Changes in cell-surface fucose-containing glycopeptides and adhesion of cultured intestinal epithelial cells as a function of cell density

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Confluent cultured intestinal epithelial cells displayed greater adhesion to the substratum than did subconfluent cells. Subconfluent and confluent cells were labelled with [³H]fucose for 24 h and the cell-surface components were released by mild Pronase treatment. After extensive Pronase digestion, cell-surface and cell-residue glycopeptides were fractionated on Bio-Gel P-6. The cell surface contained a higher proportion of lower-molecular-weight glycopeptides than the residue. No significant difference in elution pattern was found between total cell-surface glycopeptides of subconfluent and confluent cells. However, confluent cells contained almost twice as much [³H]-fucose-labelled glycopeptides that were bound to concanavalin A-Sepharose and were subsequently eluted with 20 mM-methyl α -D-glucopyranoside as subconfluent cells. When the bound glycopeptides were chromatographed on Bio-Gel P-6, it was found that confluent cells contained a larger proportion of lower-molecular-weight glycopeptides than subconfluent cells. This difference in size was eliminated after treatment of glycopeptides with sialidase. When growth of subconfluent cells was inhibited with a non-toxic concentration of retinoic acid, no significant effect on the elution pattern of [³H]fucose-labelled glycopeptides was observed on either Bio-Gel P-6 or concanavalin A-Sepharose. No significant difference was found in the total [³H]fucose-labelled glycoproteins from subconfluent and confluent cells by two-dimensional gel electrophoresis. It is suggested that the differences in [³H]fucose-labelled glycopeptides between subconfluent and confluent cells are cell-density-dependent rather than growth-dependent, and that these differences are likely to result from some changes in glycosylation mechanism(s). Furthermore, the differences in cell-surface glycopeptides may be related to the changes in the adhesion of the cells to the substratum.

We have demonstrated previously that the glycoproteins at the cell surface of confluent intestinal epithelial cells contain a larger proportion of [2-³H]mannose-labelled complex oligosaccharides than those at the cell surface of subconfluent cells. Confluent cells also displayed an increased adhesion to the substratum compared with subconfluent cells. These changes at the cell surface appeared to be dependent on cell density rather than on growth (Sasak *et al.*, 1982). Since no major difference was found between [2-³H]mannose-labelled glycoproteins of subconfluent and confluent cells by

two-dimensional gel electrophoresis, it was suggested that the observed changes in the glycopeptides may be due to differences between subconfluent and confluent cells in the biosynthesis of the carbohydrate units.

In the present paper, we have compared the cell-surface glycopeptides and glycoproteins of subconfluent and confluent intestinal epithelial cells after labelling with [³H]fucose to determine if additional density-dependent changes in carbohydrate structure occur.

Materials and methods

The source of most reagents was described previously (Sasak *et al.*, 1982). Concanavalin

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A-Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Cell culture

Rat small-intestinal epithelial cells (IEC-6 cells) were cultured in Dulbecco's modified Eagle medium (DMEM), containing 5% (v/v) foetal-bovine serum, 10 μ g of insulin/ml, 50 units of penicillin/ml, 50 μ g of streptomycin/ml and 4 mM-L-glutamine, as previously described (Quaroni *et al.*, 1979). Retinoic acid (0.1 μ g/ml in 0.1% ethanol, final concentration) was used to inhibit cell growth (Sasak *et al.*, 1982).

Labelling of cells and preparation of glycopeptides

Subconfluent growing cells, subconfluent growth-inhibited cells (about 25–30% confluent) and confluent cells grown in 100 mm dishes were labelled with L-[6-³H]fucose (5 μ Ci/ml) of sp. radioactivity 14 Ci/mmol (Amersham, Arlington Heights, IL, U.S.A.) in 10 ml of low-glucose (1 g/litre) DMEM (Gibco, Grand Island, NY, U.S.A.) supplemented with 5% dialysed foetal-calf serum, 50 units of penicillin/ml, 50 μ g of streptomycin/ml, 10 μ g of insulin/ml and 4 mM-L-glutamine. After 24 h the cells were washed with phosphate-buffered 0.9% NaCl (saline) and the cell-surface and cell-residue glycopeptides were prepared as described previously (Sasak *et al.*, 1982). Determination of radioactive sugar present in the glycopeptides after 24 h of labelling with L-[6-³H]fucose was carried out by the method of Spiro (1966). Only one radioactive spot corresponding to fucose was detected on paper chromatograms developed in ethyl acetate/pyridine/water (8:2:1, by vol.).

Other methods

Cell-detachment assay and two-dimensional slab-gel electrophoresis were carried out as previously described (Sasak *et al.*, 1982). Sialidase treatment was conducted in 50 mM-citrate/phosphate buffer, pH 5.5. Sialidase (2.5 units) from *Vibrio cholerae* (Behring Diagnostics, Somerville, NJ, U.S.A.) was added and the samples (250 μ l, final volume) were incubated for 1 h at 37°C. This enzyme preparation was not active against *p*-nitrophenyl β -D-galactopyranoside and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as substrates. Concanavalin A-Sepharose column fractionation was carried out by the method of Finne *et al.* (1980) in 5 mM-sodium acetate, pH 5.2, containing 0.1 M-NaCl and CaCl₂, MnCl₂ and MgCl₂ (1 mM each); 20 mM-methyl α -D-glucopyranoside in the above buffer was used for the elution of bound glycopeptides from the column. Recovery of radioactivity was between 87 and 95%. Two-dimensional slab-gel analysis of labelled membrane glycoproteins was performed as described previously (Sasak *et al.*, 1982).

Results

Adhesion of cells to substratum

Intestinal epithelial cells (IEC-6 cells) grown for 3 days (subconfluent) and 10 days (confluent) in culture were assayed for their adhesion to the substratum. As shown in Fig. 1, detachment of the cells increases with increasing time of trypsin treatment. About 70% of subconfluent cells could be detached from the plastic dish after 15 min of trypsin treatment. Confluent cells displayed a much higher adhesion to the substratum and in 15 min only about 5% of the cells could be detached.

Characterization of L-[6-³H]fucose-labelled glycopeptides

Subconfluent and confluent cells were incubated for 24 h with L-[6-³H]fucose and then cell-surface and cell-residue fractions were prepared by mild Pronase treatment. Under these conditions between 32 and 38% of the radioactivity in the glycopeptides was released from the cells. This fraction is referred to as cell-surface material. The total incorporation of [³H]fucose into subconfluent and confluent cells expressed per μ g of protein was approximately the same. Both cell-surface and cell-residue fractions were exhaustively digested with Pronase, and the resulting glycopeptides were fractionated on a

Fig. 1. Adhesion of IEC-6 cells to the substratum

The cells were washed with phosphate-buffered saline, pH 7.1, and then incubated on a rotary shaker at 140 rev./min for various times in phosphate-buffered saline containing 100 μ g of trypsin/ml. Both detached and attached cells were counted and the number of detached cells is expressed as a percentage of the total number of cells. Each point represents a mean of two determinations from duplicate dishes. The range of reproducibility between separate determinations was 3–24%. O, Subconfluent cells; ●, confluent cells.

Fig. 2. Comparison between cell-surface and cell-residue glycopeptides

Bio-Gel P-6 (200–400 mesh) chromatography of [^3H]fucose-labelled glycopeptides from the cell surface (—) and from the cell-residue (....) of (a) subconfluent and (b) confluent cells. The glycopeptides were eluted from the column (1 cm $\times$ 116 cm) with 0.1 M-pyridine acetate (pH 5.0), containing 0.5 mM- NaN_3 . Flow rate was 4 ml/h and 0.72 ml fractions were collected. Bovine serum albumin was used for determination of V_0 (void volume, fraction 35) and fucose for V_t (bed volume, fraction 88).

column of Bio-Gel P-6. These glycopeptides had a similar elution volume to the glycopeptides containing complex oligosaccharides previously labelled with [$2\text{-}^3\text{H}$]mannose (Sasak *et al.*, 1982). Mild alkaline treatment (50 mM-KOH/1 M- NaBH_4 , 24 h, 45°C) did not change the elution pattern of the [^3H]fucose-labelled glycopeptides. As shown in Fig. 2 the cell surface of both subconfluent and confluent cells yielded a larger proportion of lower-molecular-weight glycopeptides than the cell residue. However, no significant difference was found in the elution profile of the total [^3H]fucose-labelled cell-surface or cell-residue glycopeptides between subconfluent and confluent cells.

Fig. 3. Fractionation of the cell-surface glycopeptides. Concanavalin A-Sepharose column (1 cm $\times$ 6 cm) chromatography of [^3H]fucose-labelled glycopeptides from (a) subconfluent and (b) confluent cells. The glycopeptides not bound to the column were eluted with 5 mM-sodium acetate (pH 5.2), containing 0.1 M- NaCl and 1 mM- MgCl_2 , - MnCl_2 and - CaCl_2 each. Material bound to the column was eluted with 20 mM-methyl α -D-glucopyranoside in the eluting buffer. Fractions (3 ml) were collected. Bio-Gel P-6 column chromatography of [^3H]fucose-labelled glycopeptides from (c) subconfluent and (d) confluent cells is also shown. The glycopeptides bound (—) and unbound (....) to concanavalin A-Sepharose are shown. The conditions of the gel filtration were as described in the legend to Fig. 2. The arrows show elution with 20 mM- and 200 mM-methyl α -D-glucopyranoside, as indicated.

Concanavalin A-Sepharose analysis of glycopeptides

The cell-surface [^3H]fucose-labelled glycopeptides of subconfluent and confluent cells were

fractionated on concanavalin A-Sepharose column (Figs. 3a and 3b). About 26% of the glycopeptides from the cell surface of subconfluent cells (Fig. 3a) and 48% of the glycopeptides from confluent cells (Fig. 3b) was retained on the column and eluted with 20 mM-methyl α -D-glucopyranoside. No radioactivity was eluted from the column with 200 mM-methyl α -D-glucopyranoside. The cell-surface glycopeptides obtained from the concanavalin A-Sepharose column were then fractionated on a column of Bio-Gel P-6 (Figs. 3c and 3d). For both subconfluent (Fig. 3c) and confluent (Fig. 3d) cells, the material not bound to the column contained larger-molecular-weight glycopeptides than the bound material.

The cell-residue fractions from subconfluent and confluent cells were also analysed on concanavalin A-Sepharose column (results not shown). About 20% of the glycopeptides from subconfluent cells and about 24% of the glycopeptides from confluent cells was retained on the column and eluted with 20 mM-methyl α -D-glucopyranoside. These results indicate that, for confluent cells, the cell-surface

fraction contained a higher proportion of [3 H]-fucose-labelled glycopeptides that bound to concanavalin A-Sepharose than did the cell-residue fraction. There was no significant difference in the distribution of labelled glycopeptides between cell-surface and cell-residue fractions of subconfluent cells.

[3 H]Fucose-labelled glycopeptides from retinoic acid-treated cells

Retinoic acid treatment of the cells did not affect the pattern of [3 H]fucose-labelled glycopeptides obtained from either cell-surface or cell-residue fractions when examined on Bio-Gel P-6 before

Fig. 4. Comparison between concanavalin A-Sepharose-bound glycopeptides

Bio-Gel P-6 chromatography of [3 H]fucose-labelled cell-surface glycopeptides bound to concanavalin A-Sepharose from subconfluent (—) and confluent (....) cells. (a) Intact glycopeptides; (b) glycopeptides after sialidase treatment. The gel filtration was carried out as described in the legend to Fig. 2.

Fig. 5. Glycoproteins from subconfluent and confluent cells

Isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-slab-gel analysis of [3 H]fucose-labelled glycoproteins from (a) subconfluent and (b) confluent cells is shown. Isoelectric focusing (IF) was performed with pH 4.4–7.5 Ampholines. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS) was carried out on 7.5% slab gels from the top to the bottom of the Figure. About 250 μ g and 200 μ g of protein from subconfluent and confluent cells respectively was applied to the gels. Time of the exposure for autoradiographs was 1 month. The spot indicated by the arrow is probably an artefact owing to cracking of the gel.

and after chromatography on concanavalin A-Sepharose.

Sialidase treatment of glycopeptides

The comparison between cell-surface glycopeptides that were bound to concanavalin A-Sepharose column from subconfluent and confluent cells is shown in Fig. 4. The material isolated from confluent cells had a larger proportion of lower-molecular-weight glycopeptides than the material from subconfluent cells (Fig. 4a). After sialidase treatment of both preparations, the higher-molecular-weight glycopeptides were reduced in size and had a similar elution volume to the glycopeptides that were predominant in confluent cells (Fig. 4b).

Two-dimensional slab-gel analysis of [³H]fucose-labelled glycoproteins

Since differences in [³H]fucose-labelled cell-surface glycopeptides were found between subconfluent and confluent cells, it was important to determine whether this difference was due to the formation of new [³H]fucose-labelled glycoproteins. One-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed only seven radioactive bands, with two major labelled components that had apparent molecular weights of about 90 000 and 105 000. The autoradiograms of two-dimensional slab gels showed no major difference between [³H]fucose-labelled glycoproteins from subconfluent and confluent cells (Fig. 5).

Discussion

These results show that a higher proportion of fucose-labelled glycopeptides from the cell surface of confluent cells was bound to concanavalin A-Sepharose when compared with the glycopeptides derived from subconfluent cells. The conditions used for concanavalin A-Sepharose fractionation have been shown to separate biantennary from more highly branched *N*-linked complex oligosaccharides (Krusius, 1976; Krusius & Finne, 1977; Finne *et al.*, 1980); biantennary structures are retained on concanavalin A-Sepharose and subsequently eluted with 20 mM-methyl α -D-glucopyranoside, whereas more highly branched oligosaccharides are not adsorbed to the affinity column. It is concluded, therefore, that the cell surface of confluent intestinal epithelial cells contains a higher proportion of fucose-labelled biantennary oligosaccharides than do subconfluent cells.

The concanavalin A-Sepharose-bound fraction from the cell surface of confluent cells contained a larger proportion of lower-molecular-weight material than that of subconfluent cells. Sialidase treatment converted the high-molecular-weight glycopeptides into smaller glycopeptides, thereby indicating that

the biantennary fucose-labelled oligosaccharides appear to be less sialylated in confluent than in subconfluent cells.

Although the fucose-labelled glycopeptides from the cell surface of confluent cells are less sialylated than those of subconfluent cells, no significant difference was observed between subconfluent and confluent cells in the total [³H]fucose-labelled glycoprotein pattern by two-dimensional gel electrophoresis. The reasons for this apparent discrepancy are not entirely clear, although it should be pointed out that the cell-surface glycopeptide fraction was obtained by mild proteolytic treatment of intact cells, whereas the two-dimensional gel electrophoresis was performed on a membrane fraction obtained by differential centrifugation. Furthermore, since the number and composition of the oligosaccharide chains present in the membrane glycoproteins is not known, it is possible that the fucose-labelled oligosaccharides containing sialic acid represent only a small proportion of the total sialic acid-containing oligosaccharides. If this were the case, the migration of the glycoproteins during electrophoresis and isoelectric focusing might not be significantly affected.

When growth of subconfluent cells was completely inhibited with retinoic acid no significant difference was observed in the elution pattern of [³H]fucose-labelled cell-surface glycopeptides from concanavalin A-Sepharose and Bio-Gel P-6 compared with rapidly growing control cells. Therefore, it is suggested that the observed differences in [³H]fucose-labelled cell-surface glycopeptides between subconfluent and confluent cells, like the previously-reported differences in [³H]mannose-labelled glycopeptides (Sasak *et al.*, 1982), appear to be density-dependent.

Since confluent and subconfluent cells exhibit a very great difference in adhesion to the substratum, it seems possible that some of the changes in carbohydrate structure at the cell surface may be related to the changes in adhesion properties of the cells.

Several studies indicated a correlation between cell growth and membrane glycoprotein metabolism (Muramatsu *et al.*, 1973; Kaplan & Moskovitz, 1975; Milenkovic *et al.*, 1978). It has been shown that [³H]fucose-labelled glycopeptides from the cell surface contained a larger proportion of lower-molecular-weight material than did glycopeptides from the cell residue of human diploid fibroblasts (Muramatsu *et al.*, 1973). The cell surface of growing cells contained a larger amount of higher-molecular-weight glycopeptides than did the cell surface of non-growing cells (Buck *et al.*, 1971; Muramatsu *et al.*, 1973). The authors described these differences in cell-surface glycopeptides as growth-dependent changes.

The mechanism of cell adhesion to the substratum is still very poorly understood, although a number of investigators found some correlations between cell-surface glycoprotein alterations and changes in cell adhesion. A lectin-resistant mutant of Chinese-hamster cells (CHO) whose cell-surface glycoproteins lack terminal sugar residues was found to be very difficult to detach from the substratum by trypsin treatment. However, attachment to protein-coated glass surface was the same for the mutant and wild-type cells (Juliano, 1978). This latter author has therefore postulated that terminal carbohydrate moieties are not directly involved in the attachment of the cells to the substratum but they may modulate the proteolytic susceptibility of surface components.

Ricin-resistant clones of baby-hamster kidney fibroblasts in which cell-surface components presumably had a decreased number of galactose and *N*-acetylgalactosamine residues displayed a weaker cell-to-cell and cell-to-substratum adhesion than did control cells (Edwards *et al.*, 1976). Analysis of cell-surface glycoproteins of some ricin-resistant clones by two-dimensional gel electrophoresis showed a variety of alterations in surface macromolecule mapping (Pena *et al.*, 1979).

In other studies, a general increase or decrease in the amount of total complex oligosaccharides at the cell surface was correlated with a concomitant increase or decrease in adhesion of the cells to the substratum (Pena & Hughes, 1978; Hughes, 1979; Sasak *et al.*, 1980).

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