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SURFACE GALACTOSYL GLYCOPEPTIDES OF EMBRYONAL CARCINOMA CELLS

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SUMMARY. Surface galactosyl residues of alive embryonal carcinoma cells were tritium-labeled by the galactose oxidase NaB(3H)4 method. The labeled glycopeptides isolated from Pronase digests of the cells, were found to be similar to those prepared from endogeneously labeled embryonal carcinoma cells. They consisted mostly of high molecular weight material, and contained receptors for peanut agglutinin. It is concluded that at least a fraction of the high molecular weight glycopeptides characteristic of early embryonic cells, is displayed on the cell suface.

Teratocarcinomas are malignant tumors generally associated with the gonads. They contain a variety of cell types, distributed in a chaotic way, and clumps of a distinctive cell type called embryonal carcinoma (EC). The latter cells are the stem cells from which the differentiated cell types derive (1,2). EC cells share many biological properties with multipotential cells of normal early embryos (3). Several clonal lines of EC are therefore widely used in studies of early embryonic development, as an alternative to normal embryos (reviewed in 4-6). A class of high molecular weight fucosyl-glycopeptides, isolated after extensive Pronase digestion of [^{3}H]-L-fucose labeled cells has been defined as characteristic of EC and early embryonic cells (7). These glycopeptides disappear during *in vitro* differentiation of EC cells (7), and also from normal embryos during

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postimplantation embryogenesis (8). Similar large glycopeptides have also been found as a predominant molecular species in Pronase digests of [^{3}H] or [^{14}C] -D-galactose labelled EC cells (9). The subcellular location of these high molecular weight glycopeptides is still unknown, and it has not been established whether or not they are exposed on the cell surface.

We report here that glycopeptides recovered from cells externally labelled by the galactose-oxidase-NaB[^{3}H]$_4$ method (10) appear to be indistinguishable from those obtained from cells labelled by growth in the presence of [^{14}C]-D-galactose. Our findings show that at least part of the high molecular weight glycopeptides is indeed exposed on the cell surface, and that they contain binding sites for peanut agglutinin (PNA), a cell surface marker that disappears upon EC cell differentiation (11). These results provide further evidence for differentiation-specific changes in carbohydrate moieties exposed on the cell surface.

MATERIALS AND METHODS

CELLS. F941 is a clonal line of EC which has lost the capacity to differentiate (12). It will be referred to it as to "EC cells". Growth and harvest conditions of the cells have been published (13). Thymocytes were prepared from 4 week old male 129/Sv mice, syngeneic to the F9-41 EC line (14).

REAGENTS. NaB[^{3}H]$_4$ was purchased from the Radiochemical Center (8.6 Ci/mM), or from the Commissariat à l'Energie Atomique (10 Ci/mM). The reagent was diluted with 0.01 N NaOH to 10 mCi/ml and kept in 0.1 ml aliquots at -78°C. [^{14}C]-D-galactose, uniformly labeled (300 mCi/mM) was obtained from the Commissariat à l'Energie Atomique. Galactose oxidase, a product of Worthington (55 U/mg) or Sigma (50 U/mg) was stored at -78°C as 100 U/ml aliquots in pH 7.0 phosphate buffered saline (PBS,15). Aliquots were used only once after thawing. Peanut agglutinin (PNA) was isolated as described (16). Anti-PNA serum was raised in rabbits, 300 µl of antiserum precipitated 100 µg of PNA.

LABELING PROCEDURES. The external labelling of the cells was carried out essentially as described (10). Briefly, 0.05 ml of 10 mM unlabeled sodium borohydride in 10 mM NaOH was added to a suspension of 20-30 millions of cells in 0.45 ml of PBS pH 7.4. After 30 minutes at room temperature, the cells were washed three times with PBS pH 7.0, containing 0.001 M phenylmethylsulfonyl fluoride (Sigma). To the washed cells, suspended in 0.5 ml of the same solution, 10 units of galactose oxidase were added. Incubation was continued for 30 minutes at 37°C with occasional shaking. The cells were then washed with the same buffer containing 0.05 M D-galactose and twice with PBS pH 7.4. The washed cells were suspended in 0.45 ml of PBS pH 7.4, and mixed with 0.05 ml of tritiated sodium borohydride (0.5 mCi). After 30 minutes

at room temperature, the cells were then washed with growth medium containing 15 % fetal calf serum (Gibco), till no radioactivity was found in the supernatant. Cell viability and recovery were determined after each step of the labeling procedure.

[^{14}C]-D-galactose labeled EC cells were prepared by culturing F9-41 cells for 24 hours in the presence of 5 µCi/ml of the radioactive sugar.

ANALYTICAL PROCEDURES. Labeled products were digested extensively with Pronase (Calbiochem, 10 mg/ml, 24 hours, three times, 37°C) as described (7). Glycopeptides were analyzed by Sephadex G-50 column chromatography in 0.05 M ammonia-acetid acid buffer pH 6.0 (7). Immunoprecipitation of receptors to PNA was carried out on Triton X-100 extracts of F9-41 cells, as described (17). Briefly, 10 millions of cells were dissolved in 0.5 ml of 0.5 % Triton X-100 in PBS, at 0°C. After one hour, nuclei and cell debris were removed by high speed centrifugation. Solubilized receptors were complexed with PNA in solution (100 µg/ml) and the complexes precipitated with anti-PNA serum added in slight excess. Immune complexes were collected and washed by centrifugation. Cell viability was estimated by the Trypan blue exclusion test (13). Radioactivity was measured in an Intertechnique Scintillation spectrometer, using Unisolve (Koch-Light) as the scintillation fluid.

SUGAR ANALYSIS. Glycopeptides prepared from labeled F9-41 cells (about 30,000 cpm) were hydrolyzed by a mixture (2.5 mg) of glycosidases from Turbo cornutus (Miles) consisting in α and β galactosidase, α and β-N acetyl galactosaminidase. The reaction was carried out in 0.1 ml of 0.05 M citrate buffer pH 4.0, at 37°C, under a layer of toluene. After 24 hours, 0.2 ml of water was added, the solution was boiled for 5 minutes, centrifuged, and the supernatant was applied to Whatman 3 MM paper. The chromatogram was developped in ethyl acetate-pyridine-water (12:5:4). After drying, the paper was cut into pieces, and counted.

RESULTS AND DISCUSSION

Mouse EC cells and thymocytes were externally labelled in paralel. Thymocytes have been chosen as representatives of differentiated cells since they display terminal non-reducing galactosyl residues on their surface (18), similarly to EC cells (11). In the original cell suspension, 94 % EC cells and 97 % thymocytes were viable. Following treatment with galactose oxidase, 90 % of EC cells and 75 % of thymocytes were recovered, and less than 5 % of both cell types were killed : viability being 90 % and 95 % respectively. After labelling and extensive washing, 85 % of EC cells and 32 % of the thymocytes were recovered ; cell lethality did not increase much, viability being 83 % and 84 % respectively. The specific activity obtained was 3-3,5 x10^4 cpm/10^6 EC cells, and 2x10^4 cpm/10^6 thymocytes. Non specific labelling (i.e. incorporation of radioacti-

vity in the absence of galactose oxidase) accounted for 10-15 % of the total incorporation in the two cell types.

Paper chromatography analysis of the sugars released by extensive enzymatic hydrolysis of the glycopeptides prepared from externally labdled EC cells revealed that about 75 % of the radioactivity was associated with galactose, and 10 % with N-acetylgalactosamine. About 15 % of the radioacitivity, which was also resistant to emulsin and to acid hydrolysis (1N HCl, 4hrs, 100°C), stayed at the origin and was not identified.

The externally labelled EC cells and thymocytes were extensively digested with Pronase, and the resulting glycopeptides were analyzed by Sephadex G-50 column chromatography. Elution profiles are given in figure 1. The radioactivity eluted in fractions 65-80 presumably belong to either unreacted $NaB[^3H]_4$, or to some low molecular weight compound since a) unreacted $NaB[^3H]_4$ eluted in fractions 70-74 and, b) this peak was also found in the control experiments (in which galactose oxidase was replaced by buffer). The elution profile of material prepared from EC cells (fig. 1 top) demonstrates the existence of two main classes of glycopeptides : one of these was eluted near the excluded volume, and contained most of the radioactivity (65-70 %). The other 30-35 % were eluted in well retarded fractions, corresponding to lower molecular weights (1500-3500). Glycopeptides prepared from externally labeled thymocytes were eluted exclusively in retarded positions (fig. 1 bottom). The elution profile of externally labelled glycopeptides obtained from EC cells was thus very similar to that obtained from metabolically labelled EC cells (9), but markedly different from that of fully differentiated cells.

In order to substantiate these findings, cells from the same culture were either externally labelled with $[^3H]$ or metabolically

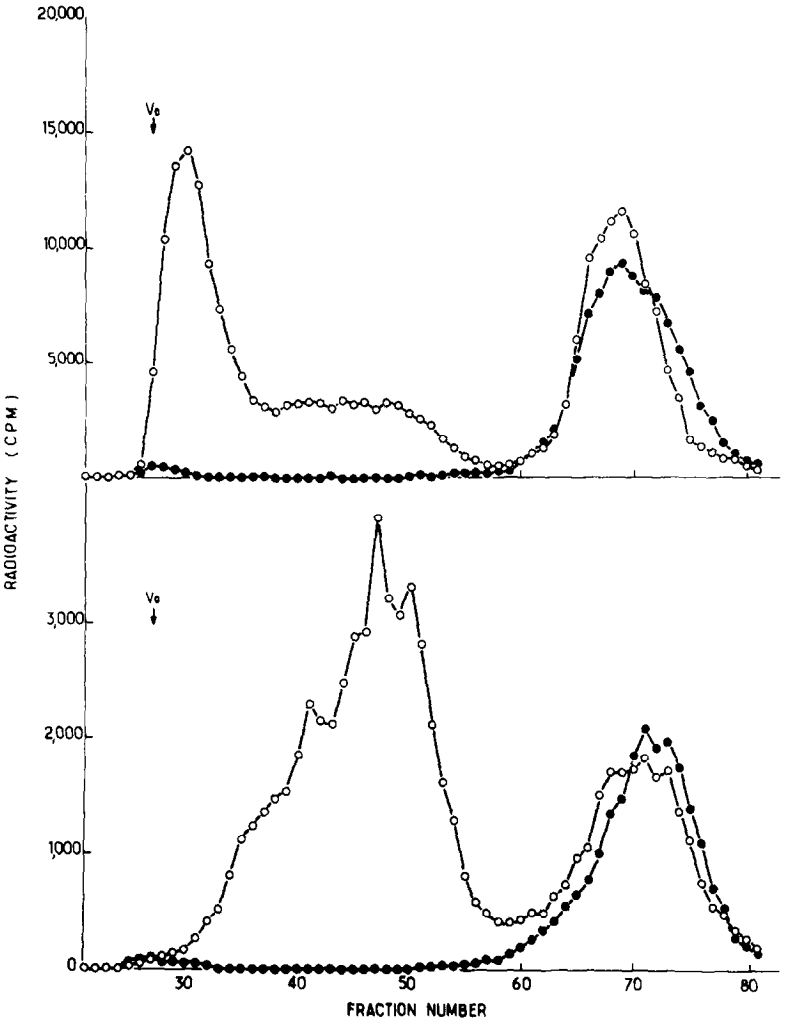

Figure 1. Elution profiles of glycopeptides prepared from externally labelled F9 cells (Top) and thymocytes (Bottom). The cells were labelled as described in materials and methods with (-o-) or without (-●-) treatment with galactose oxidase. Glycopeptides were prepared from labelled cells by extensive Pronase digestion. Chromatographic analysis was carried out on a column of Sephadex G-50 superfine (27xl.5cm, flow rate of 5 ml/hr). Dextran blue was eluted in fraction 27; IgG glycopeptides in fraction 52-53; $NaB[^3H]_4$ in fractions 70-74.

labelled with [^{14}C]-D-galactose. The [^{3}H] and [^{14}C] labelled cells were mixed, digested with Pronase, and the resulting glycopeptides analyzed as above. The overall profiles for the two isotopes were found

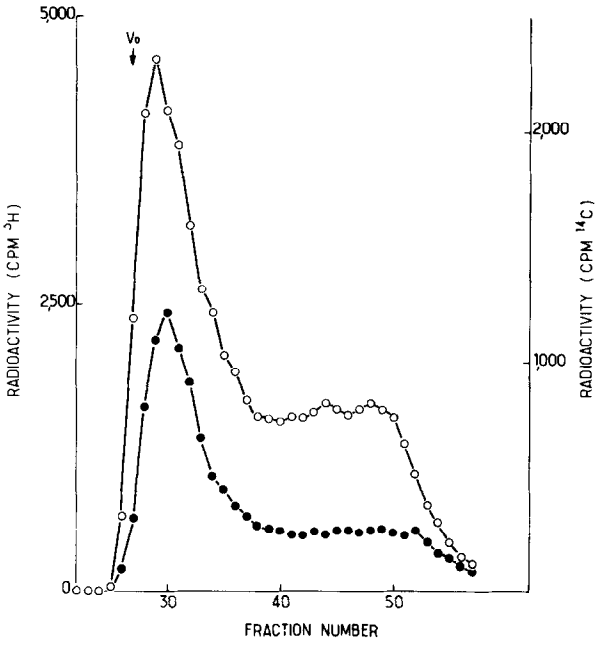

Figure 2. Elution profile of the galactosyl-glycopeptides prepared from externally labelled F9 cells (-o-) and endogenously labelled F9 cells (-●-). The experiment is described in the text. Same procedure and legend as in figure 1.

very similar (figure 2). [^{14}C] or [^{3}H] alone could not be detected in any fraction, indicating that glycopeptides containing D-galactose, carry also a significant proportion of D-galactosyl residues which are accessible to galactose oxidase on intact cells, and thus are displayed on the cell surface.

It has been previously shown that receptors to PNA (a lectin affine for non reducing terminal β-D-galactosyl residues, 14) are exposed on the surface of EC cells, and that they disappear upon cell differentiation (11). To gain further insight into the nature of the galactose labelled glycopeptides, the interaction of PNA with material extracted from externally labeled EC cells was examined. For this purpose, galactose oxidase-NaB[^{3}H]$_4$ labeled EC cells were dissolved in 0.5 % Triton X-100, and the receptors for PNA were isolated from the solubilized material by indirect immunoprecipitation. The isolated

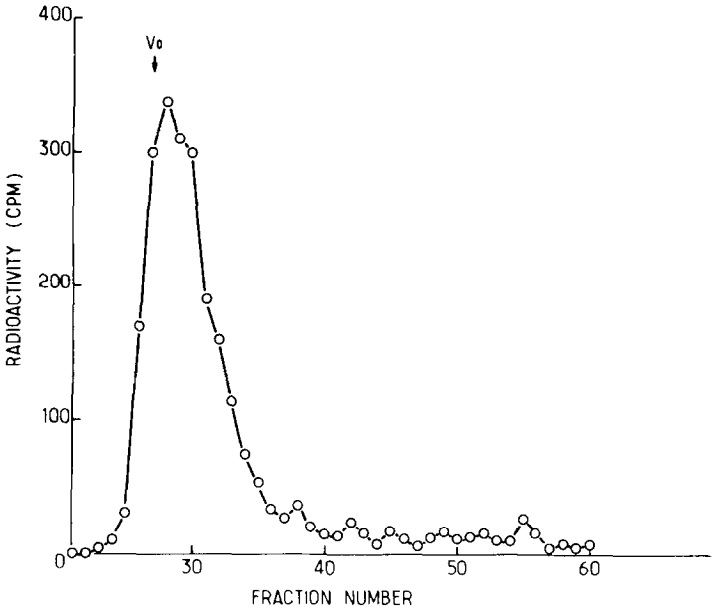

Figure 3. Elution profile of the glycopeptides prepared from receptors for PNA isolated from externally labelled F9 cells. Same column as in figure 1.

receptors were extracted with chloroform-methanol (2:1) to remove the adorbed lipids. Ten to 12 % of the radioactivity of the extract was recovered in the immune precipitate, while only 0.8 % was recovered in the presence of 0.1 M D-galactose, added during the isolation process. Glycopeptides were prepared by extensive Pronase digestion of the receptors, and were analyzed as above. The elution profile (figure 3) was nearly monophasic, and showed mostly large molecular weight glycopeptides. These results indicate that a fraction of the receptors to PNa is indeed displayed on the cell surface, and accounts for a part of the large molecular weight glycopeptides of EC cells.

From the results described in the present communication, it can be concluded that the high molecular weight galactosyl glycopeptides, which are so far characteristic or early embryonic cells (7) are at least for a part exposed on the cell surface, that these glycopeptides contain galactosyl and N-acetylgalactosaminyl residues

in an external position, and that they contain the binding sites for PNA. The quantitative decrease of these high molecular weight glycopeptides during *in vitro* differentiation of EC cells (7) can therefore be attributed to cell surface changes reflected in the disappearance of the receptors to PNA from the cell surface during the same differentiation process (11).

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