Screening of enzymatic synthesis and expression of Lewis determinants in human colorectal carcinoma

Almudena Fernández-Briera, Elisa Cuevas and Emilio Gil-Martín

1Department of Biochemistry, Genetics and Immunology. Faculty of Biology. University of Vigo. Vigo, Spain. 2Pathology Service. Complejo Hospitalario Universitario. Ourense, Spain

ABSTRACT

Background: Although colorectal carcinogenesis has been intensively studied, the published investigations do not provide a consistent description of how different carbohydrate determinants of colorectal epithelium are modified in colorectal cancer (CRC).

Objective: This study is an attempt to characterize the terminal fucosylation steps responsible for the synthesis of mono- (Lea/Lex) and difucosylated (Leb/Ley) Lewis antigens in healthy and tumour CRC tissue.

Methods: An immunohistochemical study of Lewis antigens’ expression was undertaken, along with screening of the fucosyltransferase (FT) activities involved in their synthesis, on healthy and tumour samples from 18 patients undergoing CRC.

Results: Analysis of α(1,2/3/4)FT activities involved in the sequential fucosylation of cores 1 and 2 showed significant increases in tumour tissue. Expressed as μU/mg and control vs. tumour activity (p from Wilcoxon’s test), the FT activities for Lea/Leb synthesis were: lacto-N-biose α(1,2)/α(1,4)FT, 65.4 ± 19.0 vs. 186 ± 35.1 (p < 0.005); lacto-N-fucopentose 1 α(1,4)FT, 64.9 ± 11.9 vs. 125.4 ± 20.7 (p < 0.005); Leaα1,2FT, 56.2 ± 7.2 vs. 130.5 ± 15.6 (p < 0.001). Similarly, for Leb/Ley synthesis were: N-acetyllactosamine α(1,2)/α(1,3)FT, 53.4 ± 12.2 vs. 108.1 ± 18.9 (p < 0.001); 2′-Fucosyl-N-acetyllactosamine α(1,3)FT, 61.3 ± 10.7 vs. 126.4 ± 22.9 (p < 0.001); 2′-Fucosylactose α(1,3)FT, 38.9 ± 10.9 vs. 143.6 ± 28.9 (p < 0.001); 2′-Methylactoside α(1,3)FT, 30.9 ± 4.8 vs. 66.1 ± 8.1 (p < 0.005); and Lebα1,2FT, 54.3 ± 11.9 vs. 88.2 ± 14.4 (p < 0.001).

Immunohistochemical Le expression was increased (p < 0.01 according to Wilcoxon’s test) in tumour tissue, with 84.6% of specimens being positive: 7.7% weak, 15.4% moderate and 61.5% high intensity.

Conclusions: Results suggest the activation of the biosynthesis pathways of mono- and difucosylated Lewis histo-blood antigens in tumour tissue from CRC patients, leading to the overexpression of Lea, probably at the expense of Leb.


INTRODUCTION

Conventional staging methods of colorectal cancer (CRC) are not efficient enough and fail to correct prediction of tumour progression. Therefore, it is necessary to further research on molecular and structural biomarkers of cancer cells, which may provide a better indication of malignancy evolution than staging alone, and could improve the clinical practice of CRC selecting the best treatment for each patient. In this endeavor certain glycosylated markers have proven useful, such as the carcinoembryonic antigen (CEA) and the sialyl-Lea antigen (CA 19-9), whose combined preoperative serum evaluation has been considered of interest for the management of CRC (1).

Glycosylation is a key factor modulating the structure and biological functions of glycoproteins. The importance of glycosylation is manifest a) in the fact that 50% of human proteins are glycosylated (2); and b) that about 1% of the genome of prokaryotes and eukaryotes is dedicated to encoding the highly conserved glycosylation enzymes (glycosidases and glycosyltransferases) (3).

Glycoproteins can achieve unique functional properties through certain specific alterations in the terminal composition of their glycan antennae. The fucose content of N- and mucin-type O-glycoproteins, for example, is strictly regulated during ontogeny and cell differentiation (4,5), and similarly, aberrations in the fucosylation of secreted and cell surface proteins are among the most common oligosaccharide modifications during malignant transformation (6,7). Recent evidence suggests that all these changes are not an epiphenomenon in the origin, growth and spread of cancer. Rather, fucose content is currently considered a pathophysiological effector of the malignant phenotype in gastrointestinal (8) and breast cancer (9), among others. This dynamic complexity (10) is played by the crucial role of 13 regulated and organ-specific human fucosyltransferases (FT) known.
FT are essential for the formation of the histo-blood ABH and Lewis antigens, by adding L-fucose residues to the non-reducing terminal oligosaccharides of N-/O-glycoconjugates. Thus located at the distal end of the glycan chain, fucose is exposed to the microenvironment and is therefore expected to account for a part of their distinctive properties. The biosynthesis of Lewis determinants precedes from four main precursors, designated core type 1, 2, 3 and 4 (11). Type 1 and 2 give rise mainly to the H1/H2 antigens, as well as to the monofucosylated (Leα, Leβ) and difucosylated (Leβ, Leγ) Lewis antigens (Fig. 1), all of them part of the N-/O-glycoproteins and glycolipids of the lactoseries. The terminal steps in their formation are due to the sequential action of α(1,2)-, α(1,3)- and α(1,4)FT on core 1 and core 2 precursors (Fig. 1). The two known human α(1,2)FT (EC 2.4.1.69) responsible for the initial synthesis of H blood-group antigens (H1 and H2) are encoded by the FUT1 gene (H transferase) and FUT2 gene (Se transferase). Additionally, the FUT4 to FUT7 and FUT9 genes encode FT showing the α(1,4)FT activity (EC 2.4.1.-) required to add α(1,4)Fuc to GlcNAc residues and obtain the core 1-derived (Leα/Leβ) and core 2-derived (Leβ/Leγ) antigens, respectively (10,11). However, FUT3 encodes the α(1,3)FT Lewis enzyme (EC 2.4.1.65), which exhibits both α(1,3)- and α(1,4)FT activities, and can use core 1/2 (12) and monofucosylated Leα/Leβ (13) as substrates.

The colorectal expression of mono- (Leα/Leβ) and difucosylated (Leβ/Leγ) Lewis antigens changes with time according to ontogeny, maturation and different anatomic regions of the normal colorectum (14,15). Furthermore, alterations in ABH and Lewis histo-blood group-related antigen expression are a characteristic feature of carcinomas and have been implicated in different types of intercellular interactions, such as immunological recognition and evasion, apoptosis, adhesion and invasion (11). In this context, basal levels of fucosylation in healthy colorectal tissue are relatively low, but undergo a marked increase during carcinogenesis. There is now clear evidence of neosynthesis and/or overexpression of certain ABH and Lewis antigens in the majority of cancer cells derived from colorectal epithelium (8,14,16). These findings indicate that fucosylated epitopes are typical oncofetal antigens in colorectal cancer, several of them associated with poor clinical prognosis (14-20), and useful as possible CRC biomarkers (7,21-23). This demonstrates the usefulness of molecular biology in clinic setting and highlights the importance of translational efforts to overcome the gap between basic research and clinical practice.

In sum, a) changes in H and Lewis antigen expression in colorectal carcinogenesis have been documented; b) the synthesis of these fucosylated determinants requires several tissue-specific FT acting on core 1 and core 2 precursors; and c) the kinetic alterations of some of these enzymes in CRC have recently been reported by our laboratory (13). Despite this background, contradictory data remain, and new insights in this field are required. Accordingly, we performed a screen of the synthesis and expression of mono- and difucosylated Lewis antigens, a) addressing the enzyme activity of terminal α(1,2), α(1,3) and α(1,4)FT at H1 and H2 fucosylation steps from core 1 and 2; and b) assessing the histological expression of core 1-derived (Leα and Leβ) and core 2-derived (Leβ and Leγ) Lewis determinants. Our intention was to characterize the sequential fucosylation of core 1 and core 2 structures in healthy vs. tumour CRC tissue, in order to obtain a general view of the functional status of their biosynthetic pathways. Our findings demonstrated that Leγ hyperfucosylation is important at an early stage of colorectal carcinogenesis, and thus the currently available knowledge about the molecular modulation undergone by this family of fucosylated epitopes in CRC has improved.

**Fig. 1.** Biosynthesis pathways of histo-blood H and Lewis antigens based on core type 1 (H1, Leα and Leβ) and core type 2 (H2, Leα and Leβ) precursors. –R represents the carbohydrate backbone linked to the O/N-glycoprotein or glycolipid carriers.
MATERIALS AND METHODS

Reagents

GDP-L-[\textsuperscript{14}C]-fucose (specific activity 10 GBq/mmole, 270 mCi/ mmole) and the scintillation counting mixture Ecoscint H were supplied by New England Nuclear (Ma, USA) and National Diagnostics (Ga, USA), respectively. The oligosaccharides used as exogenous acceptors, GDP-fucose, bicinechonic acid (BCA), bovine serum albumin (BSA), Dowex 2X8-400, detergents, salts and buffers, were purchased from Sigma Chemical Co. (Mo, USA). The mouse anti-human Le\textsuperscript{a}, Le\textsuperscript{b}, Le\textsuperscript{e} and Le\textsuperscript{y} monoclonal antibodies, as well as the goat anti-mouse, labelled polymer, HRP, EnVision\textsuperscript{TM} Detection System, were from DAKO (Ca, USA). All other reagents were of analytical quality.

Colorectal specimens and preparation of tissue extracts

Colorectal tissue specimens were obtained from patients with CRC undergoing surgery at the University Hospital Complex of Ourense (CHUO, Spain) after receiving approval from the appropriate local Institutional Board. After resection, 18 specimens of 1-14 cm obtained from primary colorectal carcinoma and healthy tissue (at least 10 cm distant from the tumour and without any histological trace of malignancy), were washed with ice-cold saline buffer and stored at -80 ºC until use. All relevant information, including clinical and anatomopathological features of patients and specimens (Table I), was provided by the Anatomopathological Service of CHUO, following an anonymous and strictly confidential schedule. The histopathological grading of tumours was established according to Dukes’ stages (24) and the nodal metastasis (N factor) of the TNM classification (25).

Total cell membranes were obtained as previously described (13). The protein concentration of the enzyme preparations was determined by means of the BCA protein assay, using BSA as the standard.

Fucosyltransferase assays

The standard cocktail for FT reactions contained the following reactants and final concentrations in a total volume of 50 µL: 50 mM Tris-HCl (pH 7.2), 10 mM MnCl\textsubscript{2}, 3% (v/v) Triton X-100, 100 µM GDP-L-[\textsuperscript{14}C]-fucose/GDP-L-fucose (isotopic dilution 1/200), 0.5 mM acceptor substrate (see later) and 60-100 µg of protein solution. The assays (run in duplicate) were started by adding the donor substrate GDP-L-[\textsuperscript{14}C]-fucose/GDP-L-fucose, and incubated for 90 min at 37 °C in a shaken water-bath. The reactions were stopped with 0.4 mL of cold water and immersion in an ice bath. The incubation of tumour samples and their respective controls of healthy mucosa was always conducted in parallel.

Soluble oligosaccharides corresponding to the non-reducing end of the in vivo fucosylated sequences, or alternative commercial structural analogues, were employed as exogenous acceptor substrates. As some of these oligosaccharides could act as an acceptor substrate for several FT, and vice versa, we chose a set (Table II) covering both the core 1 and 2-derived Lewis biosynthetic pathways.

The reaction products were separated from the isotopic mixture of GDP-L-fucose by ion-exchange chromatography in 3-mL Dowex 2x8-400 columns, and eluted with water in a manifold station (Waters, USA) coupled to a vacuum pump (Millipore, Spain). The eluted fucosylated oligosaccharides were collected in fractions of 0.4 mL and mixed with 5 mL of scintillation counting mixture (Ecoscint H). Radioactivity was measured on a Wallac 1409-12 Scintillator system (Boston, USA). Control assays without exogenous acceptors were carried out to evaluate endogenous non-specific radiolabelling. The enzyme activity was expressed as pmol of GDP-L-fucose transferred per min and mg of protein (µU/mg).

Immunohistochemical assays

Histological slides of tumour and healthy specimens from 18 CRC-resected patients included in the study were fixed in formalin and embedded in paraffin. Sections (4 µm) from selected tissue blocks were deparaffinised in xylene, hydrated in a graded ethanol series, and heated (microwave oven for 20 min) in 0.1 M citric acid buffer (pH 6.0) to unmask the epitopes. The endogenous peroxidase activity was eliminated with 0.5% (v/v) hydrogen peroxide.
hydrogen peroxide in methanol for 20 min. Likewise, non-specific binding was blocked with 2% BSA for 20 min. Each incubation step was followed by thorough washing of the tissue slides in PBS.

Immunohistochemical staining began with the incubation of the sections (room temperature, 30 min) with primary monoclonal antibodies against Lea (mouse anti-human Lea, isotype IgG1, dilution 1:1000), Leb (mouse anti-human Leb, isotype IgG1, dilution 1:1000), Lex (CD15, mouse anti-human granulocytes, C3D-1 clone, dilution 1:50) and Ley (mouse anti-human Ley, BM-1 clone, dilution 1:1000). After a rinse with PBS, the sections were incubated (room temperature, 30 min) with the secondary antibody bound to peroxidase (goat anti-mouse, labelled polymer, HRP, EnVision™ Detection System). The peroxidase reaction was visualized by incubating with DAB (3,3′-diaminobenzidine). Finally, after a wash with water, the sections were counterstained with Papanicolau’s haematoxylin, dehydrated in a graded ethanol series, washed in xylene and mounted in Dpex mounting medium. Negative controls were performed using PBS instead of primary antibody.

Immunohistochemical staining, detected as a brown precipitate, was evaluated by two expert pathologists belonging to the hospital partner (CHUO), who reached a final conclusion by consensus. The semiquantitative scores of antigen expression were as follows: negative (0), specimen without staining; weak (1), less than 10% of the tissue stained; moderate (2), 10-50% of the tissue stained; high (3), more than 50% of the tissue stained.

Statistical analyses

Statistical analyses were performed using SPSS v. 16.0 for Windows XP. The non-parametric Wilcoxon’s test was used for contrasting differences of enzyme activity or antigen expression between control and tumour-paired samples. Correlation between FT activity and antigen expression was carried out by means of Spearman’s test, while the comparisons with clinicopathological features were performed with the aid of the χ² test or Fisher’s exact probability test for categorical data (immunohistochemical expression), and the Kruskal-Wallis and the Mann-Whitney U test for continuous data (enzyme activity). The results were considered significant when p < 0.05.

RESULTS

Fucosyltransferase activities involved in the synthesis of core 1-derived (Lea/Leb) and core 2-derived (Lex/Ley) antigens in healthy and tumour tissue from CRC

In the present study, the FT activities involved in the synthesis of Lea/Leb and Ley/Leb antigens (Fig. 1) were evaluated using as acceptors synthetic oligosaccharides that reproduce the terminal non-reducing sequences necessary to complete the terminal steps of the biosynthesis pathway from cores type 1 and type 2 (Table II).

---

Table II. Oligosaccharides used as exogenous acceptor substrates to evaluate the enzyme activity of α(1,2)-, α(1,3)- and α(1,4)FT involved in the biosynthetic pathway of mono- and difucosylated Lewis antigens

<table>
<thead>
<tr>
<th>Core type 1</th>
<th>In vivo fucosylated sequence</th>
<th>Exogenous acceptor substrate used in the assays</th>
<th>Enzyme activity determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core type 1</td>
<td>Galβ(1,3)GlcNAcβ−R</td>
<td>Lacto-N-biose 1</td>
<td>α(1,2)FT</td>
</tr>
<tr>
<td>H₁ antigen</td>
<td>Fucα(1,2)Galβ(1,3)GlcNAcβ−R</td>
<td>Lacto-N-fucopentaose 1</td>
<td>α(1,4)FT</td>
</tr>
<tr>
<td>Le⁺</td>
<td>Galβ(1,3)[Fucα(1,4)]GlcNAcβ−R</td>
<td>Le⁺</td>
<td>α(1,2)FT</td>
</tr>
</tbody>
</table>

Core type 2

<table>
<thead>
<tr>
<th>Core type 2</th>
<th>Galβ(1,4)GlcNAcβ−R</th>
<th>N-acetyllactosamine</th>
<th>α(1,2)FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂ antigen</td>
<td>Fucα(1,2)Galβ(1,4)GlcNAcβ−R</td>
<td>2´-Fucosyllactose</td>
<td>α(1,3)FT</td>
</tr>
<tr>
<td>Le⁺</td>
<td>Galβ(1,4)[Fucα(1,3)]GlcNAc−R</td>
<td>3´-Fucosyllactose</td>
<td>α(1,2)FT</td>
</tr>
</tbody>
</table>

The structure of the oligosaccharides used correspond to the non-reducing end of the in vivo acceptors or to structural analogues of them.
For the screening of the enzymatic synthesis of the core 1 derived antigens, we determined the \( \alpha(1,2) \) - and \( \alpha(1,4) \) FT activities with lacto-\( N \)-biose 1 as the acceptor substrate in healthy and tumour specimens from 12 CRC patients. The results obtained (Table III) showed a statistically significant increase \((p < 0.005, \text{according to Wilcoxon's test})\) in \( \alpha(1,2) \) - and \( \alpha(1,4) \) FT activity in tumour tissue \((186 \pm 35.1 \mu U/mg)\) compared to healthy tissue \((65.4 \pm 19 \mu U/mg)\). Similarly, the \( \alpha(1,4) \) FT activity was assayed with lacto-\( N \)-fucopentaose 1 in healthy and tumour specimens from 15 CRC patients (Table III). The enzyme activity in tumour tissue was significantly higher than in healthy tissue \((125.4 \pm 20.7 \mu U/mg \text{ vs.} 64.9 \pm 11.9 \mu U/mg; p < 0.005, \text{according to Wilcoxon’s test})\). The last step of the Le\( ^b \) biosynthetic pathway was tested using Le\( ^a \) oligosaccharide as the acceptor substrate. In this case, the \( \alpha(1,2) \) FT activity was measured in healthy and tumour specimens from 8 CRC patients. The results (Table III) showed a statistically significant enhancement of specific activity in tumour tissue in comparison with its healthy counterpart \((130.5 \pm 15.8 \mu U/mg \text{ vs.} 56.2 \pm 7.2 \mu U/mg; p < 0.001, \text{according to Wilcoxon’s test})\).

In the case of enzymes involved in the synthesis of the core 2 derived antigens, \( \alpha(1,2) \) FT and \( \alpha(1,3) \) FT activities were evaluated together with \( N \)-acetyllactosamine as the acceptor substrate. The results of 18 tested CRC patients (Table III) showed a strong increase in enzyme activity in the tumour tissue \((108.1 \pm 18.9 \mu U/mg)\) with respect to the healthy one \((53.4 \pm 12.2 \mu U/mg)\). We analyzed the significance of this increase and found that the differences between tumour and healthy tissues were statistically significant \((p < 0.001, \text{according to Wilcoxon’s test})\). The \( \alpha(1,3) \) FT enzyme activity involved in the synthesis of Le\( ^a \) antigen was determined using the H\( ^2 \) antigen \((2’-\text{fuco-syl-N-acetyllactosamine}, 2’-\text{FNA})\) and two structural analogues \((2’-\text{fucosyllactose}, 2’-\text{FL}; \text{and} 2’-\text{methyllectose}, 2’-\text{ML})\) as acceptors in 14, 16 and 17 assays, respectively. As shown in table III, the \( \alpha(1,3) \) FT activity in tumour tissue increased when employing any of these three chosen acceptors: \(126.4 \pm 22.9 \mu U/mg \text{ vs.} 61.3 \pm 10.7 \mu U/mg\) for \( 2’-\text{FNA}; 143.6 \pm 28.9 \mu U/mg \text{ vs.} 38.9 \pm 10.9 \mu U/mg\) for \( 2’-\text{FL}; \text{and} 66.1 \pm 8.1 \mu U/mg \text{ vs.} 30.9 \pm 4.8 \mu U/mg\) for \( 2’-\text{ML}\). Likewise, in all three cases the reported increase in \( \alpha(1,3) \) FT enzyme activity was statistically significant according to Wilcoxon’s test \((p < 0.001 \text{for} 2’-\text{FNA and} 2’-\text{FL, and} p < 0.005 \text{for} 2’-\text{ML})\).

To conclude the enzymatic study of Le\( ^c \) biosynthesis, we assayed the direct fucosylation of the Le\( ^c \) and its structural analogue 3’-fucosyllactose (3’-FL) (Table III). The \( \alpha(1,2) \) FT activity on these two acceptors was increased in the tumour tissue \((88.2 \pm 14.4 \mu U/mg \text{ for} \text{Le}^c; 70.7 \pm 17.2 \mu U/mg \text{ for} 3’-\text{FL})\) with respect to the healthy one \((54.3 \pm 11.9 \mu U/mg \text{ for} \text{Le}^c; 63.2 \pm 29.5 \mu U/mg \text{ for} 3’-\text{FL})\). The enhancement for Le\( ^c \) acceptor was statistically significant \((p < 0.001 \text{according to Wilcoxon’s test})\).

**Immunohistochemical expression of core 1-derived (Le\( ^a / \text{Le}^b \)) and core 2-derived (Le\( ^c / \text{Le}^e \)) antigens in healthy and tumour tissue from CRC**

The immunohistochemical expression of Le\( ^a / \text{Le}^b \) and Le\( ^c / \text{Le}^e \) antigens was analysed in tumour and healthy-paired specimens from 12/10 and 12/13 CRC-resected patients, respectively. The cell expression of all of them showed up as a brown and granular staining with the same cell and tissue distribution (Fig. 2).

### Table III. Fucosyltransferase activities involved in the biosynthesis of mono- and difucosylated Lewis antigens derived from core type 1 (Le\( ^a / \text{Le}^b \)) and core type 2 (Le\( ^c / \text{Le}^e \)) precursors

<table>
<thead>
<tr>
<th>Acceptor substrate</th>
<th>Enzyme activities assayed</th>
<th>Healthy tissue (( \mu U/mg ))</th>
<th>Tumour tissue (( \mu U/mg ))</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Core type 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacto-( N )-biose 1, ( n = 12 )</td>
<td>( \alpha(1,2) ) FT ( \alpha(1,4) ) FT</td>
<td>65.4 ± 19.0</td>
<td>186 ± 35.1</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Lacto-( N )-fucopentaose 1, ( n = 15 )</td>
<td>( \alpha(1,4) ) FT</td>
<td>64.9 ± 11.9</td>
<td>125.4 ± 20.7</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Le( ^a ), ( n = 8 )</td>
<td>( \alpha(1,2) ) FT</td>
<td>56.2 ± 7.2</td>
<td>130.5 ± 15.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Core type 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( N )-acetyllactosamine, ( n = 18 )</td>
<td>( \alpha(1,2) ) FT ( \alpha(1,3) ) FT</td>
<td>53.4 ± 12.2</td>
<td>108.1 ± 18.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2’-Fucosyllactose, ( n = 16 )</td>
<td>( \alpha(1,2) ) FT ( \alpha(1,3) ) FT</td>
<td>38.9 ± 10.9</td>
<td>143.6 ± 28.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2’-Methyllectose, ( n = 17 )</td>
<td>( \alpha(1,3) ) FT</td>
<td>30.9 ± 4.8</td>
<td>66.1 ± 8.1</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>2’-Fucosyl-( N )-acetyllactosamine, ( n = 14 )</td>
<td>( \alpha(1,2) ) FT</td>
<td>61.3 ± 10.7</td>
<td>126.4 ± 22.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Le( ^c ), ( n = 18 )</td>
<td>( \alpha(1,2) ) FT</td>
<td>54.3 ± 11.9</td>
<td>88.2 ± 14.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>3’-Fucosyllactose, ( n = 15 )</td>
<td>( \alpha(1,2) ) FT</td>
<td>63.2 ± 29.5</td>
<td>70.7 ± 17.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values of specific activity are expressed as \( \mu U/mg \) (pmol/min per mg of protein) and correspond to means ± SEM (standard error of the means). They are the result of duplicate and independent determinations carried out in parallel with healthy and tumour specimens from \( n \) CRC patients. The statistical comparisons were performed according to Wilcoxon’s test. NS: Not significant.
The expression scores (Fig. 3) indicated that healthy mucosa showed a high level of Le\(a\) expression (3) in a remarkable number of specimens (58.3%), while the remainder showed moderate (2) (16.7%) or negative (0) expression (25%). Simultaneously, in the tumour tissue specimens the most important changes were as follows: negative staining in 33.3% of cases, weak expression in 8.3% of cases, and a reduction in the percentage of cases with a high level of expression (41.7%). No change in the number of specimens with moderate expression (16.7%) was observed. The study of Le\(b\) expression revealed specimens of healthy mucosa showing the antigen with an equal distribution among negative (30%), moderate (30%) and high (40%) expression. Meanwhile, among the specimens of CRC tumour tissue, Le\(b\) expression showed the same profile as in healthy tissue, with the exception of a decrease in the number of specimens with negative expression (falling to 20%) and the scoring of weak staining in 10% of them. When these tumour and healthy Le\(a\) and Le\(b\) percentages of expression were compared using Wilcoxon’s test, we found no statistically significant differences.

As for the core 1-derived antigens, the Le\(a\) expression in the control tissue distributed in 50% of specimens with weak (1) expression, 25% with moderate (2) expression and 25% with negative (0) expression. In the case of tumour CRC specimens, 66.7% of them showed negative Le\(a\) expression, 25% moderate and 8.3% high (3) expression. Wilcoxon’s test indicated that these differences of expression were not statistically significant. However, the results of the immunohistochemical study of Le\(x\) antigen in 13 CRC patients differed. Negative expression (61.5%) was predominant in healthy specimens (23.1% weak, and 7.7% moderate/high expression), whereas in tumour specimens Le\(x\) expression was greatly increased, with 84.6% of 13 patients studied being positive: 7.7% weak intensity, 15.4% moderate and 61.5% of specimens showing high

---

Fig. 2. Immunohistological expression of core 1-derived Le\(a\)/Le\(b\) antigens (A and B, respectively) and core 2-derived Le\(x\)/Le\(y\) antigens (C and D, respectively). A. Le\(a\) expression in a type specimen of tumour tissue. B. Le\(b\) expression in a type specimen of tumour tissue. C. Le\(x\) expression in a type specimen of tumour tissue. D. Le\(y\) expression in a type specimen of tumour tissue. Magnification x10 in all cases.
Le\textsuperscript{a} expression. Wilcoxon’s test indicated that the increase in Le\textsuperscript{a} expression in tumour tissue was statistically significant (p < 0.01).

Relationship between fucosyltransferase activity or antigen expression and the clinicopathological features of CRC patients and specimens

Having studied the functional activity of the biosynthetic pathways of mono- and difucosylated Lewis antigens in tumour and healthy-paired CRC specimens, as well as their histological expression, we decided to investigate the correlation between these variables and the most important clinicopathological features of CRC patients and specimens (Table I). The results of this statistical study detected no statistical correlation (r < 0.7) between the degree of expression of either Le\textsuperscript{a}/Le\textsuperscript{b} or Le\textsuperscript{y}/Le\textsuperscript{y} antigens in each specimen and the corresponding enzyme activity of the biosynthetic FT involved. Similarly, comparison of antigen expression, as well as their biosynthetic enzyme activities, with the macroscopic and microscopic characteristics of the tumours (anatomopathological stage, degree of differentiation, lymph node metastasis, existence of disease recurrence and post-operative CEA levels) showed no statistical significance in either case.

DISCUSSION

Initially discovered on red cells, ABH and Lewis-related determinants show a much wider distribution in humans,
being mainly present on foetal epithelia and reexpressed in carcinomas (11). Derived from core 1 and 2 precursors, their structural difference lies in the position of the glycosidic bond between Gal and GlcNAc residues in the non-reducing terminals of glucide chains of protein and lipid carriers (Fig. 1). This positional isomerism allows accessibility to fucosylation by different FT, and therefore the products of their action also differ (H₁/Le⁰/Le⁵ from core 1, and H₁/Le⁰/Le⁵ from core 2). The work herein discussed focused on the functional characterization of terminal fucosylation steps of mono- (Le⁰, Le⁵) and difucosylated (Le⁵, Le⁷) Lewis antigens in healthy and tumour-paired tissue from CRC-resected patients. Additionally, from histological sections of patients undergoing CRC surgical resection (Fig. 2), we evaluated the immunohistochemical expression of these antigens (Fig. 3).

The screening of FT activities necessary for the terminal steps of core 1-derived Le⁵/Le⁰ and core 2-derived Le⁰/Le⁵ synthesis showed a generalized activation in tumour tissue of CRC patients. As table III shows, the activity of all FT evaluated resulted increased in a statistically significant way. In this regard, a corresponding enhancement of Le⁰ and/or Le⁵ expression in tumour tissue would be expected. However, the expression levels of these core 1-derived antigens were not modified in relation to the malignant transformation of CRC tissue (Fig. 3), in spite of previous evidence showing a correlation between the increased expression of Le⁰ and the Dukes’ stage of each specimen (19,26). On the contrary, other studies have reported the uniform Le⁰ distribution throughout the colon before and after the occurrence of CRC (27,28), or even its reduced expression in parallel with the appearance of colorectal malignancy (16,20). In any case, our apparent increase of α(1,2)FT activity in the tumour colonic tissue is in line with histological (29) and α(1,2)FT cDNA-transfected rat colon cell lines (30) studies, and with them we suggest that since this key enzyme acts on core 1, it could be responsible for regulating the expression of both Le⁰ and Le⁵ antigens, in competition with the α(1,4) FT for this common acceptor (29).

The synthesis of colon Le⁵ (and Le⁷) antigen is completed by other α(1,2)FT, which directly fucosylates Le⁰ to Le⁵ (and Le⁷ to Le⁹) (13). The genetic origin of this α(1,2) FT remains uncertain, but all evidence suggests that both activities are associated with the FUT3 gene encoding the α(1,3/4)FT or Lewis enzyme (31,32). The determination of this enzyme activity using Le⁰ as acceptor showed a statistically significant increase in tumour tissue, of the same magnitude as the abovementioned FT activities (Table III). It has been reported that increased cellular Le⁰ fucosylation reduces Le⁰ expression in favour of Le⁵ (29). Conversely, in 8 patients in our cohort we found coexpression of both antigens in the healthy and/or tumour tissue from CRC patients, thus indicating that not all the pool of Le⁰ epitope was transformed into Le⁵, and that the direct fucosylation of Le⁰ to Le⁵ is not a specific feature of the neoplastic status, as previously suggested (33,34).

The results of this study are consistent with the activation of FT responsible for the biosynthesis of Le⁵ and Le⁷ antigens. This fact is relevant, since to date no functional characterization of the terminal steps of their biosynthetic pathway has been undertaken. However, this activation is not the only variable that controls the production of the end products of the pathway—the activity of upstream acting enzymes should also be considered. In the synthesis of gangliosides in melanoma and neuroblastoma, for example, there is competition between sialyltransferases and FT for the same substrates (35). Similarly, our observations in CRC tumour tissue are consistent with the enhancement of core 1 sialylation and channelling of the corresponding associated antigens (i.e. sialyl-Le⁰), (36), to the detriment of their fucosylated counterparts studied herein. So, core 1 would be channeled into sialyl-Le⁰, which would deprive colorectal mucosa of both the direct precursor of Le⁰ (core 1) and the intermediary to render Le⁰ (H ). In line with this hypothesis, it has been reported that the enhancement of α(2,3)sialyltransferases and α(1,3/4)FT is one of the principal mechanisms leading to increased sialyl-Le⁰ (and sialyl-Le⁵) expression in different neoplasias (37).

Regarding the core 2-derived Le⁰/Le⁵ antigens, it is reasonable to propose that activated FT activities (Table III) led to the up-regulation of Le⁰ at the expense of decreased Le⁵ (Fig. 3). The overexpression of Le⁰ may indicate that the predominant activity is that of α(1,2)FT (Fig. 1). This possibility is supported by previous evidence giving this enzyme (H transferase) a key regulatory role in the up-regulation of H₃ and Le⁰ antigens in tumour tissue of CRC patients (38). Moreover, the assay of α(1,3)FT responsible for the synthesis of Le⁰ showed the existence of a statistically significant increase in H₃ fucosylation, thus rendering the Le⁰ antigen, as previously reported (39). This was corroborated by the negative expression found in control and tumour tissue of four CRC patients tested for H₃. As pointed out with antigens derived from core 1, the biosynthesis of antigens derived from core 2 includes the transformation of Le⁰ into Le⁵ by the action of α(1,2) FT Lewis enzyme. The statistically significant increase of this α(1,2)FT activity in tumour tissue of CRC, along with the reduction in Le⁰ expression and the increase in Le⁵, suggest that this enzyme may be partially responsible for the Le⁰ overexpression reported by us and others, and that the enhancement of this FT activity may be closely linked to colorectal carcinogenesis (33).

Several authors have found no significant differences in the expression of Le⁰ in CRC tumour tissue in comparison with healthy mucosa (40). However, our reported change in Le⁰ expression is consistent with those of previous studies (27,28,41,42) which have justified the classical consideration of this determinant as an oncofetal antigen (43) and a premalignant biomarker of distal CRC (18,40). In this regard, it should be noted that tumour biomarkers are not only monitors for diagnosis or monitoring of patients, but represent real characteristics of cancer cells. Thus, the
mechanisms leading to the expression of any tumour marker should be disclosed, since this knowledge will accelerate and make more plausible its translation to clinical therapy.

Taken as a whole, the results of this study confirm the activation of the biosynthetic pathways of mono- and difucosylated Lewis histo-blood antigens in tumour tissue of CRC-responded patients. The kinetic activation of core-2 derived pathway allows us to hypothesize that increased activity on N-acetyllactosamine (core 2) would essentially result from the overactivity of tumour α(1,2)FT (H transferase, 38). Consequently, the activation of this enzyme stimulated the enhanced expression of oncofetal H2 epitope (44), and the overproduction of H1 activated the α(1,3)FT enzyme (this enzyme acts on H2 in preference to core 2 as a substrate) leading to the overexpression of Leα antigen, probably at the expense of Leα (and H2) determinants. In other words, the malignant transformation of the colon causes positive modulation of the biosynthesis pathway of Lewis antigens derived from core 2, which promotes the production of Leα instead of Leα. The functional significance of Leα (and Leα) up-regulation has been linked to the stimulation of cancer propagation and metastasis, since this epitope is expressed by adhesive and cell motility glycoproteins (45), and has been found in glycan homophilic interactions involved in the aggregation of tumour cells or in their adhesion to the vascular endothelium (4). In addition, a role for Leα (Leα and others) in the resistance of epithelial cells to apoptosis has been suggested (11). Based on this evidence, it is not surprising that treatment with anti-Leα antibodies has been found to slow down or even cause regression of colon tumours in animal models (46).

The cohort included in this study was large enough to confirm the activation of the biosynthetic Leα/Leα and Leα/Leα pathways, as well as the Leα overexpression, but not to find significant correlations with the standard clinicopathological features of specimens and patients. The inconsistency between the expression of Lewis antigens and the specific activity of FT involved in their synthesis, as well as their discrepancies with clinicopathological features, could be explained by the low size of the cohort and the variation among individuals. Also, we must keep in mind that scores of antigen expression derived from an immunohistochemical semiquantitative evaluation, while in mind that scores of antigen expression derived from an immunohistochemical semiquantitative evaluation, while immunohistochemical analysis of regional distribution. Am J Pathol 1989;135:111-9.


31. Chandrasekaran EV, Jain RK, Rhodes JM, et al. Expression of blood group Lewis α determinant from Lewis a: Association of this novel fucosyltransferase found in colorectal carcinoma may be encoded by a bi00014a032 fucosyltransferase. Biochemistry 1995; 34: 4748-56. DOI: 10.1021/bi00014a032