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Processing of Asparagine-Linked Oligosaccharides Is an Early Biochemical Marker of the Enterocytic Differentiation of HT-29 Cells

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The inability of HT-29 cells to undergo an enterocytic differentiation when grown in a glucose-containing (Glc^+) medium has been recently correlated to an overall impairment of N-glycan processing. These results were obtained using confluent HT-29 cells in which the differentiation characteristics are fully expressed under differentiation permissive conditions (glucose-deprived medium, Glc^-). Whether these changes of N-glycan processing appear progressively during the cell growth or are already present from the beginning of the culture was investigated in this work by comparing the actual status of N-glycan processing in both exponentially growing Glc^+ and Glc^- HT-29 cells. Under these conditions, HT-29 cells do not express any characteristics of enterocytic differentiation, even when grown in differentiation permissive conditions. We show here that the conversion of high-mannose to complex glycoproteins is, however, severely reduced in HT-29 cells grown in differentiation non-permissive conditions (HT-29 Glc^+) whatever the phase of growth studied. In contrast, HT-29 cells grown in differentiation permissive conditions (HT-29 Glc^-) display a normal pattern of N-glycan processing in both the exponential and the stationary phase of growth. We also show that both growing and confluent HT-29 Glc^+ cells accumulate Man_{9-8} $GlcNAc_2$ species, thus suggesting that there is an important regulatory point at this level. We therefore conclude that the N-glycan processing may be used as an early biochemical probe for the enterocytic differentiation of HT-29 cells. Whether these early changes result from an early metabolic regulation or are the consequence of a genetic control remains to be studied.

Key words: N-glycan processing, cell differentiation, enterocytes, colon cancer, cell growth

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Recent data demonstrate that the human colon cancer cell line HT-29 is a very powerful system for the study of the dynamics of cell differentiation as the same cells may exhibit a typical enterocytic differentiation when grown in a glucose-deprived medium or will remain totally undifferentiated when glucose is present during all the time in culture [1–4]. It is only when cells grown in a glucose-deprived medium reach confluency that both morphological and enzymatical characteristics of differentiated cells appear, i.e., a polarization of the cell monolayer with an apical brush border, and the presence in the brush border membrane of hydrolase activities, namely sucrase-isomaltase (SI), dipeptidylpeptidase IV (DPP-IV), aminopeptidase N (APN), alkaline phosphatase (Alk P) [1–4]. Studies on SI, the most specific and sensitive enzymatic marker of the enterocytic differentiation of HT-29 cells, have shown that the posttranslational processing of this protein, and especially its glycosylation, is impaired in undifferentiated HT-29 cells [5]. More recently, we have demonstrated that alterations of protein glycosylation are not restricted to some specific proteins but that the overall N-glycan processing is deficient in confluent undifferentiated HT-29 cells, whereas differentiated cells exhibit a classical pattern of N-glycosylation [6]. Therefore, N-glycan processing may be considered as a marker of differentiation, at least in this model.

The main changes in N-glycosylation observed in confluent undifferentiated cells may be summarized as follow: 1) D-[2-^{3}H]-mannose (Man) incorporation into glycoproteins is 10 times lower; 2) the conversion of high mannose into complex glycopeptides is greatly decreased; 3) this decreased conversion could be a consequence of an impairment of the reaction catalyzed by mannosidase I, as substantiated by the accumulation of Man_{9-8}-$GlcNAc_2$-Asn species [6].

One of the most puzzling questions dealing with the differentiation process is to know how and when cells are committed to differentiate. The availability of a cell culture system such as HT-29 cells, in which the state of cell differentiation may be predicted and modulated by a simple manipulation of the culture medium, provides an answer to this type of question. In this paper, exponentially growing HT-29 cells were cultured in glucose-free (Glc^-) or glucose-containing (Glc^+) medium, i.e., in differentiation-permissive or differentiation-non-permissive conditions, respectively. The N-glycan processing was studied in each condition by using D-[2-^{3}H]-mannose labeling techniques. We demonstrate that the previously described alterations of N-glycan processing associated with confluent undifferentiated HT-29 cells are already present in exponentially growing HT-29 cells that will not differentiate later (HT-29 Glc^+) but not present in HT-29 cells that will differentiate when the cells will reach confluency (HT-29 Glc^-).

MATERIALS AND METHODS

Cells and Culture Conditions

HT-29 cells [7] were cultured as previously reported [5]. The same cells were grown in a medium containing 25 mM glucose (HT-29 Glc^+ cells:differentiation-non-permissive conditions) or without glucose containing 2.5 mM Inosine (HT-29 Glc^- cells:differentiation-permissive conditions). The cells were used after confluency (18–20 days) when HT-29 Glc^- cells exhibit a typical enterocytic differentiation and during the early phase of growth (5 days) when the differentiation criteria are not yet expressed in HT-29 Glc^- cells [2,4].

Preparation of Cellular Extracts and Enzyme Assays

Cell homogenates were prepared according to a method described elsewhere [8]. Sucrase (EC 3.3.1.48), aminopeptidase N (EC 3.4.11.2), and dipeptidylpeptidase IV (EC 3.4.14.5) activities were measured in the cell homogenate according to previously described methods [9–11]. The activities are expressed as milliunits (mU) per mg of proteins. One unit is defined as the enzymatic activity that hydrolyzes 1 μmole of substrate per min at 37°C.

Cell Labeling and Glycopeptide Chromatography

Metabolic labeling of the cells was performed using D-[2-^{3}H]-mannose (20 Ci/mmol, The Amersham Radiochemical Centre, Amersham, Buckinghamshire, UK) for either short (10 min, 400 μCi/ml pulse experiments) or long (24 h, 40 μCi/ml) periods. During pulse-chase experiments the medium was supplemented with 5 mM unlabeled mannose and 5 mM unlabeled fucose.[1] At the end of the incubation period the cell homogenates were extracted successively with chloroform-methanol (2:1 v/v) and chloroform-methanol-water (10:10:3, v/v/v). The resulting glycoprotein pellet was extensively digested with 2 mg/ml pronase (grade CB, Calbiochem France, Meudon) at 60°C for 24 h under a toluene atmosphere [12]. Glycopeptides obtained after pronase digestion were fractionated on a column of Bio-Gel P6 (1 × 130 cm, 200–400 Mesh, Bio-Rad France, Paris) in a 0.1 M pyridine acetate buffer, pH 5.0, containing 5 mM sodium azide at a flow rate of 5 ml/h as earlier reported [13]. The void volume and the included volume of the column were determined using bovine serum albumin (BSA) and mannose, respectively. Four fractions (I, II, III, and IV) were usually recovered. The elution of fraction I in the void volume of the column was not due to an incomplete pronase digestion since a second pronase digestion did not change the elution pattern of this fraction. Each fraction was pooled, lyophilized, and then treated with 2.5 mU of endo-beta-N-acetylglucosaminidase H (endo H) from streptomyces griseus (endo H, Miles France, Paris) in 100 μl of 50 mM citrate buffer, pH 5.0, under a toluene atmosphere for 18 h at 37°C [14]. The samples were boiled for 3 min and then fractionated on the same Bio-Gel P6 column as described above [13]. Oligosaccharides released by endo H treatment were further digested with 1.5 U of Jack bean alpha-mannosidase (Boehringer, Mannheim, FRG). In all cases two products were formed that correspond to free tritiated mannose and to the disaccharide mannosyl (beta 1,4) N-acetylglucosamine [13].

Oligosaccharide Chromatography

Oligosaccharides released by endo H treatment were fractionated by HPLC in a Varian model 5000 liquid chromatograph equipped with a column of 5 μ aminospher-isorb (Société Française de Chromatographie, Neuilly-Plaisance, France) as previously reported [15]. Oligosaccharides were fractionated in the presence of ^{14}C labeled oligosaccharide standards. To ascertain the nature of the oligosaccharide that is transferred from the dolichol to the polypeptide chains, the cells were incubated for 5 min with 500 μCi of D-[2-^{3}H]-mannose.

Oligosaccharides linked to the dolichol recovered in the chloroform/methanol/water (10:10:3, v/v/v) extract were analyzed as previously reported [6]. Briefly, oligosac-

[1] All sugars except fucose are of the D-configuration.

charides were released from the dolichol by a mild acid hydrolysis with 0.01 M HCl for 45 min at 90°C [16]. After neutralization and desalting, the oligosaccharides were fractionated on HPLC as described above. In both growing HT-29 Glc^- and HT-29 Glc^+ cells, the oligosaccharide Glc_3-Man_9-$GlcNAc_2$ was shown to be present as previously observed in confluent cells [6].

Conversion of [^{3}H]-Mannose

Tritiated glycopeptides were hydrolyzed either with 0.05 M H_2SO_4 for 8 h at 100°C or with glacial acetic acid and 5 M H_2SO_4 in a ratio 95:5 (v/v) for 4 h at 80°C. The recovery of fucose was similar under both hydrolytic conditions. After desalting, both hydrolysates were spotted and run for 40 h on Schleicher and Schüll 2043b paper in 1-butanol/pyridine/0.1 M HCl (5:3:2, by vol.) [6].

RESULTS

1. The growth and differentiation characteristics of HT-29 cells allow studies on the early events of enterocytic differentiation.

HT-29 cells were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 2.5 mM inosine without (differentiation-permissive conditions) or with (differentiation-non-permissive conditions) 25 mM glucose. Growth curves were established on these two cell populations and are displayed in Figure 1. In

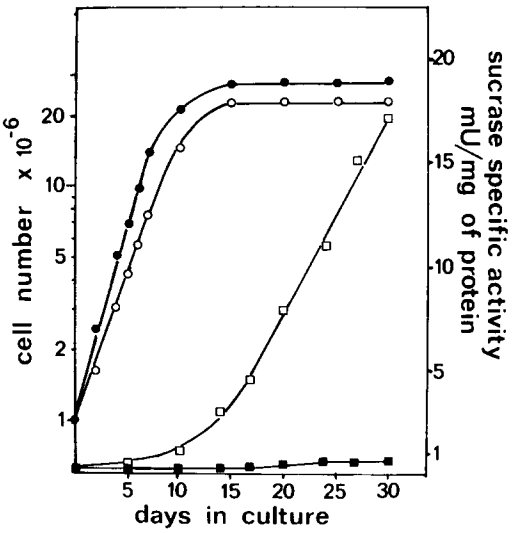

Fig. 1. Growth curve and sucrase-specific activity in HT-29 Glc^- and HT-29 Glc^+ cells as a function of the time in culture. HT-29 Glc^- (○—○) and HT-29 Glc^+ (●—●) cells were seeded at 1×10^6 cells per 25 cm^2 flask and the cell number was counted at day 2,4,5,6,7,8,10,14,17,25, and 30. Results are expressed as the number of cells per 25 cm^2 flask. Note that the growth of the two cell populations is very similar with a doubling time of 20 hours and 26 hours for HT-29 Glc^+ and HT-29 Glc^- cells, respectively. Sucrase-specific activity was measured in HT-29 Glc^- (□—□) and HT-29 Glc^+ (■—■) cells at the indicated times described in Materials and Methods. DPP-IV (EC 3.4.14.5) and APN (EC 3.4.11.2) (not displayed in this figure) gave the same pattern as sucrase (EC 3.3.1.48) i.e., no change in HT-29 Glc^+ cells and a growth dependent increase of their specific activity in HT-29 Glc^- cells.

the same experiment, enzymatic activities were measured as described in Materials and Methods. In HT-29 Glc^- cells, the activities of DPP-IV, APN (not shown), and sucrase (Fig. 1), which were absent or low in the exponential phase of growth, progressively increased after the cells had reached confluency. No such increase could be detected in HT-29 Glc^+ cells.

Immunofluorescence and transmission electron microscopy studies have shown that HT-29 cells are not polarized during the first 6–7 days in culture whatever the glucose content of the culture medium [4]. However, a functional brush border appears after 10 days in HT-29 Glc^- cells, whereas HT-29 Glc^+ cells remain unpolarized (data not shown).

Such a modulatable cellular system allows prediction of how the cells will be differentiated after confluency even though they are undistinguishable, on the basis of the reported criteria, during the exponential phase of growth. Whether these cells can be distinguished at this stage by other criteria, known to be associated with cell differentiation such as the characteristics of N-glycan processing, is further analyzed here.

2. Incorporation of D-[2-^{3}H]-mannose into glycoproteins is lower in undifferentiated HT-29 cells, whatever the phase of growth.

HT-29 Glc^+ and HT-29 Glc^- cells were pulse-labeled for 10 min at day 5 (exponential phase) and at day 20 (stationary phase). Glycopeptides were extracted and purified as described in Materials and Methods. Radioactivity was counted in each sample, and the results are displayed in Table I. The incorporation of D-[2-^{3}H]-mannose in stationary HT-29 Glc^- and HT-29 Glc^+ cells is similar to our previous results [6] with a tenfold lower incorporation in undifferentiated HT-29 Glc^+ cells. This difference is also observed during the exponential phase of growth, except that growing HT-29 Glc^- cells incorporate twice as much D-[2-^{3}H]-mannose into glycoproteins than their confluent counterpart. Therefore the difference between HT-29 Glc^- and HT-29 Glc^+ cells is increased in growing as compared to confluent cells.

3. Undifferentiated HT-29 Glc^+ cells accumulate high-mannose species during the exponential and the stationary phases of growth.

After a 24 h labeling period using D-[2-^{3}H]-mannose, glycopeptides from HT-29 Glc^- and HT-29 Glc^+ cells were extensively digested with pronase and fractionated on a column of Bio-Gel P6 (Fig. 2). In the two cell populations, four peaks were resolved. In each case only peak IV was sensitive to endo-H treatment. Furthermore, this peak exhibited a high affinity for Con A Sepharose, whereas peaks I, II, and III were not

TABLE I. Comparison of the Quantitative Incorporation of D-[2-^{3}H]-Mannose Into Glycoproteins in Both Growing and Confluent HT-29 Glc^- and HT-29 Glc^+ Cells*

	dpm/mg Protein[a]	
	HT-29 Glc^-	HT-29 Glc^+
Growing cells	30,700 ± 3,500	1,500 ± 125
Confluent cells[b]	17,500 ± 2,000	1,700 ± 140

*Whole homogenates were prepared as described in Materials and Methods. Glycopeptides were obtained by a 24 h pronase digestion. The radioactivity of the total glycopeptide fraction was determined on aliquots of the samples to be loaded on the Bio-Gel P6.

[a]Results are given as dpm in the total glycopeptide fraction per mg of protein of the whole cell homogenates and were derived from 3 separate independent 10 min pulse experiments.

[b]Similar results were obtained in another independent set of experiments previously published [6].

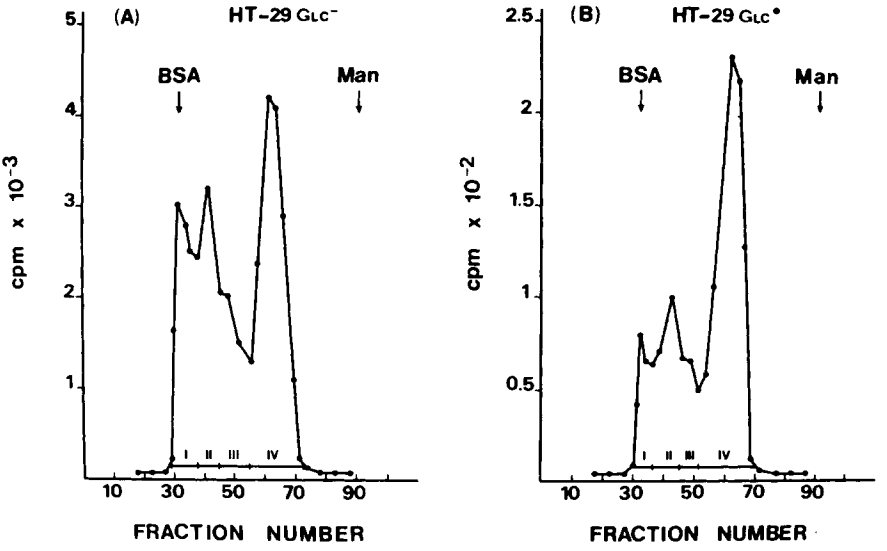

Fig. 2. Gel filtration of tritiated mannose-labeled cellular glycopeptides. Growing HT-29 Glc$^-$ (A) and HT-29 Glc$^+$ (B) cells were incubated for 24 h with 40 μCi/ml of D-[2-^{3}H]-mannose in their respective culture medium. At the end of the labeling period, cell homogenates were prepared, a lipid extraction was performed as described in Materials and Methods. The resulting pellet was exhaustively digested with 2 mg/ml of pronase for 24 h. The glycopeptides obtained were fractionated on a column of Bio-Gel P6 (1 x 130 cm) eluted with 0.1 M pyridine acetate, pH 5.0. One ml fractions were collected. The radioactivity of 100 μl aliquots was determined, and fractions I, II, III, and IV were pooled separately and freeze-dried. The void and included volumes were determined using bovine serum albumin (BSA) and mannose (Man), respectively.

TABLE II. Comparison of the Distribution of [^{3}H]-Mannose Labeled Glycopeptides Derived From Bio-Gel P6 Separation*

	HT-29 Glc$^-$ (%)[b]		HT-29 Glc$^+$ (%)[b]	
	Growing	Confluent[a]	Growing	Confluent[a]
Fraction I	18	25	14	14
Fraction II	24	17	20	9
Fraction III	11	20	6	14
Fraction IV	47	38	60	63

*Growing and confluent HT-29 Glc$^-$ and HT-29 Glc$^+$ cells were labeled and the glycopeptides were prepared as detailed in Figure 2.

[a]Similar results were obtained in another independent set of experiments previously published [6].

[b]Results are expressed as the percentage of each glycopeptide species relative to the total radioactive glycopeptides recovered on the Bio-Gel P6 column.

retained on this immobilized lectin (data not shown). Distribution of ^{3}H-mannose glycopeptides from growing HT-29 Glc$^-$ and HT-29 Glc$^+$ cells obtained in Figure 2 was compared to the values derived from the corresponding confluent cells (Table II). Whatever the situation considered (i.e., growth or confluency) HT-29 Glc$^+$ cells exhibited a higher percentage of radioactivity in high-mannose fraction than HT-29 Glc$^-$ cells.

4. The alteration of high-mannose processing is an early event in undifferentiated HT-29 Glc$^+$ cells.

In order to explain the accumulation of high-mannose species in HT-29 Glc$^+$ cells, the N-glycan processing in exponentially growing HT-29 Glc$^-$ and HT-29 Glc$^+$ cells was followed using pulse-chase experiments. Previous studies by us [6] and others [17] have shown that post-confluent differentiated cells display a high level of ^{3}H-mannose conversion into ^{3}H-fucose. This is also true for exponentially growing cells (Table III). Taking into account the high level of ^{3}H-mannose conversion into ^{3}H-fucose, the pulse-chase experiments were therefore carried out in the presence of 5 mM unlabeled fucose, a condition in which no more conversion could be detected.

After a 10 min pulse with D-[2-^{3}H]-mannose, the cells were incubated with 5 mM unlabeled mannose and 5 mM fucose for various periods of time. At each time the radioactivity associated with lipid-linked oligosaccharides (Fig. 3A,B), high-mannose glycopeptides (Fig. 3C,D), and complex glycopeptides (Fig. 3E,F) was measured. The nature of the radioactivity associated with labeled glycopeptides was analyzed and was found to be exclusively associated with mannose. In HT-29 Glc$^-$ cells the fate of ^{3}H-mannose was similar to what was expected for a classical pathway of N-glycosylation [18].

The biosynthesis of labeled lipid-linked oligosaccharides and their transfer to high-mannose species was qualitatively similar in both growing HT-29 Glc$^-$ and HT-29 Glc$^+$ cells (Fig. 3A–D). In contrast, a low level of radioactivity was recovered in complex N-glycans from HT-29 Glc$^+$ as compared to HT-29 Glc$^-$ cells (Fig. 3E,F). Therefore, the ratio: complex/high-mannose N-glycans was lower in growing HT-29 Glc$^+$ than in growing HT-29 Glc$^-$ cells. A comparison with the results derived from confluent cells [6] indicates that whatever the phase of growth, the complex N-glycan biosynthesis is similarly impaired in HT-29 Glc$^+$ cells (Fig. 3G,H). In contrast, the biosynthesis of complex N-glycans is not altered in either growing or post-confluent HT-29 Glc$^-$ cells.

High-mannose species were studied in the endo H sensitive fractions from the 24 h labeling experiments. Results of high performance liquid chromatography (HPLC) analysis of the different high-mannose types are displayed in Table IV. These results clearly demonstrate that growing HT-29 Glc$^+$ cells accumulate Man_{9-8}-$GlcNAc_2$ species in the same range than the corresponding confluent cells. Therefore the mechanism underlying the impairment of N-glycan trimming in growing HT-29 Glc$^+$ cells seems to be due to an accumulation of Man_{9-8}-$GlcNAc_2$ glycopeptides, as previously shown in confluent HT-29 Glc$^+$ cells.

TABLE III. Percentage of Conversion of D-[2-^{3}H]-Mannose Into [^{3}H]-Fucose During a 24 h Labeling Experiment of Growing HT-29 Glc$^-$ and HT-29 Glc$^+$ Cells in the Different Glycopeptide Species Resolved by a Bio-Gel P6 Column Chromatography

	%[a]	
	HT-29 Glc$^-$	HT-29 Glc$^+$
Fraction I	57	66
Fraction II	69	50
Fraction III	60	63
Fraction IV	0	0

[a]Results are expressed as the percentage of radioactivity found in fucose after a 24 h labeling period using D-[2-^{3}H]-mannose as precursor.

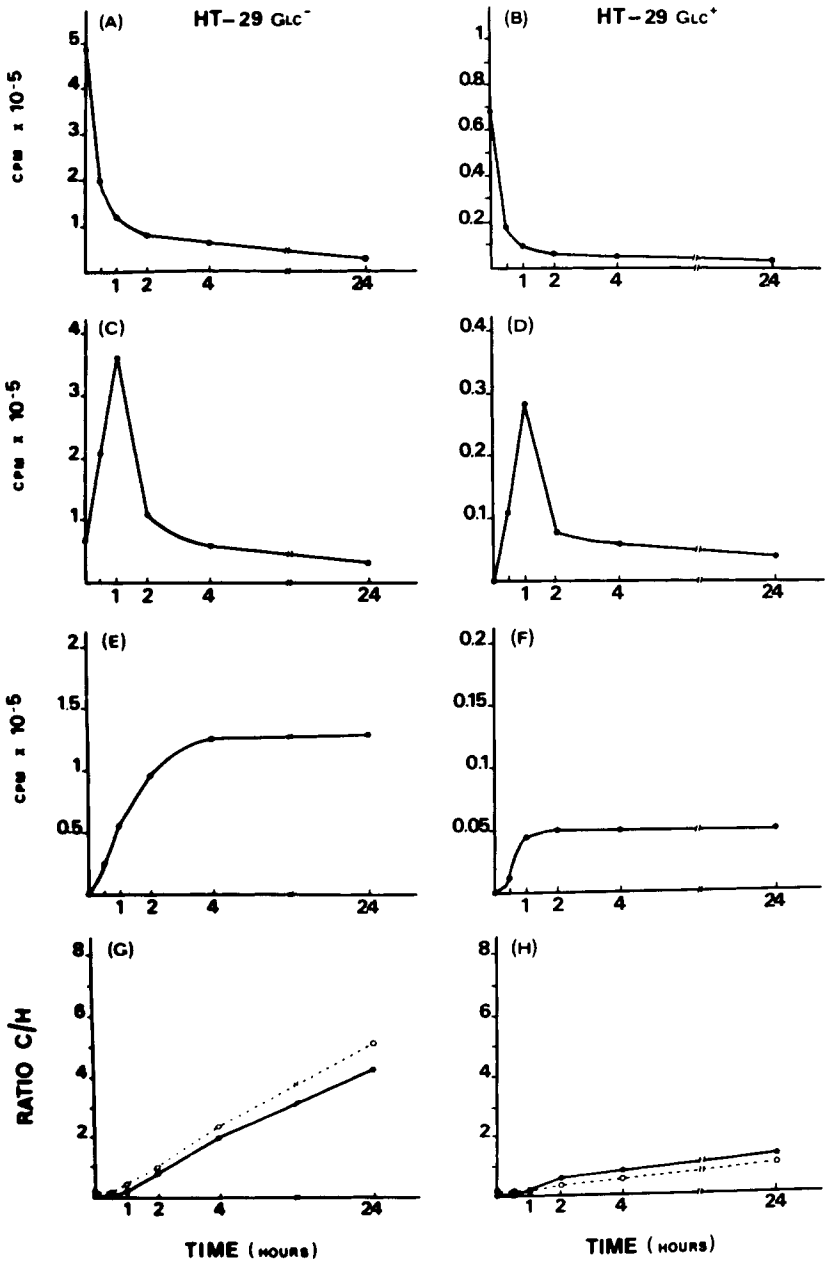
(A) HT-29 Glc⁻
(B) HT-29 Glc⁺
(C)
(D)
(E)
(F)
(G)
(H)
CPM x 10⁻⁵
RATIO C/H
TIME (HOURS)
TIME (HOURS)

Fig. 3.

TABLE IV. Comparison of Labeled High-Mannose Oligosaccharides After HPLC Fractionation in Growing and Confluent HT-29 Glc⁻ and HT-29 Glc⁺ Cells

Oligosaccharide species[a]	HT-29 Glc⁻		HT-29 Glc⁺	
	Growing	Confluent[b]	Growing	Confluent[b]
Man_{9-8}-GlcNAc[c]	44	34	60	57
Man_7-GlcNAc	16	18	12	18
Man_6-GlcNAc	25	19	16	20
Man_5-GlcNAc	14	29	12	5

[a]The results are given as molecular species, i.e., each value corresponds to the ratio:

$$\frac{\text{Radioactivity measured in the considered fraction}}{\text{number of mannose units}}$$

The results represent the percentage of each molecular species relative to the total of the high-mannose oligosaccharide species.
[b]Similar results were obtained in another independent set of experiments previously published [6].
[c]The sum of Man_9-GlcNAc plus Man_8-GlcNAc species, resolved by HPLC, is displayed in order to clarify the presentation of the results.

DISCUSSION

The results presented in this paper demonstrate that the N-glycan processing is independent of the phase of growth of HT-29 cells but strongly depends on the ability of cells to differente when they reach confluency. In HT-29 cells grown in differentiation-permissive conditions, there is a normal transfer of labeled oligosaccharides from the lipid donor to the acceptor peptides. The high-mannose species are then quantitatively converted to complex glycopeptides, demonstrating that these cells exhibit a classical N-glycan processing, whatever the phase of growth. In contrast, HT-29 cells grown in differentiation non-permissive conditions do not synthesize high amounts of complex N-glycopeptides in both the exponential phase and the stationary phase of growth. From these results it can be concluded that alterations in N-glycan processing are present very early in HT-29 Glc⁺ cells, i.e., in cells that will not differentiate.

Fig. 3. Fate of tritiated mannose incorporated in the different cell populations during a pulse-chase experiment. Growing HT-29 Glc⁻ (**left panel**) and HT-29 Glc⁺ (**right panel**) were pulsed for 10 min with 400 μCi/ml of D-[2-^{3}H]-mannose, as described in Materials and Methods, in their respective culture medium. The chase was performed for 0.5, 1, 2, 4, and 24 h in the presence of 5 mM unlabeled mannose and 5 mM unlabeled fucose in the original medium. At each time, the lipid-linked oligosaccharides were extracted with chloroform/methanol/water (10:10:3, by vol), dried under a stream of nitrogen, and the radioactivity of this lipid fraction determined (**A,B**). The glycopeptides obtained after pronase digestion were fractionated on a column of Bio-Gel P6. Fractions exhibiting the same Kav as fraction IV (high-mannose glycopeptides) were treated with endo H. The radioactivity recovered in the high-mannose fraction (fraction IV, endo H-sensitive) was plotted (**C,D**). The sum of the radioactivity recovered in the complex glycopeptides (fractions I, II, and III) was plotted in **E,F**. At each time the ratio of the radioactivity in complex glycopeptides/high-mannose glycopeptides (C/H) was derived from data in C–F. The values of this ratio are shown in **G,H**. In the bottom row of the figure, the dotted lines correspond to the results obtained previously with the corresponding confluent cells [6]. In each graph, the 0 of the abscissa corresponds to the beginning of the chase after a 10 min pulse. Note that an efficient transfer of high-mannose-associated radioactivity occurs from the lipid donor to the protein backbone in both cell populations. In contrast, the conversion of high-mannose into complex type chains is greatly reduced in HT-29 Glc⁺ as compared to HT-29 Glc⁻ cells, whatever the growth phase.

It should be pointed out that there is a higher proportion of high-mannose species in growing as compared to confluent differentiated HT-29 Glc^- cells. These results are consistent with previous findings by other groups on several cell systems [19–21], i.e., that there is a higher proportion of high-mannose species in growing or low density as compared to confluent or high-density cells. However, in growing HT-29 Glc^- cells, the higher proportion of high-mannose species is not associated with an impairment of their processing to complex glycopeptides. This would strongly suggest that besides the growth-related change in the proportion of high-mannose glycoproteins, which has also been observed in previously published studies [22–24], there is another phenomenon, namely the impairment of the N-glycan processing that is directly related to the differentiation program of HT-29 cells. In these cells, the step that appears to be defective is the conversion of Man_{9-8} $GlcNAc_2$ into Man_{7-6-5} $GlcNAc_2$ species. The fact that this conversion is severely decreased in both growing and confluent HT-29 Glc^+ cells raises the question of the regulation of this enzymatic reaction. Further studies should investigate whether the observed alterations in the N-glycan processing result from a genetic control or from an early metabolic regulation.

Our results demonstrate that although the two cell populations are indistinguishable during the exponential phase of growth according to morphological and enzymatical criteria, it is still possible to distinguish them by using a biochemical tool, namely the N-glycan processing. Therefore, at least in HT-29 cells, the N-glycan processing appears to be a sensitive and early biochemical probe of the ability of these cells to differentiate. Whether changes in the N-glycan processing can be used in other systems as a marker for cell commitment remains to be analyzed.

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