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Fucosyltransferase III and sialyl-Le^x expression correlate in cultured colon carcinoma cells but not in colon carcinoma tissue

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The potential contribution of fucosyltransferases to the overexpression of sialyl-Le^x antigen was investigated in the colon carcinoma cell line HT-29 and in human colon carcinoma tissue. In HT-29 cells as well as in normal or malignant colonic tissues Fuc-TIII, Fuc-TIV, Fuc-TVI but not Fuc-TV nor Fuc-TVII were detectable after RT-PCR. Sodium butyrate treatment of HT-29 cells increased (to about 200%) and DMSO treatment decreased (to about 20%) the expression of sialyl-Le^x. This modulation of sialyl-Le^x was concomitant with the analogous increase/decrease of mRNA of Fuc-TIII but not Fuc-TIV. Fuc-TVI was not detectable by Northern blotting in HT-29 cells. In six human colon carcinomas which exhibited strong overexpression of sialyl-Le^x, the expression of Fuc-TIII-mRNA was the same or lower than in the corresponding normal colonic tissue. Thus Fuc-TIII expression may be affecting the expression of the sialyl-Le^x moiety in HT-29 cells but not in human colon carcinoma tissue.

Keywords: colon carcinoma associated mucins, sialyl-Le^x, fucosyltransferases, AM-3

Introduction

The carbohydrate antigens sialyl-Le^x as well as sialyl-dimeric-Le^x (sialyl-Le^{x}Le^x) belong to the modified blood group antigens overexpressed in colonic cancer (reviewed in [1, 2]). This observation has gained particular significance since it has been shown that sialyl-Le^{x}Le^x moiety is expressed more frequently and in larger amounts in metastatic tissue than in the primary colonic tumours, thus lending support to the hypothesis that the overexpression of this tumour antigen may be related to the process of metastasis [3–5]. Subsequent cell adhesion experiments showed that the sialyl-Le^x moiety is the ligand for P-selectin [6] as well as for E-selectin [7], which triggers the process of granulocyte extravasation [8, 9]. These findings have supported the concept that sialyl-Le^x antigen might be initiating the extravasation of tumour cells and thus enhancing the metastatic process [10]. Indeed, the expression of this antigen is an unfavourable prognostic factor in patients with colonic [11, 12] and bladder carcinoma [13].

Notwithstanding the functional significance of sialyl-Le^x, the common lesions(s) causing the strong overexpression of this antigen in the majority of colon carcinomas has not been identified. As the carriers of sialyl-Le^x-groups in colonic tissue the mucins MUC1 and MUC2 have been identified [14, 15]. Since their expression usually does not change after malignant transformation of colonic tissue, the overexpression of sialyl-Le^x appears to be mainly due to a more frequent presence of these groups on the mucin core [16]. This alteration could be due to an increased activity of one of the enzymes operative in the biosynthesis of the sialyl-Le^x moiety.

In the course of biosynthesis of this antigen the type 2 carbohydrate chain, which is preferentially synthesized in colon cancer cells [17], is modified by the α2-3-sialyltransferase(s) as the penultimate and the α1-3-fucosyltransferase(s) as the ultimate step in the biosynthetic sequence [18]. The α1-3 fucosylation step in the

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sialyl-Le^x synthesis can be catalysed *in vitro* by several genetically distinct transferases. The transfection of COS cells with either Fuc-TIII [19], Fuc-TV [20] or Fuc-TVI [21] or of CHO cells with Fuc-TIV [22] resulted in expression of the normally absent sialyl-Le^x antigen on the cell surface. These results suggested that one of these fucosyltransferases could be determining the level of sialyl-Le^x expression in tumour cells. Which transferase is essential for the biosynthesis of the sialyl-Le^x moiety in normal or carcinomatous colonic tissue is not yet known. Previous studies suggested that the Fucα1-3GlcNAc transferase activity is higher in carcinoma than in the normal colonic tissue [23–25]. These enzymatic assays did not, however, unequivocally relate the increase of activity to a defined gene product. However, the recent cloning of Fuc-TIII [26], Fuc-TIV [19, 23, 27, 28], Fuc-TV [20], Fuc-TVI [21, 29] and Fuc-TVII [30] enables the expression of each gene product to be investigated at the mRNA level.

In the present work the expression of the fucosyltransferases potentially involved in sialyl-Le^x biosynthesis was compared at the mRNA level with the expression of this antigen in modulated HT-29 colon carcinoma cells and in human colonic tissues. The data indicate that the alterations of Fuc-TIII-mRNA expression only in cultured cells parallel those of sialyl-Le^x expression in cell lines while in colon carcinoma tissue factors other than Fuc-TIII determine the level of this antigen.

Materials and methods

Cell culture conditions and tissues

HT-29 cells were obtained from the ATCC. They were grown in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2 and passaged once a week. They were mycoplasma-free, as seen by testing with the fluorescent dye 4,6-diamino-2-phenylindole·2HCl (DAPI, Serva, Heidelberg, Germany).

Tumour tissue or normal colonic tissue collected at least 10 cm from the tumour was obtained at the time of hemicolectomy and RNA was isolated immediately.

Isolation of RNA for RT-PCR and Northern blotting

Total RNA from HT-29 cells grown at half confluence was isolated with RNAzolB (Biotecx Laboratories Inc., Huston, TX) according to the manufacturer's instructions. Total RNA from the tissue was extracted with guanidinium isothiocyanate, as described previously [31]. For RT-PCR 20 μg of each RNA preparation were treated with 200 U RNase-free DNase (Boehringer, Mannheim, Germany) for 2 h at 37 °C in 50 mM Tris buffer, pH 6.5, containing 10 mM $MgCl_2$, 20 U RNase inhibitor (Boehringer, Mannheim, Germany) and 10 mM DTT (Gibco BRL, Gaithersburg, MD, USA). After digestion the DNase was inactivated at 65 °C for 5 min. For Northern blotting polyadenylated RNA was enriched with Dynabeads (Dynal, Hamburg, Germany) as recommended by the manufacturer.

Detection of fucosyltransferases in the RT-PCR reaction

RT-PCR was carried out as described elsewhere [32]. Here 2.5 μg of total RNA were reverse-transcribed in 50 μl volume containing 2.5 μl original hexanucleotide solution as primer and 500 U MMLV reverse transcriptase (MMLV RT, Superscript, Gibco) according to the supplier's recommendations (Gibco). The reverse transcriptase was denatured at 100 °C for 10 min and a cDNA equivalent of 100 ng RNA was amplified in a reaction volume of 25 μl containing 1 × GeneAmp PCR buffer (Perkin Elmer, Cetus, Norwalk, CT, USA), 0.5 U AmpliTaq DNA polymerase (Perkin Elmer), 250 μM of dATP, dGTP, dCTP and dTTP and 50 ng of each primer. Prior to amplification the sample was heated to 92 °C for 5 min. Amplification was performed for 40–45 cycles at 92 °C, 1.5 min; 72 °C, 3.5 min; and 72 °C for 7 min after the last cycle in a Hybaid Omnigene apparatus. A negative control consisting of a 25 μl reaction mix without cDNA was included in each amplification of cDNA. Contamination by genomic DNA was excluded by PCR amplification of DNase-digested RNA. The primers used to detect Fuc-TIII/Fuc-TV have been published previously [33]. Under the described conditions they yield a 244 bp amplimer of Fuc-TIII and a 283 bp amplimer of Fuc-TV [33]. The primers for Fuc-TIV and Fuc-TVI have been described by Weston *et al.* [29] and yield amplimers of 319 bp and 280 bp respectively. The primers amplifying only Fuc-TV were 5′CCA GGG CTT ATG GCA GTG GAA CCT G3′ (forward, bases 139–169) and 5′GAC ATG GTG AGA TTG AAG TAT CCG3′ (reverse, bases 489–512). They gave an amplimer of 374 bp. The primers for Fuc-TVII were 5′CCA GCC CAC GAT CAC CAT CCT TGT C3′ (forward, bases 129–153) and 5′TGC CGC TCC TGG AAG TTG CTG ACC 3′ (reverse, bases 525–548) and they yielded an amplimer of 420 bp, unique for Fuc-TVII [30].

Northern blotting

Northern blots were performed using Nytran membranes (Schleicher and Schüll, Dassel, Germany) and overnight capillary blotting from 1% agarose gels. The blotted RNA was immobilized by UV irradiation (240 mJ) in a Stratalinker (Stratagene, Heidelberg, Germany). Prehybridization was carried out overnight at 42 °C in the presence of 50% formamide according to a standard procedure [34] with denatured herring sperm DNA (Promega, Madison, WI, USA). Hybridization was carried out for 15–20 h at 42 °C with 2 × 10^6 cpm per ml ^{32}P-labelled DNA probe. Autoradiography was performed using Kodak X-Omat cassettes with an intensifying screen at −70 °C. The autoradiogramms were evaluated with a Hoefer scanning

densitometer (Hoefer, San Francisco, CA, USA), and a median value of at least three experiments was calculated. For testing of the integrity and the amount of the applied mRNA β-actin probe or a polydT-probe were used. The actin probe was a 550 bp long *Eco* RI/*Xho* I gene fragment, cloned into a Bluescript vector (Stratagene). The random primed labelling of the excised fragment was performed with the Boehringer kit (Boehringer, Mannheim, Germany), as recommended by the manufacturer. The polydT probe (48 nucleotides), obtained from TIB MOL (Berlin, Germany), was ^{32}P-labelled with terminal transferase (Boehringer) according to the protocol of the manufacturer. The gene-specific probes for Fuc-TIII, Fuc-TIV and Fuc-TVI were amplimers with the length 260 bp, 435 bp and 280 bp respectively, obtained as described previously and shown to specifically detect each transferase [29].

Modulation of sialyl-Le^x-expression in vitro

HT-29 cells were grown in medium either with or without addition of the modulating agent. The cell density at the time of harvesting or assays in a half confluent state (3×10^5 cells cm^{-2}) was obtained. Sodium butyrate was from Fluka (Neu Ulm, Germany), DMSO was from Merck (Darmstadt, Germany) and phorbol myristate acetate (TPA), dexamethasone, retinoic acid and staurosporine were from Sigma (Deisenhofen, Germany).

Monoclonal antibodies

Monoclonal antibody AM-3 (IgM) has been produced as described previously [35]. It is directed against the monomeric sialyl-Lewis^x group [36]. The monoclonal antibody CSLEX-1 (IgM) was kindly donated by Dr Terasaki (UCLA, School of Medicine, University of California, Los Angeles, CA).

Detection of sialyl-Le^x-expression on the cell membrane by ELISA

The expression of sialyl-Le^x on HT-29 cells was carried out by ELISA as described previously [37]. The obtained ELISA signal was expressed per 10^5 cells, after cells seeded in parallel had been trypsinized and counted. The experiments were repeated at least three times and the mean values ± SD were calculated.

Quantification of the antigen in the medium

The relative content of sialyl-Le^x epitope in the cell medium was assayed in a sandwich ELISA, with purified AM-3 antibody as the catcher and peroxidase-conjugated AM-3 antibody as tracer as described [14].

FACS analysis of the modulated cells

HT-29 cells were fixed in 1.5% paraformaldehyde solution in PBS, washed and incubated with anti-sialyl-Le^x antibody at 4 °C for 30 min, washed again in PBS, and then stained with FITC-goat anti-mouse IgM conjugate. Alternatively, fluorescein-labelled AM-3 antibody was used in a one step procedure. Cells were analysed for positive staining by FACS-Scan (Beckton-Dickinson, Heidelberg, Germany).

Immunohistochemistry and semiquantitative evaluation of staining

The immunohistochemical detection of sialyl-Le^x was carried out with the ABC method as described [38] and evaluated by two independent observers. The intensity was graded as negative (0), low to moderate (1) or strong (2). The fraction of expressing cells was divided into: none (0), <25% stained cells (1), 25–49% of stained cells (2) and 50–100% of stained cells (3). The product of these two variables (range 0–6.00) was used as a measure of the overall sialyl-Le^x expression and was described as negative (0), low (1–2) moderate (3–4) or strong (5–6).

Results

1. Detection of fucosyltransferases in HT-29 cells and in human colonic tissue by means of RT-PCR

The reverse transcriptase-polymerase chain reaction revealed that HT-29 cells express Fuc-TIII, Fuc-TIV and Fuc-TVI transferase. Fuc-TV and Fuc-TVII were absent in HT-29 cells (DMSO or butyrate-treated or non-treated), (Fig 1).

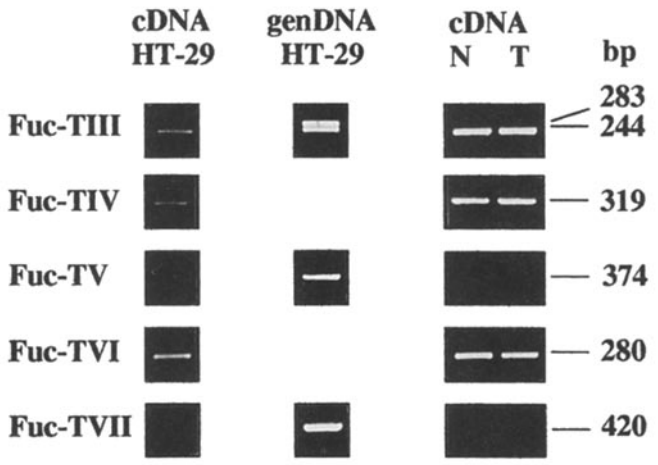

Figure 1. Detection of Fuc-TIII, Fuc-TIV and Fuc-TVI transferase but not Fuc-TV nor Fuc-TVII in HT-29 cells and in the normal and carcinomatous colonic tissue by RT-PCR. As template cDNA from HT-29 cells or from normal (N) or tumour (T) tissue was used. Genomic DNA from HT-29 cells was used to demonstrate that Fuc-TV and Fuc-TVII are detectable under the applied conditions. Aliquots of the amplimers were electrophoresed on agarose gels and stained with ethidium bromide. The sizes of the amplified products are indicated in base pairs (bp). Human tissue results are representative for seven patients.

The lack of expression of these enzymes in HT-29 cells is not the result of culture conditions since they were also not detectable in the normal or carcinomatous tissue of seven investigated patients.

2. Modulation of sialyl-Le^x expression in HT-29 cells with sodium butyrate or DMSO

Preliminary experiments indicated that the expression of sialyl-Le^x in the membrane of HT-29 cells is not dependent on the growth time of cells. At confluency however, a lower ELISA signal per cell than at half confluency was observed, probably due to the diminished sialyl-Le^x epitope accessibility (not shown). To avoid the variability due to different cell density, in all experiments the cell density at the time of evaluation corresponded to half confluency.

The treatment of HT-29 cells for 48 h with 2 mM sodium butyrate induces an increase in sialyl-Le^x-expression on the cell surface to 197 ± 17% while incubation in 1.5% DMSO for 1 week reduced the expression to 22 ± 6% (Fig. 2). The alterations in the medium were 176 ± 18% (sodium butyrate) and 32 ± 6% (DMSO) of the control value, respectively.

Staining either with AM-3 or CSLEX-1 and evaluation in FACS confirmed the data obtained by ELISA. The mean fluorescence intensity detected with AM-3 antibody increased after treatment with sodium butyrate to 186 ± 10% and it decreased after treatment with DMSO to 24 ± 5% of the control value. The corresponding values obtained with CSLEX-1 antibody were 228 ± 7% and 49 ± 6% respectively (Fig. 2).

The incubation with TPA (up to 0.2 μM), retinoic acid (up to 1 μM) or staurosporine (up to 0.1 μM) did not have any effect on sialyl-Le^x expression on HT-29 cells (data not shown).

3. Alterations of Fuc-TIII- and Fuc-TIV mRNA expression in HT-29 cells after sodium butyrate or DMSO treatment

Northern blotting with gene-specific probes showed that after treatment of cells with sodium butyrate the Fuc-TIII-mRNA expression is 300 ± 120% of the control. By contrast, treatment with DMSO caused a decrease in Fuc-TIII mRNA expression to 33 ± 12% of the control value.

The steady state concentration of Fuc-TIV-mRNA increased to 200 ± 100% or to 220 ± 90% after treatment with sodium butyrate or DMSO, respectively (Fig. 3). Fuc-TVI transcript was not detected in mRNA extracted from HT-29 cells (not shown). Since the applied probe reacted in Southern blot with the expected DNA fragments as published previously [29], this result was interpreted to indicate a low expression of Fuc-TVI in colon carcinoma cells.

The probing of the same membranes with a radioactive labelled poly-dT-probe revealed that β-actin expression

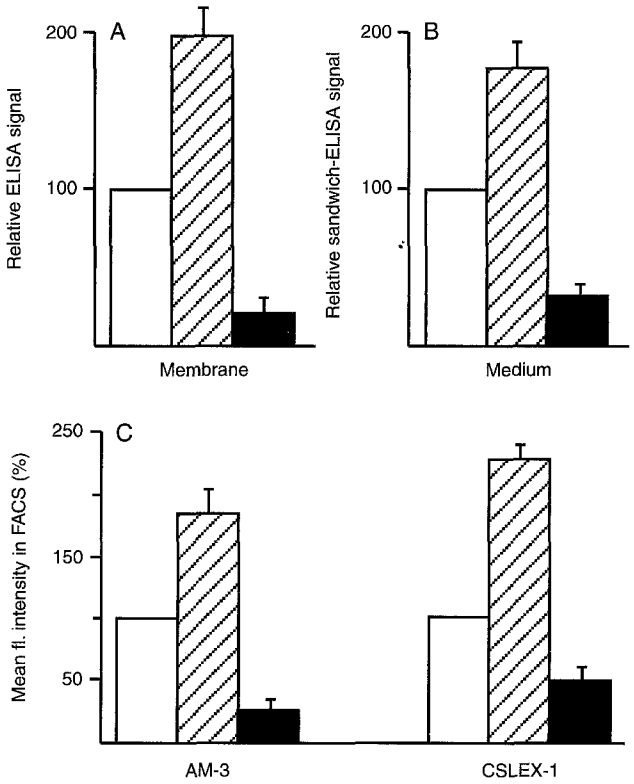

Figure 2. (A) Effect of sodium butyrate (diagonal bars) (2 mM, 48 h) or DMSO (solid columns) (1.5%, 7 days) treatment on the expression of sialyl-Le^x antigen in HT-29 cells, determined on the surface of adherent HT-29 cells by ELISA with the AM-3 antibody and (B) in the cell medium, determined by sandwich ELISA with AM-3 antibody as catcher and AM-3-peroxidase conjugate as tracer. (C) Detection of sialyl-Le^x-antigen on the cell surface of sodium butyrate-treated or DMSO-treated cells by FACS, with either AM-3 or CSLEX-1 antibodies. Bars represent average values of three experiments ± SD. Results obtained with cells grown in standard medium (open columns) were taken as 100%.

was not affected by DMSO but was slightly upregulated by sodium butyrate (Table 1).

Thus only the steady state concentration of the Fuc-TIII mRNA correlated with the expression of sialyl-Le^x in HT-29 cells suggesting that the amount of this enzyme may be determining the level of sialyl-Le^x expression in these cells.

4. Expression of Fuc-TIII and sialyl-Le^x antigen in normal and carcinomatous colonic tissue

Expression of sialyl-Le^x was not detectable with the AM-3 antibody in normal colonic tissue but was strong in six of seven investigated colon carcinoma samples of the same patients (Fig 4 and Table 2). The expression of Fuc-TIII-mRNA was normal or decreased in these samples (Table 2). In one patient Fuc-TIII was not detectable in the tumour tissue. This was not due to mRNA degradation since the amount of RNA of the housekeeping enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH) in

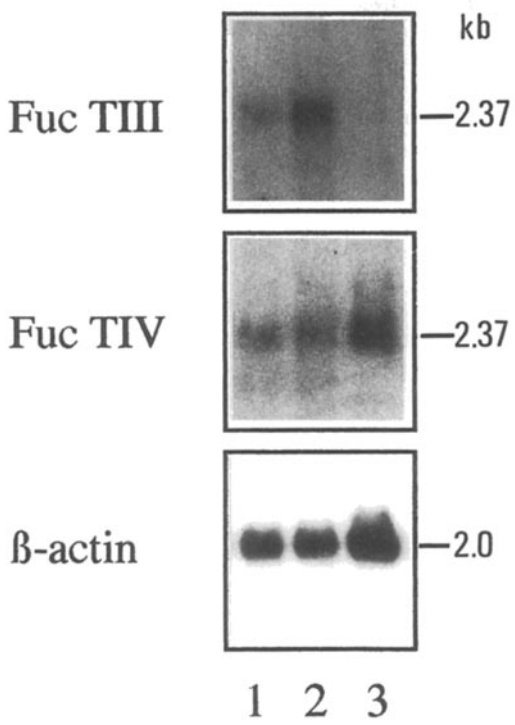

Figure 3. Northern blotting of mRNA (approximately 10 μg per lane) extracted from HT-29 cells grown in a standard medium (lane 1) or medium supplemented with 2 mM sodium butyrate for 48 h (lane 2) or with 1.5% DMSO for 7 days (lane 3). PCR-amplimers radioactively labelled by random priming were used as probes. The size of the detected transcripts is given in kilobases (kb). A β-actin probe, used to test the quality and the relative amount of the mRNA indicates that a larger amount of mRNA was applied to lane 3. Representative blots of three to four independent experiments for each probe.

Table 1. Gene expression in HT-29 cells treated with sodium butyrate or DMSO expressed as ratio to nontreated control. The signal in each lane was related to poly-dT signal. The expression of β-actin was checked with a polydT probe. Number of experiments is given in brackets. Mean values ± SD.

	Cells treated with:	
mRNA	*Sodium butyrate*	*DMSO*
Fuc-TIII	3.00 ± 1.20 (4)	0.33 ± 0.12 (3)
Fuc-TIV	2.00 ± 1.00 (3)	2.20 ± 0.90 (5)
β-actin	1.70 ± 0.30 (4)	1.50 ± 0.70 (6)

both tissues were comparable (Table 2). Thus there is no correlation between the increase of sialyl-Le^x antigen expression and the expression of Fuc TIII-mRNA after malignant transformation of the colon. Malignant transformation does not appear to affect the expression of β-actin (Table 2).

Table 2. Ratio of gene expression in colon carcinoma to normal colonic tissue. The relative amounts of total mRNA in each lane were assayed with a polydT probe. The expression of sialyl-Le^x was semiquantitatively determined by immunohistochemistry with AM-3 antibody.

			Sialyl-Le^x expression tissue:	
Patient no.	*β-actin*	*Fuc-TIII*	*Tumour*	*Normal*
143	1.0	0.6	6	ND
154	0.8	0.9	6	ND
155	0.9	0.1	1	ND
160	–	ND	6	ND
163	1.7	1.0	6	ND
164	0.7	0.2	6	ND
165	1.6	0.9	6	ND

ND, not detectable; –, not determined, GAPDH expression in this sample was not altered.

Discussion

In the present work the question was addressed *in vitro* and *in vivo* of whether the overexpression of sialyl Le^x in colon carcinoma cells is related to the expression of the known fucosyltransferases potentially involved in the last step of the biosynthesis. In the HT-29 cell line the expression of the sialyl-Le^x-antigen was modulated with sodium butyrate or DMSO and the expression of the fucosyltransferases monitored at the mRNA level.

Sodium butyrate is a differentiating agent shown here to induce approximately a doubling of sialyl-Le^x expres-

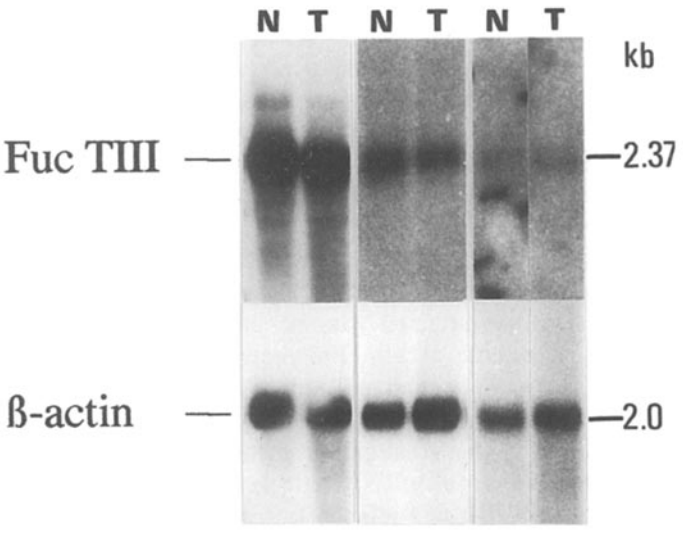

Figure 4. Northern blots of mRNA isolated from normal and malignant colonic tissue of three patients, hybridized with a Fuc-TIII probe (upper panels, 2.37 kb). The quality of applied mRNA was tested with a β-actin probe on the same blot (lower panels, 2.0 kb).

sion, detectable with the monoclonal antibody AM-3 or CSLEX-1 on the cell surface of HT-29 cells as well as on glycoproteins secreted into the medium. Another differentiating agent, DMSO, had a suppressing effect on sialyl-Le^x expression while retinoic acid or TPA had no influence at all. The discordant effects of differentiating agents on sialyl-Le^x expression *in vitro* corroborate previous data *in vivo* [35] indicating that the expression of sialyl-Le^x is independent of the differentiation process.

The decrease in expression after treatment with DMSO, which is known to activate protein kinase C [39] as well as the lack of influence of another activator of protein kinase C, TPA, the inhibitor, staurosporine, suggest further that the signal transduction mediated by protein kinase C is not necessary for regulation of sialyl-Le^x expression.

One of the reasons for sialyl-Le^x overexpression may be an increased activity or amount of an enzyme involved in its biosynthesis. This concept is supported by recent examples of induction of sialyl-Le^x synthesis by transfection of cells with cDNA coding for a single fucosyltransferase [40]. Several gene products with α1–3 fucosyltransferase activity detected in human tissues [20, 21, 29, 30] were shown to synthesize the sialyl-Le^x antigen. Further, it has been shown that the fucosyltransferase activity is consistently increased in sera of colon cancer patients but it decreases after tumour resection [41]. The present work indicates that HT-29 cells as well as the human colonic tissue express only Fuc-TIII, Fuc-TIV and Fuc-TVI but not Fuc-TV or Fuc-TVII. Of these, only Fuc-TIII ('Lewis enzyme') with α1–3 and α1–4 transferase activity was affected by either modulator in a manner corresponding to the changes in the expression of the sialyl-Le^x-antigen. These data are in line with the previous observations made *in vitro* [19] and are compatible with the hypothesis that the alterations of Fuc-TIII contribute to the altered sialyl-Le^x expression in modulated HT-29 cells.

This mechanism does not operate in the human colonic tissue, where the expression of sialyl-Le^x is barely detectable in normal tissue and is usually very strong in colonic cancer. The expression of Fuc-TIII mRNA in tumour was either similar to or lower than in the normal tissue of six investigated patients and in one patient it was not detectable at all. The recent data on Fuc-TIII enzyme activity in human normal and carcinomatous colonic tissue correspond to the present Northern blot results [23]. If the assumption is made that the steady state concentration of mRNA reflects the enzyme activity, then the Fuc-TIII is unlikely to cause sialyl-Le^x-overexpression in colon cancer. *In situ* hybridization with Fuc-TIII probes combined with simultaneous immunohistochemical detection of sialyl-Le^x in the same cells is necessary to confirm that both species are indeed independently expressed. A recent report by Yago *et al.* [42] suggested that in leukaemic cells Fuc-TIII, Fuc-TV or Fuc-TVI can be candidates for the enzymes which form the sialyl-Le^x-determinant. The present data show that Fuc-TV is absent from colon carcinoma cells. Fuc-TVI was detected in colon carcinoma cells by RT-PCR but not by Northern blot, indicating that this enzyme is expressed at a very low level. Fuc-TIII is detectable in colon and colon carcinoma but its alterations are not responsible for the increased expression of sialyl-Le^x determinant in colonic tumours. Vavasseur *et al.* [43] found that the activity of α1–3/4 fucosyltransferase *in vitro* remains constant and very low during progression from the adenomatous to the carcinomatous phenotype while another α1–3 fucosyltransferase is expressed. Similarly, Dahi *et al.* [44] also implicated that an additional α1–3 fucosyltransferase activity may be induced during colon carcinogenesis: the activities of α1–3 fucosyltransferase and α1–4 fucosyltransferase correlate in the normal but not in the carcinomatous colonic tissue supporting the assumption that in colon cancer a yet unknown α1–3 fucosyltransferase is operative. Thus it is possible that a hitherto not characterized fucosyltransferase determines the level of sialyl-Le^x expression in colon carcinoma tissue. Alternatively, another mechanism than fucosylation may be responsible for the overexpression of the sialyl-Le^x antigen. The present data are compatible with either hypothesis.

Acknowledgements

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