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**Original article**

# N-Glycosylation modification of proteins is an early marker of the enterocytic differentiation process of HT-29 cells

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**Summary** — The human colon cancer cell line HT-29 remains totally undifferentiated when glucose is present in the culture medium (HT-29 Glc$^+$), while the same cells may undergo typical enterocytic differentiation after reaching confluence when grown in glucose-deprived medium (HT-29 Glc$^-$). Recently, we demonstrated a deficiency in the overall N-glycan processing in confluent undifferentiated cells, whereas differentiated cells follow a classical pattern of N-glycosylation. The main changes in N-glycosylation observed in confluent undifferentiated cells may be summarised as follows: 1) the conversion of high mannose into complex glycopeptides is greatly decreased; 2) this decreased conversion could be a consequence of an accumulation of $Man_{9-8}$–$GlcNAc_2$-Asn high mannose species. Whether these changes in N-glycan processing appear progressively during cell culture or are already present from the beginning of the culture was investigated in this study by comparing the actual status of N-glycan processing in exponentially growing HT-29 Glc$^-$ and HT-29 Glc$^+$ cells. Under these conditions, HT-29 Glc$^-$ cells do not exhibit any characteristics of differentiation. The conversion of high mannose into complex glycoproteins is severely reduced in HT-29 Glc$^+$ cells, regardless of the growth phase studied. In contrast, HT-29 Glc$^-$ cells display a normal pattern of N-glycan processing in both growth phases. We therefore conclude that N-glycan processing may be used as an early biochemical marker of the enterocytic differentiation process of HT-29 cells.

**N-glycan trinning / high mannose type chain / enterocytic differentiation / colon cancer / early biochemical marker**

**Résumé — La N-glycosylation des protéines est un marqueur précoce de la différenciation entérocytaire des cellules HT-29.** *Les cellules HT-29, dérivées d'un adénocarcinome humain d'origine colique et cultivées en présence de glucose (HT-29 Glc$^+$) demeurent indifférenciées alors que les mêmes cellules, cultivées en absence de glucose (HT-29 Glc $^-$) experiment, après confluence cellulaire, une différenciation entérocytaire typique. Cette étude réalisée après confluence a permis*

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*de mettre en évidence différentes altérations dans le métabolisme des N-glycannes des cellules HT-29 Glc+, qui ne sont pas observées dans les cellules HT-29 Glc −: 1) la maturation des chaînes oligomannosidiques en chaînes de type complexe est fortement réduite dans ces cellules; 2) une forte accumulation de chaînes oligomannosidiques comportant 9 à 8 résidus de mannose peut être détectée. De plus, le modèle HT-29 permet d'étudier les événements biochimiques précoces qui se mettent en place avant l'acquisition de la différenciation des cellules HT-29 Glc −. En effet, en phase exponentielle de croissance, les cellules HT 2A Glc − et HT 2A Glc+ sont phénotypiquement indifférenciées, malgré leur devenir différent après confluence.*

***métabolisme des N-glycannes / chaîne oligomannosidique / différenciation entérocytaire / cancer colique / marqueur biochimique précoce***

## INTRODUCTION

The HT-29 cell line (Fogh and Trempe, 1975) derived from a human colon adenocarcinoma, is an excellent model for the study of the dynamics and positioning of the elements involved in cell differentiation. In fact, the same cells can undergo typical enterocytic differentiation when they are grown without glucose (HT-29 Glc−), or remain totally undifferentiated when cultured in the presence of glucose (HT-29 Glc+) (Zweibaum *et al,* 1985). It is only after confluence has been reached that the morphological and enzymatic characteristics of differentiation appear in HT-29 Glc− cells. Recently, we demonstrated that the entire protein glycosylation process is markedly perturbed in confluent undifferenciated cells, whereas the differentiated cells undergo classical glycosylation at the same growth stage (Ogier-Denis *et al,* 1988). It remains important to determine whether the modifications of co- and post-translational mechanisms precede the expression of the morphological and enzymatic characteristics of HT-29 Glc− cell differentiation or not. The HT-29 system, for which the stage of diffentiation can be predicted and modulated simply by modifying the culture medium, enables an approach to be made to these types of questions. Thus, we have studied N-glycosylation of HT-29 cells during the exponential growth phase.

## MATERIALS AND METHODS

### *Cells and cell culture*

HT-29 cells were cultured in the presence of 2.5 mmol/l inosine in the absence of glucose (permissive condition of differentiation) or with 25 mmol/l glucose (non-permissive condition of differentiation). Cells were used after reaching confluence (d 20) when HT-29 Glc− cells underwent typical enterocytic differentiation, and during the exponential growth phase (d 5) when the differentiation markers were not yet expressed in HT-29 Glc− cells.

### *Metabolic labelling and chromatography*

Cells were radiolabelled with D-[2–3H]–mannose (20 Ci/mmole, The Amersham Radiochemical Centre, UK) for 10 min (400 μCi/ml) and 24 h (40 μCi/ml). During pulse-chase experiments, 5 mmol/l of non-radioactive mannose and 2 mmol/l of non-radioactive fucose were added. After metabolic labelling, the cellular glycoproteins were extracted successively by a mixture of chloroform-methanol (2:1, v/v) and a mixture of chloroform-methanol-water (10:10:3, v/v/v), and then exhaustively digested with pronase (2 mg/ml; CB grade, Calbiochem, France)

at 60 °C for 24 h under a toluene atmosphere (Ogier-Denis *et al,* 1988). The glycopeptides obtained were then chromatographed on a Bio-Gel P6 column (1 x 130 cm, 200–400 mesh; Bio-Rad, USA), according to the technique of Codogno *et al* (1985). Each fraction was treated with 2.5 mU of *Streptomyces griseus* endo-β-N-acetylglucosaminidase H (endo H) (Miles, France) in 100 µl of citrate buffer , pH 5.0, for 18 h at 37 °C under a toluene atmosphere (Tarentino and Maley, 1974). The samples were heated at 100 °C for 3 min and chromatographed on a Bio-Gel P6 column.

### *High performance liquid chromatography (HPLC)*

The oligosaccharides released by endo H were fractionated by HPLC (Varian model 5000 equipped with a 5µ aminospherisorb column). The samples were co-eluted with oligosaccharide standards radiolabelled with [$^{14}$C]-mannose. The dolichol-linked oligosaccharide chains, extracted in the chloroform-methanol-water (10:10:3, v/v/v) phase, were analysed as reported by Roméro and Herscovics (1986). Briefly, the oligosaccharides were separated from dolichol by controlled acid hydrolysis (0.01 mol/l HCl for 45 min at 90 °C). After neutralization and desalting, the oligosaccharides were fractionated by HPLC as described above. Regardless of the cell population, the oligosaccharides $Glc_3$–$Man_9$–$GlcNAc_1$ were detected both during the exponential growth phase and after reaching cellular confluence.

## RESULTS

### *Undifferentiated cells accumulated a high proportion of high mannose chains during exponential and stationary growth phases*

The glycopeptides, obtained from metabolically labelled (D-[2–$^3$H]–mannose for 24 h) HT-29 cells following exhaustive Pronase digestion of the glycoproteins, were chromatographed on a Bio-Gel P6 column (table I). Four fractions were identified in the 2 cell populations. Only fraction IV was sensitive to the enzymatic action of endo H and was strongly retained on a column of immobilized concanavalin A. The distribution of the glycopeptides isolated from HT-29 cells during their exponential growth phase was comparable to that found for confluent cells (table I). Regardless of the growth phase considered, HT-29 Glc$^+$ cells expressed a very high proportion of high mannose chains (fraction IV).

### *Alteration of the processing of high mannose chains is an early event in undifferentiated cells*

In order to understand more fully the accumulation of high mannose species in HT-29 Glc$^+$ cells, N-glycan biosynthesis and processing in HT-29 cells during the exponential growth phase were followed by pulse-chase experiments. After a 10 min pulse labelling with D–[2–$^3$H]–mannose, the cells were incubated in the presence of 5 mmol/l of non-radioactive mannose for 0–24 h; 2 mmol/l of non-radioactive fucose was also added to the culture medium in order to eliminate all interconversion of tritiated mannose into tritiated fucose, which had been previously demonstrated (Ogier-Denis *et al,* 1989). At each time interval, we determined the radioactivity associated with the dolichol-linked oligosaccharides (fig 1A and 1B), the glycopeptides bearing the high mannose chains (fig 1C and 1D) and the glycopeptides containing complex-type chains (fig 1E and 1F). The ratio of the radioactivity associated with the complex chains to that of the high mannose chains was calculated for the 2 cell populations during both of the cell growth phases considered (fig 1G and 1H). The biosynthesis of lipid intermediates and their transfer onto the protein backbone were qualita-

**Table I.** Distribution of the different glycoprotein fractions obtained after chromatography on a Bio-Gel P6 colum. HT-29 Glc$^-$ and HT-29 Glc$^+$ cells, exponentially growing and confluent, were metabolically labeled with D–[2–$^3$H]–mannose for 24 h. The glycopeptides obtained after Pronase digestion were separated by chromatography on a Bio-Gel P6 column. a : similar results were obtained previously for confluent cells (Ogier-Denis *et al*, 1988). Exp: exponential growth phase: Conf: confluent cells.

| *Fractions* | *HT-29 Glc$^-$* | | *HT-29 Glc$^+$* | |
|---|---|---|---|---|
| | *Exp* | *Conf* [a] | *Exp* | *Conf* |
| Fraction I | 18 | 25 | 14 | 14 |
| Fraction II | 24 | 17 | 20 | 9 |
| Fraction III | 11 | 20 | 6 | 14 |
| Fraction IV | 47 | 38 | 60 | 63 |

tively similar in the 2 cell populations during the exponential growth phase (fig 1A and 1D). However, HT-29 Glc$^-$ cells undergo classic maturation of the high mannose chains into complex chains, whereas HT-29 Glc$^+$ cells express only a very weak proportion of complex chains (fig 1E and 1F). This deficiency in the mechanism of high mannose side chain maturation was confirmed by analysing the ratio of the radioactivity incorporated into the complex and the high mannose chains (fig 1G and 1H). The nature of the high mannose chains obtained following metabolic labelling (D–[2–$^3$H]–mannose for 24 h), was determined by HPLC after treatment with endo H. The results clearly demonstrate that the undifferentiated HT-29 Glc$^+$ cells amass a high proportion of $Man_{9-8}$–$GlcNAc_2$–Asn species, regardless of the growth phase studied (Ogier-Denis *et al*, 1989).

## DISCUSSION

The results presented herein demonstrate that the N-glycosylation process is independent of the growth phase in HT-29 cells, but depends greatly upon the capacity or incapacity of the cells to differentiate after reaching confluence. In HT-29 Glc$^-$ cells, oligosaccharide transfer follows the classical pathway from dolichol to the polypeptide backbone, independently of the growth phase. The high mannose chains formed are thus quantitatively transformed into complex-type chains, as has been demonstrated for many eukaryote cells (Kornfeld and Kornfeld, 1985). In contrast, HT-29 Glc$^+$ cells accumulate, in spite of their ability to engage in a classical mechanism for the biosynthesis of lipid intermediates, a very high proportion of high mannose chains. In addition, the maturation of the latter into complex-type chains is severely altered, regardless of the growth phase considered. The modifications of the N-glycosylation process are thus present very early in HT-29 Glc$^+$ cells. One of the most striking differences between the HT-29 system and other cell models described by other authors is that the N-glycan metabolism is independent of cell density and growth, but is directly linked to the differentiation program of HT-29 Glc$^-$ cells. In HT-29 Glc$^+$ cells, the deficient metabolic step involves the conversion of high mannose chains, $Man_{9-8}$–$GlcNAc_2$–Asn into $Man_{7-6-5}$–$GlcNAc_2$–Asn. The fact that this transformation is altered in undifferentiated

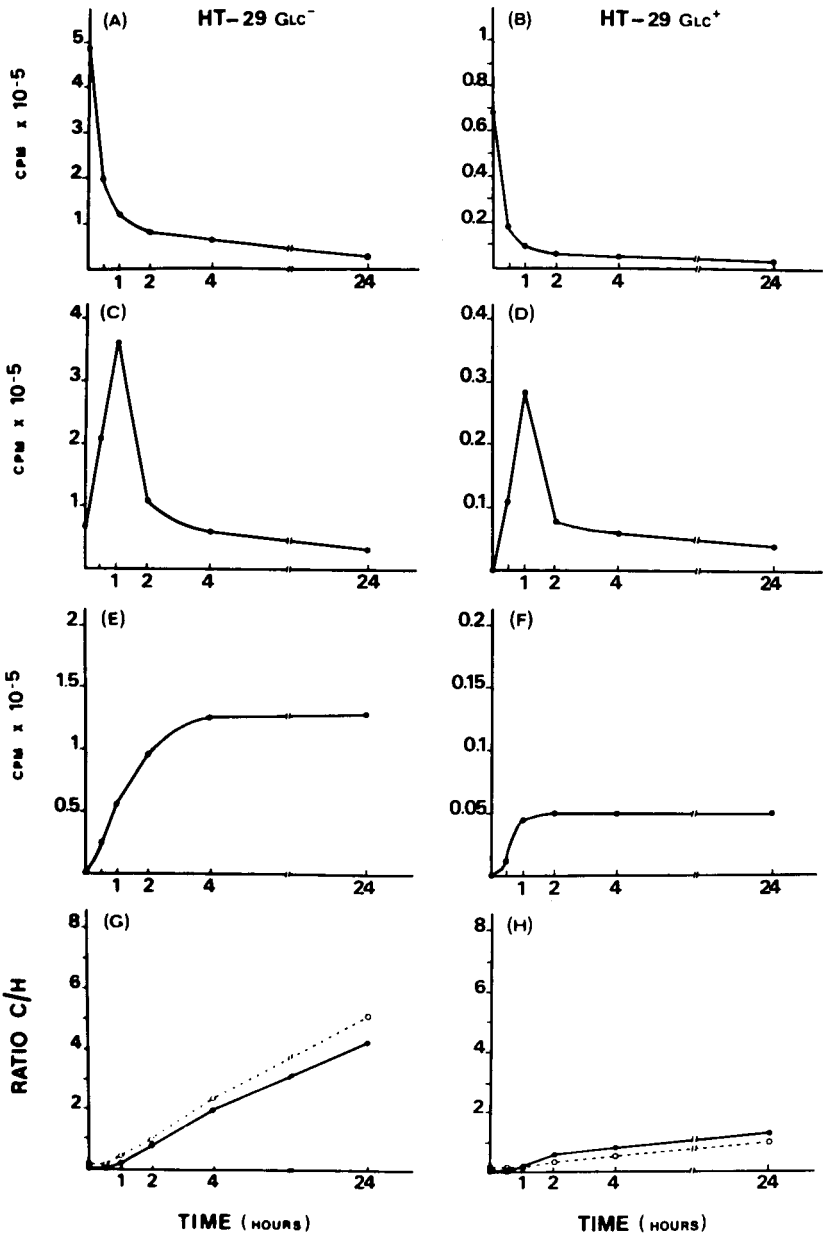


**Fig 1.** Fate of tritiated mannose incorporated in the different cell populations during a pulse-chase experiment. Growing HT29 Glc⁻ (left panel) and HT-29 Glc⁺ (right panel) cells were pulsed for 10 min with 400 μCi/ml of D-[2-$^3$H]-mannose, as described in the *Materials and Methods* section. The chase was performed for 0.5, 1, 2, 4 and 24 h in the presence of 5 mmol/l unlabelled mannose and 2 mmol/l unlabelled fucose. At each time, the lipid-linked oligosaccharides were extracted and the radioactivity of this lipid fraction determined (first row, A–B). The glycopeptides obtained after Pronase digestion were fractionated on a Bio-Gel P6 column. The radioactivity recovered in the high mannose fraction (fraction IV endo H-sensitive) was plotted in the 2nd row (C–D). The radioactivity recovered in the complex fractions was plotted in the 3rd row (E–F).In the last row, we report the ratio of the radioactivity in complex/high mannose glycopeptides (ratio C/H) in exponential cells, and the dotted lines correspond to the results obtained previously with corresponding confluent cells (Ogier-Denis *et al,* 1988).

HT-29 Glc+ cells, regardless of the growth phase considered, raises the question as to the regulation of this enzymatic reaction. Future studies will reveal whether the alterations observed in the N-glycosylation process result from a regulation of the genetic control or an early regulation of N-glycan metabolism. Although the 2 cell populations are morphologically and enzymatically indistinguishable during the exponential growth phase, it is now possible to differentiate between them using an early biochemical marker, *i e,* N-glycosylation of proteins. Thus, the N-glycosylation mechanism seems to be a sensitive and early indicator of the capacity of HT-29 cells to enter into the complex pathway of cellular differentiation.

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