed for a wide variety of tumor cells as compared with the corresponding normal cells,[14] although in some virus-transformed cells, total sialic acid levels are decreased as compared to those of the untransformed cells.[15] Bosmann, Case and Morgan[16] found that cell surface sialic acid accessible to neuraminidase is increased in transformed cells compared to control cells. Warren, Zeidman and Buck[17] and Van Beek, Smets and Emmelot[15b] also showed that various transformed and tumor cells had more highly sialylated glycoproteins at the cell surface compared to those of normal cells. Yogeeswaran, Stein and Sebastian[18] demonstrated that the amount of sialic acid exposed on the cell surface is increased in high lung-metastasizing F10 cells as compared to those in low lung-metastasizing F1 cells. Lloyd *et al.*[13a] examined cell surface glycoproteins of various human tumor cells and found that cell surface glycoproteins of melanomas, which are highly metastatic, are substituted with sialic acid but that astrocytomas and carcinomas had few glycoproteins labeled by the galactose oxidase–NaB^3H_4 method. The results obtained in the present study are consistent with these previous findings that cell-surface glycoproteins in highly tumorigenic cells are highly sialylated. Further work will be necessary to examine the physiological role of the sialylated major glycoprotein of the plasma membrane of AH-66 cells.

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