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GLYCOPEPTIDES OF HUMAN TERATOMA STEM CELLS

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Abstract - Glycopeptides of human teratoma cells were studied. Two glycopeptide fractions (Fractions A and C) obtained from pronase digests of the human teratoma stem cells of line PA 1 were analyzed. Fraction A glycopeptides were of large size (M_r > 7400). They could be labeled by supplying the cells with ^{3}H-mannose, ^{3}H-fucose, ^{3}H-galactose, ^{3}H-glucosamine, ^{3}H-glucose and ^{35}S-sulfate. Acid hydrolysates of Fraction A contained at least mannose and fucose. A part of Fraction A glycopeptides could be obtained by affinity chromatography on Concanavalin A-Sepharose in a form virtually free of ^{35}S-sulfate; at least these glycopeptides may represent molecules different from glucosaminoglycans. A pulse-chase experiment with ^{3}H-mannose suggested that Fraction A glycopeptides may derive their mannose units from Fraction C glycopeptides, which were of the same molecular size (850-2200 daltons) as the common oligomannosyl type asparagine glycopeptides, and which had also labeling characteristics typical to the oligomannosyl glycopeptides. Fraction A glycopeptides were probably present on the surface of the PA 1 cells, as they could be released by mild trypsin treatment of the living cells.

INTRODUCTION

Teratocarcinomas are germ line tumors containing embryonal carcinoma stem cells, which can differentiate into a variety of cell types (1). There is considerable interest in the oligosaccharide-containing surface antigens and binding sites for lectins of these cells; these molecules appear to experience remarkable changes during the differentiation of the cells (2-7). Similar changes occur also in developing mouse embryos (8). Muramatsu et al. have recently described large differentiation sensitive fucosyl glycopeptides in mouse teratoma stem cells, which may well be structurally unique among mammalian glycoprotein glycans (9).

In the present communication we show that stem cells of human teratoma (cell line PA 1), described recently by Zeuthen et al.[1] contain large surface glycopeptides which may be similar to those of the mouse teratoma stem cells.

We also demonstrate that the PA 1 cells contain glycopeptides resembling asparagine linked oligomannosyl glycans in their gel filtration and their labeling characteristics. Finally, we show that the large glycopeptides of the human cells may be derived from the oligomannosyl type glycopeptides.

MATERIALS AND METHODS

Cell culture

The human teratoma stem cells, line PA 1, of ovarian origin, were kindly provided by Dr. J. Zeuthen (University of Aarhus). The cells were grown in Eagle's minimum essential medium supplemented with 10 % foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin under 5 % CO_2 at 37°C. The cells were replated, 5 x 10^6 cells per 90 mm dish, every third or fourth day.

Labeling of the cells and preparation of the pronase digests

To PA 1 cells, which had been replated two days earlier, was added 10 ml of medium containing 3.5 µCi/ml of 2-^{3}H-mannose (Amersham, Buckinghamshire, 12 Ci/mmol). The cells were harvested after 3 h of labeling. Unlabeled control cultures contained 14 x 10^6 cells at time of harvest. The radioactive medium was removed and the cells were washed twice with phosphate buffered saline (PBS) and scraped with a rubber policeman into 2.5 ml of Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. Sodium dodecyl sulfate (250 µl of 0.1 % solution) was added and the dissolved cells were heated for 3 min at 100°C.

For pronase digestion the dissolved cells were treated during 48 h with four portions of 125 µl of an autodigested 1 % pronase solution. The digestion was carried out at 60°C (10) under toluene. At the end of the hydrolysis toluene was blown off with nitrogen and the reaction mixture was heated for 3 min at 100°C and stored at -20°C. Before use the digest was lyophilized.

The labeling with 6-^{3}H-fucose (New England Nuclear, Dreieichenhain, 12 Ci/mmol) was carried out essentially in the same way as with ^{3}H-mannose. The labeling time was 48 h.

For labeling with 1-^{3}H-galactose the cells were supplied with 10 ml of the medium containing 25 µCi/ml of the precursor (Amersham, 22 Ci/mmol). The cells were harvested after 3 h, and processed essentially in the same way as the mannose labeled cells. The labeling with 6-^{3}H-glucosamine hydrochloride (Amersham, 15 Ci/mmol) was carried out in the same way as with ^{3}H-galactose. Labeling with 2-^{3}H-glucose was carried out with 5 ml of medium containing 13 µCi/ml of the precursor (Amersham, 7 Ci/mmol) for 48 h.

For labeling with radioactive sulfate the cells were incubated with 100 µCi/ml of carrier free aquous ^{35}S-sulfate (Amersham) for 24 h, and the labeled cells were processed as above.

Pulse-chase labeling with 2-^{3}H-mannose

Five culture dishes of cells were labeled for 3 h with 5 ml of medium containing 5 µCi/ml of 2-^{3}H-mannose. The radioactive medium was then removed and the cells were washed with PBS. The cells of two cultures were analyzed at this time point. Fresh, non-labeled medium was added to the rest of cultures and the label was chased for 16 h. At this time point the cells were collected for analysis.

Gel filtration

The glycopeptides were gel filtrated in columns of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) (200-400 mesh, 1 x 80 cm) equilibrated with 0.2 M pyridine acetate, pH 5.0, containing 0.02 % NaN_3. The elution rate was about 3 ml/h, and fractions of 1 ml were collected. Small samples of each fraction were analyzed for radioactivity. The molecular weights of the glycopeptides were estimated by calibrating the columns with Blue Dextran 2000 (Pharmacia, Uppsala), with a chymotryptic glycopeptide GC-4 (M_r = 3238) from immunoglobulin of bovine colostrum (11), with an octasaccharide of the following structure, Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→2Manα1→3] Manβ1→4GlcNAc (12) (M_r = 1441), and with free ^{3}H-mannose. The unlabeled octasaccharide and the immunoglobulin glycopeptide GC-4 were gifts from Drs. J. Montreuil, A. Cheron and G. Strecker, Université des Sciences et Techniques de Lille I. We labeled the octasaccharide marker by reducing it with NaB^3H_4, and the GC-4 glycopeptide by a treatment with galactose oxidase (EC 1.1.3.9) and NaB^3H_4 as described before (13); the labeled products were purified by gel filtration on Bio-Gel P-6.

Gel filtration of glycopeptides on a column (1 x 80 cm) of Bio-Gel P-30 (100-200 mesh) was carried out in the same way as on Bio-Gel P-10 columns.

The glycopeptides were desalted by gel filtration on Bio-Gel P-2 columns (1 x 60 cm) eluted with 0.2 M pyridine acetate buffer, pH 5.0. For preparative purposes the glycopeptides were recovered from the eluates by lyophilization.

Affinity chromatography on Concanavalin A-Sepharose

The glycopeptides were fractionated on columns (1 x 15 cm) on Concanavalin A-Sepharose (ConA-Sepharose) (Pharmacia). The method used is based on the pioneering work of Ogata et al. (14). The columns were equilibrated with 0.01 M Tris-HCl buffer solution, pH 7.5 containing 100 mM NaCl and 1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$, as well as 0.02 % NaN_3. The sample was applied to the column in the equilibration buffer, and the elution was also started with this solution, which eluted the non-retained glycopeptides (Fraction ConA I). Subsequently, 10 mM α-methyl-mannoside, and still later 300 mM α-methyl-mannoside were added to the eluting solvent in order to recover the retained glycopeptides (Fractions ConA II and ConA III, respectively). The elution rate was about 7 ml/h; fractions of 1.2 ml were collected and small aliquots of each fraction were analyzed for radioactivity. The lectin column was standardized with ^{3}H-labeled heparin of M_r = 6000-20 000 (New England, Nuclear) and with the ^{3}H-labeled immunoglobulin glycopeptide GC-4.

Monosaccharide analysis

The presence of mannose was established by hydrolysis of ^{3}H-mannose labeled glycopeptides with 1 N hydrochloric acid at 100°C for 4 h. The hydrolysate was subjected to paper chromatography on Whatman No 1 paper by co-chromatographing it with a mixture of unlabeled mannose, galactose, fucose and xylose (20 µg each). The solvent system used was n-butanol/acetic acid/water, 4/1/5 (15). The developed chromatogram was cut into stripes 1 cm wide, and these were assayed for radioactivity in a liquid scintillation counter.

Hydrazinolysis

Glycopeptide samples were dried before hydrazinolysis at 0.1 mmHg over P_2O_5 for 48 h. They were then treated with 0.2 ml of anhydrous hydrazine (Pierce, Rockford, Ill.) at 105°C for 48 h in Teflon capped tubes under nitrogen (13). At the end of the reaction hydrazine was removed by repeated evaporation with toluene in a nitrogen flush. The dry residue was re-N-acetylated under conditions described by Kobata (16); 200 µl of saturated, aqueous $NaHCO_3$ were added and the solution was treated three times with 10 µl portions of acetic anhydride for 10 min at 23°C. At the end of the reaction the tube was heated at 100°C for 2 min. The reaction mixture was analyzed by gel filtration on Bio-Gel P-10.

Preparation of cell surface digests from living cells

Washed cells labeled with ^{3}H-glucose were simultaneously detached from the culture dish and trypsin digested by a treatment with 0.25 % trypsin solution (Orion, Helsinki) in PBS for 7 min at 20°C. Cell viability was controlled by the trypan blue exclusion test; 98 % of the cells were viable. The trypsinate was separated by centrifugation and digested with pronase.

Determination of radioactivity

Radioactivity was determined in a Wallac 8100 scintillation counter using a xylene-Triton X-114 cocktail (8:3, v/v). Quenching was occasionally checked with an external standard.

RESULTS

Isolation of labeled glycopeptides of PA 1 cells

The cells were labeled metabolically with ^{3}H-mannose, ^{3}H-glucosamine and ^{3}H-galactose for 3 h. Pronase digests of the labeled cells were prepared and the glycopeptides of these digests were fractionated by gel filtration on calibrated columns of Bio-Gel P-10 (Fig. 1). From each gel filtration experiment four major preparations, called Fractions A, B, C and D were pooled. The cut between Fractions A and B corresponds to a molecular weight of 7400 daltons, that between B and C to 2200 daltons, and that between C and D to 850 daltons (Fig. 1). Fractions A, B and C proved to be glycopeptides, whereas Fraction D represented small molecules other than glycopeptides. Fraction A derived relatively little label from ^{3}H-mannose, but it become heavily labeled from ^{3}H-glucosamine and ^{3}H-galactose in the relatively short labeling experiments shown in Fig. 1.

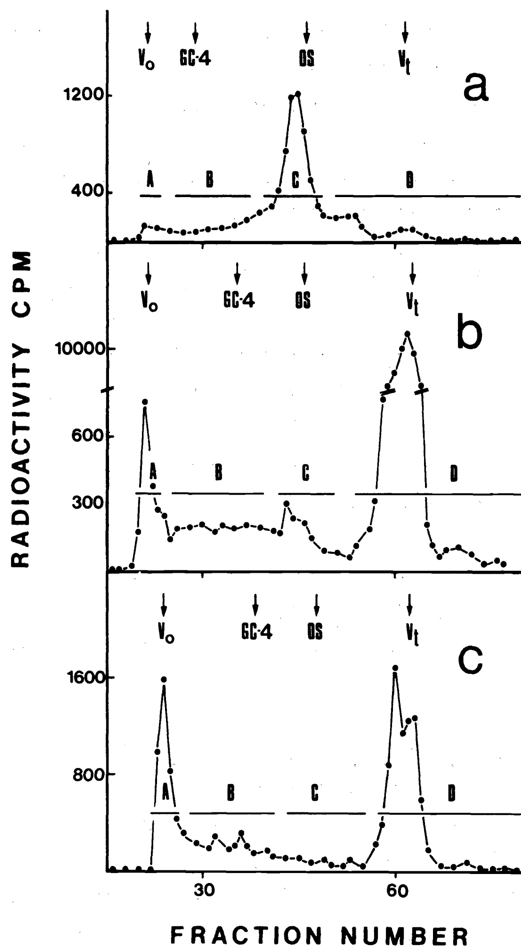

Fig. 1. Gel filtration profiles of PA 1 cell glycopeptides.
a) Glycopeptides labeled with 2-^{3}H-mannose.
b) Glycopeptides labeled with 6-^{3}H-glucosamine.
c) Glycopeptides labeled with 1-^{3}H-galactose.
The columns of Bio-Gel P-10 were calibrated with Blue Dextran 2000 (V_o), a chymotryptic glycopeptide (GC-4) of 3238 daltons from IgG (11), an octasaccharide (OS) of 1441 daltons from urine (12), and with free mannose (V_t).

Labeling experiments analogous to those described in Fig. 1 were carried out with PA 1 cells also by using ^{3}H-fucose and ^{35}S-sulfate as oligosaccharide precursors. These precursors were incorporated into Fraction A and Fraction B glycopeptides, but little or no label was incorporated in Fraction C glycopeptides (data not shown).

<u>Characterization of Fraction A glycopeptides</u>

Fraction A glycopeptides were large molecules, and they survived repeated and prolonged digestions with pronase without a change in their gel filtration profile. The pronase treatment was without any effect even when applied to desialylated Fraction A. The removal of any sialic acid units eventually

present is known to improve proteolysis (17). As Fraction A glycopeptides incorporate label from ^{35}S-sulfate, it is obvious that they may contain glucosaminoglycans. However, chromatographic experiments on Concanavalin A-Sepharose (14) showed that Fraction A almost certainly contains also glycopeptides different from glucosaminoglycans. A significant fraction (15-23 % of total carbohydrate) of Fraction A molecules was retained by Concanavalin A-Sepharose and could be eluted only by using 10 mM α -methyl-mannoside as eluant. In contrast, virtually all Fraction A glycopeptides labeled with ^{35}S-sulfate were eluted in the non-retained fraction; only 3 % of the label was retained and could be eluted in 10 mM α -methyl-mannoside.

The ability to bind to Concanavalin A suggested the presence of mannose in Fraction A glycopeptides, and mannose was indeed found in monosaccharide analysis of these glycopeptides. Some of the tritium label was found also as ^{3}H-fucose. The conversion of mannose label into fucose label has been observed in many other cells (18).

Fraction A glycopeptides were subjected to hydrazinolysis under conditions known to liberate intact oligosaccharides while destroying the peptide parts of glycoproteins and glycopeptides (13). Fig. 2 shows that most of the label of Fraction A survived the treatment as large molecules eluting at the void volume from a column of Bio-Gel P-10. This finding suggests that large oligosaccharides are present in Fraction A.

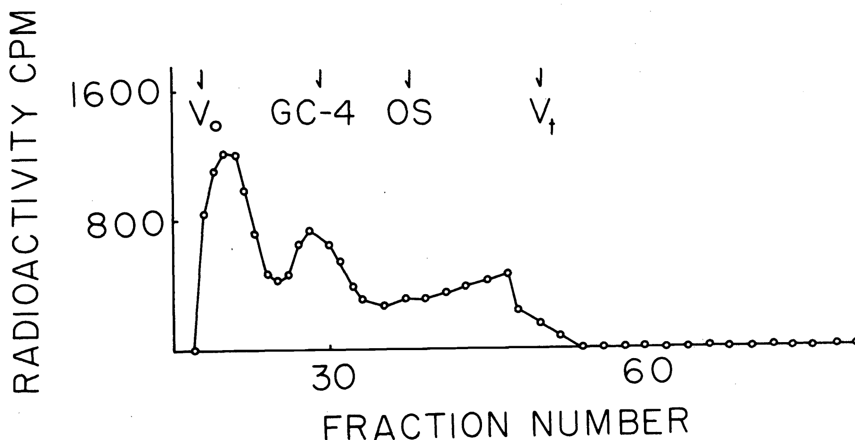

Fig. 2. Gel filtration profile of ^{3}H-fucose oligosaccharides liberated from Fraction A by hydrazinolysis (48 h, 105°C). The Bio-Gel P-10 column was calibrated as in Fig. 1.

Characterization of Fraction C glycopeptides

The Fraction C glycopeptides of PA 1 cells ranged in size between 850 and 2200 daltons (Fig. 1). They incorporated rapidly much label from ^{3}H-mannose, and also from ^{3}H-glucosamine (Fig. 1). In contrast, label from ^{3}H-galactose, ^{3}H-fucose and ^{35}S-sulfate incorporated into Fraction C only to a small extent. Our most recent experiments with endo-β-N-acetylglucosaminidase H liberated the glycans from Fraction C. The reactivity with this enzyme, the size and the fast labeling with ^{3}H-mannose and ^{3}H-glucosamine are compatible with the

notion that Fraction C glycopeptides represent oligomannosyl type glycans, which are commonly found linked to asparagine of membrane and soluble glycoproteins (19).

'Pulse-chase' labeling of PA 1 cell glycopeptides with ^{3}H-mannose

PA 1 cells were 'pulsed' with ^{3}H-mannose for 3 hours, after which the labeled precursor was removed and a part of cells was processed for analysis. The rest of the cells was incubated further for 16 hours in an unlabeled medium, after which the 'chased' cells were processed for analysis. The analysis consisted of exhaustive pronase digestion and gel filtration of the labeled glycopeptides.

Table I shows that the amounts of label in the large glycopeptides of Fraction A (and Fraction B) had doubled during the 'chase' period. In contrast, the label of the glycopeptides of Fraction C had decreased substantially. This experiment suggests that the oligosaccharides of Fraction A (and those of Fraction B) may derive their mannose residues from the glycans of Fraction C.

TABLE I. 'Pulse-chase' labeling of PA 1 cells with ^{3}H-mannose

Glycopeptide Fraction	Cells at the end of 3 h 'pulse'	Cells at the end of 16 h 'chase'
	% of label	% of label
A (M_r > 7400)	6.0	13.9
B (M_r = 7400-2200)	19.0	36.3
C (M_r = 2200-850)	75.1	49.8
Total glycopeptides	100.0[a)]	100.0

a) Fig. 1a shows that almost all radioactivity of the cells represents glycopeptide label.

Presence of A- and B-glycopeptides at the surface of PA 1 cells

The localization of the Fraction A glycopeptides of PA 1 cells was studied by mild trypsin treatment of the living cells. The trypsinate from cells labeled with ^{3}H-glucose was found to contain glycopeptides of Fraction A and B after pronase digestion. This suggests that glycoproteins containing the glycopeptides of Fraction A and Fraction B are present also at the cell surface.

DISCUSSION

According to the present data human teratoma cells of line PA 1 contain large size surface glycans, which are linked to proteins. These glycans could be labeled by supplying the cells with ^{3}H-mannose, ^{3}H-fucose, ^{3}H-galactose, ^{3}H-glucosamine and ^{35}S-sulfate. They were shown to contain at least mannose and fucose. The high level of incorporation obtained with ^{3}H-galactose and ^{3}H-glucosamine suggests that even these monosaccharides may be present in

Fraction A glycopeptides. The 'pulse-chase' experiment with ^{3}H-mannose seems to indicate that the mannose units of Fraction A glycopeptides originate from Fraction C glycopeptides, which in turn resemble the common asparagine glycopeptides of oligomannosyl type.

The large mannose glycans of PA 1 cells are unlikely to represent 'macro-glycolipids' (20), because mannose is a rare constituent in mammalian ceramide glycolipids (20,21).

Mannose-containing large oligosaccharides similar to those of Fraction A are rare in mammalian cells. Only keratan sulfate (22), some glycopeptides of red blood cells (23,24) and the fucosyl glycopeptides of mouse teratocarcinoma cells (9) are of comparable size. It would be of considerable interest if the large glycopeptides of mouse teratocarcinoma cells would turn out to be even structurally similar to the large surface glycopeptides of the human teratoma stem cells. The finding (9) that the large glycopeptides of the mouse teratocarcinoma cells disappear during cell differentiation, makes it interesting to see if a similar change will take place also during the differentiation of the PA 1 cells.

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