Glycoconjugate Journal (1996) **13:** 115–121

Differentiation-dependent expression of human β-galactoside α2,6-sialyltransferase mRNA in colon carcinoma CaCo-2 cells

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Received 5 June 1995, revised 10 August 1995

We have previously documented a dramatic elevation in the activity of α2,6-sialyltransferase towards Galβ1,4GlcNAc (EC 2.4.99.1) (α2,6ST) in CaCo-2 cells maintained in culture for several days after confluence to elicit a high degree of enterocytic differentiation phenotype. Northern analysis performed with a probe complementary to a region of human α2,6ST mRNA common to all known transcripts demonstrated that the expression of α2,6ST mRNA in CaCo-2 cells increased with the degree of differentiation. When probes complementary to 5′-untranslated exons (Y + Z or X) previously identified in transcripts isolated from human placenta and from several human lymphoblastoid cell lines were used, no hybridization signal with mRNA of CaCo-2 cells was found, as reported for the mRNA of hepatoma cell line HepG2 (Wang XC, Vertino A, Eddy RL, Byers MG, Jani-Sait SN, Shows TB, Lau JTY (1993) *J Biol Chem* **268**: 4355–61). These results support the notion that the major α2,6ST transcript of CaCo-2 cells was the hepatoma isoform or a new one, so far unreported. Consistent with the differentiation-dependent increase in α2,6ST-mRNA expression, an elevation of the reactivity with *Sambucus nigra* agglutinin of differentiated CaCo-2 cell-surface was observed, indicating an enhanced α2,6-sialylation of membrane glycoconjugates.

Keywords: α2,6-sialyltransferase, CaCo-2 cells, enterocyte-differentiation, α2,6-sialylation, *Sambucus-nigra* agglutinin

Abbreviations: α2,6ST, Galβ1,4GlcNAc α2,6-sialyltransferase; α2,3ST, Galβ1,4GlcNAc α2,3-sialyltransferase; α2,3(O)ST, Galβ1,3GalNAc α2,3-sialyltransferase; SNA *Sambucus-nigra* agglutinin; Dig-SNA, digoxigenin-conjugated *Sambucus-nigra* agglutinin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ALP, alkaline phosphatase; DPP-IV, dipeptidylpeptidase-IV; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; BSA, bovine serum albumin.

Introduction

The addition of sialic acid to glycoproteins and glycolipids occurs through the action of sialyltransferases, a family of 10–12 or more enzymes which can be distinguished by their catalytic specificity for acceptor structures and the isomeric linkage formed by the sialic acid with the glycan acceptor [1]. On N-linked glycans of glycoproteins, formation of sialyl linkages are usually mediated by two different sialyltransferases: β-galactoside α2,6 sialyltransferase (EC 2.4.99.1) (α2,6ST) and β-galactoside α2,3 sialyltransferase (EC 2.4.99.6) (α2,3ST) [2]. α2,6ST Expression differs dramatically in various tissues; within the same tissue remarkable changes have been observed during development and cell differentiation [3–10]. In previous works, we have demonstrated that in human and rat intestine α2,6ST activity is onco-developmentally regulated. Indeed: (i) in human colorectal cancer specimens the enzyme activity is usually higher than that assayed in adjacent colon mucosa [11]; (ii) in cells from fetal and newborn rat colon the level of activity is particularly high but dramatically declines at the

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weaning time [10, 12]; (iii) in rat small-intestine the maximum of activity is detectable in poorly differentiated cells of the crypt bottom [12]; (iv) in human colon carcinoma CaCo-2 cells the cell-associated and medium-released α2,6ST activity increases with the degree of cell differentiation [13]. As a consequence of the increased α2,6ST activity, colon cancer tissues actually express an increased degree of α2,6-sialylated membrane glycoconjugates [14, 15]. This modification might be crucial for the ability of tumour cells to invade the neighbouring tissues and to form metastasis. In fact, colon cancer cells with a high expression of α2,6 sialylated glycans are more tumorigenic and metastatic in mice [16–19], and colon primary tumours express α2,6ST activity lower than that of metastatic disseminations [20].

Recently, the expression of human α2,6ST mRNA in various cell types has been investigated by Wang *et al.* [21], resulting in the proposal that tissue-specific expression of α2,6ST depends on the use of alternative promoters. A large transcript, containing two 5′-untranslated exons (Exon Y+Z), has been isolated from several human cell types and very likely it represents the 'constitutive' isoform of human α2,6ST mRNA [21, 22]; a distinct transcript containing the 5′-untranslated exon X, but not exons Y+Z, has been isolated from various human lymphoblastoma cell lines of the B-lineage [21, 23]; finally, none of these exons was found to be present in the major transcript isolated from human hepatoma HepG2 cells [21, 24].

To our knowledge, neither the expression of the α2,6ST mRNA in human intestinal cells nor its regulation during the differentiation process has been investigated. To investigate this point, we took advantage of the fact that human colon carcinoma CaCo-2 cells differentiate spontaneously *in vitro* when maintained in culture after the confluence for an extended period of time [25]. In fact, post-confluent CaCo-2 cells exhibit apical/basolateral polarity and an elevated expression of brush border hydrolases [25, 26]. In this study, we report that the differentiation-dependent increase in α2,6ST activity is associated with an accumulation of an mRNA isoform lacking exons Y+Z and exon X and that such an accumulation resulted in an increased expression of α2,6-sialylated glycans on the cell surface.

Materials and methods

Materials

N-Acetyllactosamine (Galβ1,4GlcNAc), Galβ1,3GalNAc-*O*-benzyl, unlabelled CMP-NeuAc and human transferrin were from Sigma. CMP-[^{14}C]NeuAc and [^{32}P]ATP were from Amersham (Aylesbury, UK). 3′-sialyllactosamine and 6′-sialyllactosamine were from Oxford GlycoSystems. [^{14}C]NeuAcα2,3Galβ1,3GalNAc-*O*-benzyl was synthesized in our laboratory from Galβ1,3GalNAc-*O*-benzyl and CMP-[^{14}C]NeuAc by using as an enzyme source the cell extract of a subpopulation of HT-29 cells which express a high α2,3(O)sialyltransferase activity. Digoxigenin-conjugated *Sambucus-nigra* agglutinin (Dig-SNA) and digoxigenin detection kit were from Boheringer (Mannheim, Germany).

Cells

Human colon cancer CaCo-2 cells (gift from Dr Giovanna Bises, Istituto Nazionale della Nutrizione, Rome, Italy) were routinely grown in DMEM (Gibco, Paisley, UK) supplemented with 20% FCS containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were seeded at a density of 8×10^4 cells cm^{-2}, refed three times a week, and harvested either just after the confluence (confluent cells) or after 3–4 weeks of maintaining in culture when many domes (about 40 cm^{-2}) were visible in the layer (post-confluent cells). After two washings with PBS, cells were detached with a cell scraper, collected by centrifugation and conserved at −80 °C until use. Culture conditions of human hepatoma HepG2 cells and of human B lymphoblastoid cell line Louckes were reported elsewhere [21].

Differentiation marker enzymes

Alkaline phosphatase (ALP) activity was determined in cell homogenates by the ALP Kine test (p-nitrophenylphosphate kinetic method) as described by the manufacturer (Sclavo Diagnostic, Milan, Italy). Dipeptidyl peptidase IV (DPP-IV) activity was determined in cell homogenates according to Nagatsu *et al.* [27] using glycyl-L-proline-4-nitroanilide as substrate. For the sucrase activity, the cell membranes were prepared by subjecting the homogenate to centrifugation at 100 000 × *g* for 1 h; the method was that described by Dahlqvist [28]. Proteins were determined by the Lowry method [29] with BSA as standard.

Sialyltransferase assays

Sialyltransferase activities were measured in the whole cell homogenates in the range of linearity with respect to time and enzyme concentration according to published procedures as detailed below. Radioactivity incorporated on endogenous acceptors (i.e. in the absence of exogenous acceptors) was subtracted. α2,3ST and α2,6ST: 80 mM Na-cacodylate buffer, pH 6.5, 10 mM $MnCl_2$, 0.5% Triton X-100, 250 μg (13 mM) of *N*-acetyllactosamine, 80 μM CMP-[^{14}C]NeuAc (37 dpm pmol^{-1}), and 50–100 μg of homogenate proteins in a final volume of 50 μl. After 2 h of incubation at 26 °C, samples were diluted with 1 ml of water, boiled for 3 min in order to degrade CMP-NeuAc and filtered through a 0.45 μm membrane. Separation of NeuAcα2,3Galβ1,4GlcNAc from the corresponding NeuAcα2,6- isomer was achieved by HPLC as previously described [19].

α2,3(O)ST: assay conditions were as above but 5 mM Galβ1,3GalNAc-*O*-benzyl was used as an acceptor. After 1 h at 37 °C, reactions were stopped by the addition of 1 ml of water, boiled and filtered as above. The reaction mixture was analysed by HPLC on an NH_2 Lichrosorb column (Merck, Darmstadt, Germany) eluted isocratically in 80:20 acetonitrile:KH_2PO_4 and the radioactivity coeluting with authentic NeuAcα2,3Galβ1,3GalNAc-*O*-benzyl was taken as a measure of the enzyme activity.

Kinetics studies

Homogenates of confluent and post-confluent cultures of CaCo-2 cells were used as enzyme sources. Both homogenates were used at the same protein concentration. The acceptor substrate was asialotransferrin and the conditions those previously reported [11] with 275 000 dpm of CMP-[^{14}C]NeuAc. CMP-NeuAc concentration ranged between 10 and 640 μM. Incubation was for 1 h at 26 °C. The radioactivity incorporated on endogenous substrates was subtracted. Intercepts with X and Y axis of the Lineweaver-Burk plot were calculated by the least square method.

Northern analysis

Total RNA was extracted from cell pellets by the guanidine isothiocyanate method. Ten μg of each sample were electrophoresed on a 1% agarose gel containing 6.4% formaldehyde [30], transferred to Zetabind (Cuno Laboratories, Inc.) nylon membrane and subjected to standard blot analysis [31]. The [^{32}P]-labelled probe for coding region of human α2,6ST was generated by single strand PCR amplification of a portion of the coding region of exon II of human α2,6 ST cDNA. The PCR reaction contained 1 μl of a *Eco* RI/*Eco* RI fragment spanning two-thirds of human α2,6ST coding domain cloned into Blue Script SK+ (clone pBSHS1) as template, 20 pmol of oligonucleotide HST-p4 (5′-AACTTGATGCCTGGTCC-3′) as primer, 50 μCi α-[^{32}P]dATP, 5 nmol each dCTP, dGTP, dTTP and 2 U of Taq polymerase in a final volume of 20 μl. PCR amplification was performed for six cycles of a step programme (95 °C 1 min, 37 °C 1 min, 72 °C 1 min). Labelled probes for exons X and Y+Z were prepared by PCR amplification as above using PCR-amplified fragments of the appropriate cDNA clones as templates [21]. Oligonucleotides HL-p20 (5′-TAG-TAGTTCTACGACGT-3′) and HL-p7 (5′-CTCAGCAC-GGGGATGGACAC-3′) were used as primers for X and Y+Z, respectively.

Staining with digoxigenin-conjugated SNA

Confluent or post-confluent CaCo-2 cells, grown on glass coverslips, were washed twice with PBS, then fixed with 2% paraformaldehyde, 0.1% glutaraldehyde in PBS for 1 h at room temperature [32]. Cells were stained with 1 μg ml^{-1} Dig-SNA as described [15].

Results

Differentiation of CaCo-2 cells

In order to verify that our cultures of CaCo-2 cells maintained in culture for 25 days after the confluence (post-confluent cells) had differentiated, we compared the activities of ALP, sucrase and DPP-IV of post-confluent cells with those of cells harvested at confluence (confluent cells) which are considered to be poorly differentiated cells. The remarkably enhanced activity of the three hydrolytic enzymes as well as the high density of domes in the monolayer of post-confluent cells indicated that these cells had actually reached well differentiated status (Table 1).

Sialyltransferase activities

Consistent with previous results [13], the α2,6ST activity was found to increase several fold upon cell differentiation, whereas the α2,3ST activities acting on Galβ1,4GlcNAc and on Galβ1,3GalNAc-*O*-benzyl remained unchanged (Table 2). These results support the notion that a specific differentiation-dependent regulation of α2,6ST occurs in this colon cancer line.

Expression of α2,6ST mRNA

In order to ascertain whether the elevated activity of α2,6ST in well differentiated CaCo-2 cells was dependent on a higher expression of the mRNA, a blot of RNAs from confluent and post-confluent CaCo-2 cells was hybridized with a probe complementary to the exon II of human α2,6ST mRNA, which is common to all known isotypes of transcripts [21]. As Fig. 1A shows, a weak hybridization signal was observed in mRNA from confluent cells (undifferentiated cells) whereas the hybridization to mRNA of post-confluent cells (differentiated) was much stronger; in this case the signal intensity was similar to that obtained with mRNA of human hepatoma cells (HepG2) and of one lymphoblastoid cell line (Louckes),

Table 1. Brush-border enzyme activities in confluent and post-confluent CaCo-2 cells.

Enzyme	*Confluent cells*	*Post-confluent cells*
	(mU per mg protein)	
DPP-IV	86.6 ± 15.1	483.3 ± 129.9
ALP	57 ± 4.0	170 ± 15.6
Sucrase	8.3 ± 2.7	52.8 ± 13.8

The activities of DPP-IV, ALP and sucrase were determined as described in the text and expressed as mU per mg of proteins. One unit of DPP-IV and sucrase is defined as the activity that hydrolyses 1 μmol of substrate per min at 37 °C. ALP activity is expressed in arbitrary units. Values are means ± SD of three different cell cultures.

Table 2. Sialyltransferase activities in confluent and postconfluent CaCo-2 cells.

Enzyme	*Product*	*Transferase activity*	
		Confluent cells	*Post-confluent cells* (*pmol mg^{-1} h^{-1}*)
Gal:α2→6(N)ST	NeuAcα2,6Galβ1,4GlcNAc	850	9230
Gal:α2→3(N)ST	NeuAcα2,3Galβ1,4GlcNAc	251	237
Gal:α2→3(O)ST	NeuAcα2,3Galβ1,3GalNAcα-Bn	206	210

Transferase activities were assayed in duplicate using homogenates as the enzyme source as described in the Experimental section. The values of transferase activities correspond to the incorporation into exogenous acceptor minus that of the endogenous substrate. Means of two different batches of cells.

both of which are known to express high levels of α2,6ST mRNA [21]. No difference in the signal with β-actin probe was observed in mRNA from undifferentiated and differentiated cells.

As mentioned in the Introduction three isotypes of human α2,6ST mRNA have been isolated which diverge for the presence or absence of exons upstream from the translated sequence. In Louckes cells the transcript with the untranslated exon X is the major one but even the transcript containing exons Y+Z is present [21]. To obtain information on the mRNA isotype(s) of CaCo-2 cells two blots identical to that shown in Fig. 1A were hybridized with probes complementary to either exons Y+Z or X (Fig. 1B and C). As expected, RNA from Louckes cells gave a strong hybridization signal with probe for X and a weak signal with probe for Y+Z. In contrast, RNA from undifferentiated and differentiated CaCo-2 cells failed to hybridize with either probe, similar to the transcript of HepG2 cells in which exons Y+Z or X have not been found to be present [21].

We compared the results of densitometry analysis of Northern blot shown in Fig. 1A (normalized with the internal β-actin standard) with the α2,6ST enzyme activity measured in cell homogenates of the same cell preparation used for RNA extraction. The post-confluent cells seem to have a greater proportion of enzyme activity relative to message than the other cells (Fig. 2).

Kinetics of α2,6ST

Elevation in α2,6ST mRNA level in differentiated CaCo-2 cells suggests that an increased synthesis of α2,6ST is the basis of the increased activity. To gain insights into this point, we compared the kinetic parameters of α2,6ST in undifferentiated and differentiated CaCo-2 cells. As shown in Fig. 3, the enzyme from the differentiated and undifferentiated cells displays very similar K_m values (90 and 77 μM). On the contrary, the V_{Max} of the enzyme from differentiated cells was remarkably higher (270 pmol h^{-1} *versus* 27 pmol h^{-1}). These data are consistent with the hypothesis that differentiated CaCo-2 cells contain a higher number of enzyme molecules qualitatively identical to those of undifferentiated cells.

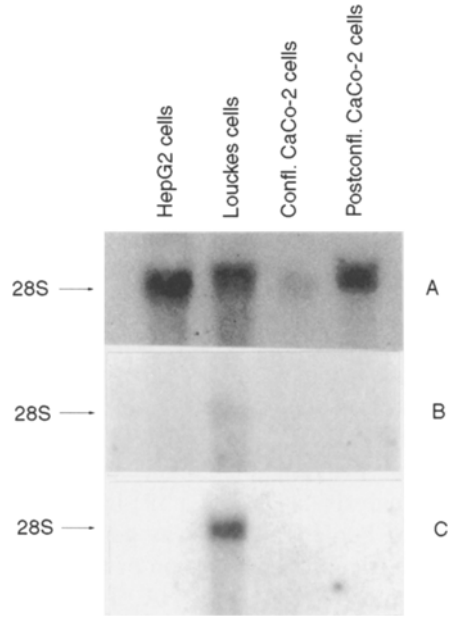

Figure 1. Expression of α2,6ST mRNA in undifferentiated CaCo-2 cells (confluent cells), differentiated CaCo-2 cells (post-confluent cells), HepG2 cells and Louckes cells. Northern blots with RNA from the indicated cells were hybridized with a probe for the coding region of exon II of human α2,6ST (Panel A). Two blots like that of panel A were hybridized with probes for exon Y+Z (Panel B) and exon X (Panel C). The position of 28S ribosomal RNA is indicated.

Reactivity of CaCo-2 cells with Sambucus nigra agglutinin

To assess whether the increased expression of α2,6ST in differentiated CaCo-2 cells resulted in a concomitant modification of membrane glycoconjugates, the presence of α2,6-sialylated sugar chains on confluent and post-confluent cultures of CaCo-2 cells was investigated using the NeuAcα2,6Gal/GalNAc-specific lectin SNA conjugated with the hapten digoxigenin [33]. As Fig. 4A reveals, the Dig-SNA reactivity of confluent CaCo-2 cells was, in general, very weak. In contrast, layers of post-confluent CaCo-2 cells (Fig. 4B) were heavily stained by Dig-SNA. The non-uniform pattern of staining indicates

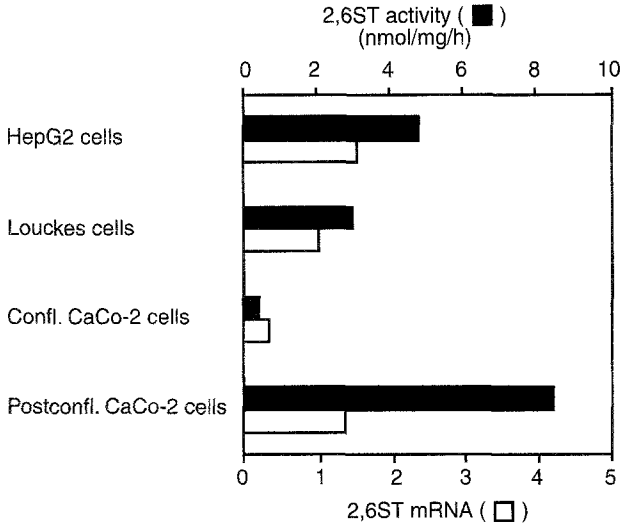

Figure 2. Comparison between the α2,6ST activity and the expression of the corresponding mRNA. α2,6ST activity was measured in homogenates of the same cell preparations used for the RNA extraction as described in the text. The intensity of the bands of Fig. 1A was normalized to that of the corresponding β-actin mRNA.

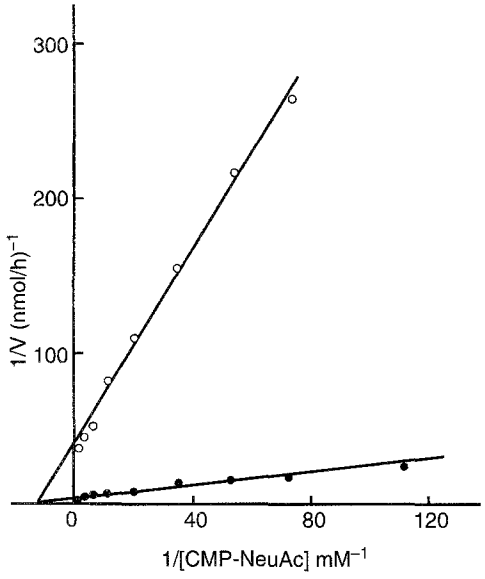

Figure 3. Kinetic analysis of α2,6ST. Intercepts with X and Y axis of the Lineweaver-Burk plot were obtained mathematically by varying the donor substrate concentration as described in the Methods section. Enzyme sources were homogenates of confluent (○) and post-confluent CaCo-2 cells (●).

that α2,6-sialylated sugar chains are not evenly expressed by CaCo-2 cells and suggests that even the α2,6ST activity is not uniformly distributed among the cells of the culture. In both confluent and post-confluent cultures of CaCo-2 cells, Dig-SNA reactivity is clearly associated with cell membranes but a fine, granular staining in the cytoplasm may also be observed.

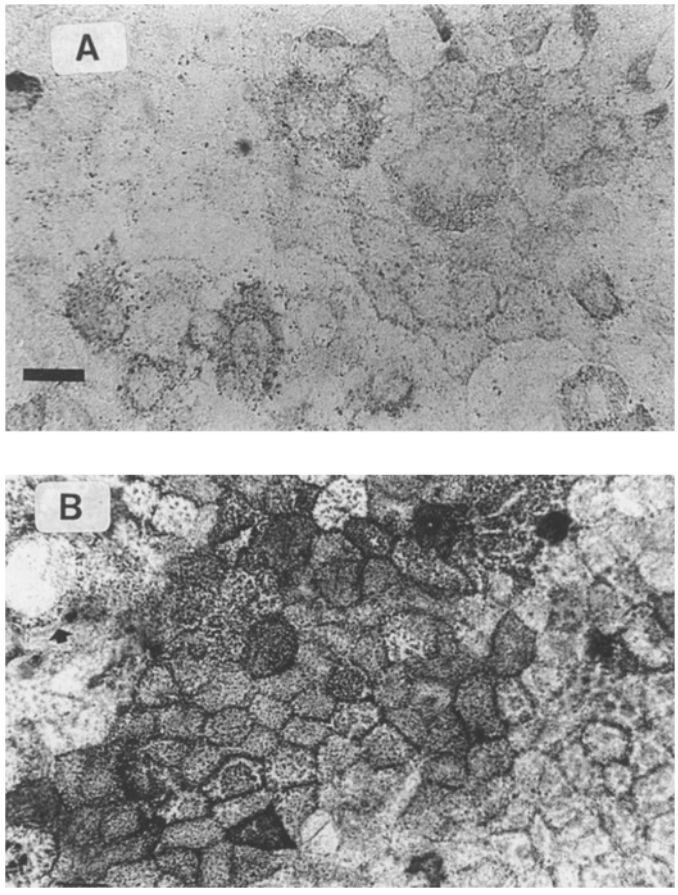

Figure 4. Dig-SNA staining. Confluent (A) and post-confluent CaCo-2 cells (B) were stained with digoxigenin-conjugated SNA. A small dome (arrow) is visible in the monolayer of post-confluent cells. Pictures were taken at the same magnification. Scale bar 30 μm.

Discussion

Current results indicate that mRNA accumulation is the cause of the differentiation-dependent enhancement of α2,6ST activity in differentiated CaCo-2 cells. This is probably a consequence of the transcriptional activation of the gene resulting in an increased number of sialyltransferase molecules. Consistent with this notion, we found that upon differentiation the V_{max}, but not the K_m, of α2,6ST for the donor substrate was increased ten-fold (27 *versus* 270 pmol h^{-1}). Previously, we have established that the K_m for the donor substrate of α2,6ST from some human colon-carcinoma specimens is 50 μM [11]. This value is very close to that calculated here for α2,6ST of CaCo-2 cells.

A good correlation between the α2,6ST activity and α2,6-sialylation of cell membranes had previously been reported in various colon cancer cell lines and in colon cancer tissues [14, 15, 19, 34]. Similarly, the differentiation of CaCo-2 cells results in an elevation of the

reactivity with *Sambucus nigra* agglutinin of cell membranes, indicating an enhanced α2,6-sialylation of membrane glycoconjugates. Post-confluent CaCo-2 cells display a non-uniform pattern of Dig-SNA reactivity. This observation is consistent with those indicating that other markers of differentiation, such as sucrase-isomaltase, are expressed according to a mosaic pattern in late post-confluent CaCo-2 cells [35].

Current results also reveal that the α2,6ST mRNA from CaCo-2 cells does not hybridize with probes for exons Y+Z and X. Although, on the basis of Northern analysis it cannot be excluded that minor amounts of transcript(s) containing the Y+Z or X were present, our results support the notion that the major 'CaCo-2 transcript' does not correspond to the constitutive form but is similar to the HepG2-cell transcript or is a fourth form, so far unreported. To date, there is no information on the structure of the α2,6ST transcript in human intestinal cells. In rat the expression has been extensively studied as a function of the development in both liver and intestinal cells [10]. At birth, rat liver expresses low level of a larger 4.7 kb transcript (representing the constitutive form of rat α2,6ST mRNA) [10], whereas at the weaning time, concomitant with the large increase of the α2,6ST level, and in adult liver the expression of a 4.3 kb transcript becomes predominant [9, 10]. In rat small intestine an opposite pattern of developmentally regulated α2,6ST expression occurs: the level of the enzyme is very high before weaning and is associated with the expression of the 4.3 kb transcript, but it drops to a very low level after weaning [10] and in adult rat intestine only the 4.7 kb form has been identified [9]. These results indicate that in rat both the amount and size of the α2,6ST mRNA change during the development process and that the expression of 4.3 kb and 4.7 kb transcript is associated with a high and low level of α2,6ST, respectively. Since the genomic organization and transcriptional mechanisms of α2,6ST in human and rat are apparently highly conserved, it can be suggested that the expression of a transcript lacking the exons Y+Z or X represents the intestinal-restricted production of α2,6 ST mRNA at a defined-stage of enterocyte differentiation. It has been proposed that poorly differentiated CaCo-2 cells correspond to cells of embryonic intestine, whereas well differentiated CaCo-2 cells correspond to those of fetal colon [36]. The reappearance of fetal phenotypes is one of the more common events in tumour cells, thus it is possible that colon cancers express the 'CaCo-2 transcript' and that the high α2,6ST enzyme activity assayed in differentiated CaCo-2 cells and in several colon cancers is associated with the enhanced expression of such a transcript. The level of other glycosyltransferases appears to be regulated during cell differentiation by the expression of different transcripts diverging at the 5′-end because they are initiated by different promoters. A good example has been offered by the research of Shaper's group on β1,4-galactosyltransferase in mammary gland during lactation and during murine spermatogenesis [37, 38].

As for the regulation of α2,6ST expression during the differentiation of intestinal cells, it should be considered that these cells may differentiate along two main lineages: enterocytes and mucus-secreting cells. Lesuffleur *et al.* [39, 40] carried out an *in vitro* model of differentiation along the mucus-secreting lineage of colon carcinoma HT-29 cells based on the resistance to 10^{-5} M methotrexate treatment. We observed that α2,6ST activity drops dramatically in 10^{-5} M methotrexate-resistant HT-29 cells in comparison to untreated cells [41]. All together these results suggest that the expression of such a glycosyltransferase does not merely depend on the differentiation process, but is related to the achievement of structural and functional specializations which accompany the maturation of distinct populations of intestinal cells.

Even though aberrant expression of glycosyltransferases and the concomitant alteration of membrane carbohydrates are very common events in tumour cells [42], very little is known about the molecular mechanisms which link the expression of glycosyltransferases with that of genes involved in the regulation of cell proliferation, namely oncogenes and tumour suppressor genes. Colon carcinogenesis is a multi-step process comprising several mutation events [43, 44]; one is represented by the activation of the *K-ras* oncogene [45]. A possible link between neoplastic transformation and α2,6ST expression is provided by the observation that transformation of mouse fibroblast by activation with *ras* oncogenes results in an increased expression of α2,6ST mRNA and enzyme activity [46, 47].

Further investigations are required to characterize the 'CaCo-2 transcript' of α2,6ST and to establish whether it is expressed in cells from normal intestinal mucosa and/or in cells from colon carcinomas.

Acknowledgements

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, Consiglio Nazionale delle Ricerche–Progetto Finalizzato Applicazioni Cliniche Ricerca Oncologica, and MURST 60%, Rome (to F.S.C.), and National Institutes of Health grant GM38193 (to J.T.Y. Lau).

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