Biosynthesis and expression of the Sda and sialyl Lewis x antigens in normal and cancer colon

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The carbohydrate determinants Sda and sialyl Lewis x (sLex) both result from substitution of an α2,3-sialylated type 2 chain: the first with an N-acetylgalactosamine (GalNAc) β1,4-linked to Gal and the second by an α1,3-linked fucose on N-acetylgalcosamine. The Sda antigen is synthesized by Sda β1,4-N-acetylgalactosaminyltransferase II (β4GalNAcT-II), which is downregulated in colon cancer, whereas sLex is a cancer-associated antigen. In view of the possible competition between β4GalNAcT-II and the fucosyltransferases (FucTs) synthesizing the sLex antigen, we investigated whether β4GalNAcT-II acts as a negative regulator of sLex expression in colon cancer. β4GalNAcT-II cDNA, when expressed in LS174T colon cancer cells, induces the expression of the Sda antigen, a dramatic inhibition of sLex expression on cell membranes, and the replacement of sLex with the Sda antigen on 290 kDa glycoproteins. Unexpectedly, in colorectal cancer tissues, the sLex antigen is regulated mainly by (i) Sda and sLex antigens are expressed by different glycoproteins of 340 and 290 kDa, respectively; (ii) the activity of 4GalNAcT-II undergoes a variable degree of downregulation in colon cancer tissues, the Slex antigen is regulated mainly by the total FucT activity on 3'-sialylactosamine acceptors and that β4GalNAcT-II can inhibit sLex expression in an experimental model, although not in colon cancer tissues.

Key words: N-acetylgalactosaminyltransferase/fucosyltransferases/Sda antigen/sialyl Lewis x antigen/colon cancer

Introduction

Oligosaccharide chains covalently linked to glycoproteins and glycolipids regulate a variety of interactions among cells and also between cells and their environment (Varki 1993). Such structures are synthesized through the coordinated action of different glycosyltransferases and undergo profound alterations in pathologic processes, including cancer (Hakomori 1989; Dall’Olio 1996; Dennis et al. 1999). The histo-blood group antigenic determinant Sda is expressed on erythrocytes and found in secretions of approximately 95% of individuals of Caucasian origin. It is formed by an α2,3-sialylated type 2 chain, substituted in the O-4 position of galactose (Gal) with an N-acetylgalactosamine (GalNAc) residue, according to the structure Siaα2,3(GalNAcβ1,4)Galβ1,4GlcNAc-R (Blanchard et al. 1983; Donald et al. 1983). The enzyme responsible for the addition of the immunodominant GalNAc residue is Sda β1,4-N-acetylgalactosaminyltransferase II (β4GalNAcT-II). This enzyme was first described in guinea pig kidney (Serafini-Cessi and Dall’Olio 1983), successively in human kidney (Piller et al. 1986), and subsequently identified in very large amounts in the colon of different species such as human (Malagolini et al. 1989), rat (Dall’Olio et al. 1990), and pig (Malagolini et al. 1994). The cDNA of β4GalNAcT-II was first isolated from murine cytotoxic T lymphocytes (Smith and Lowe 1994), whereas the full-length cDNA of human β4GalNAcT-II was first cloned from the Caco2 colon cancer cell line, independently by our group (Lo Presti et al. 2003) and by others (Montiel et al. 2003). The human gene encodes at least two different transcripts (Figure 1) sharing exons 2–11, although differing for exon 1. Exon 1S and 1L encode the amino terminal portion of the short and of the long forms of β4GalNAcT-II, respectively. The long-form peptide is peculiar due to the very long cytoplasmic domain (67 residues), whereas the cytoplasmic domain of the short form has a more conventional length of seven residues.

In colon cancer, β4GalNAcT-II undergoes a variable degree of downregulation (Malagolini et al. 1989; Dohi et al. 1996). In cancer tissue of some patients, it retains a high level of activity, whereas in others it is almost completely switched off. It is well known that ectopic expression of sialyl Lewis x antigen (sLex) greatly favors the metastatic ability of cancer cells (Hasegawa et al. 1993; Matsu et al. 2004) by allowing the interaction of cancer cells with cell adhesion molecules of the selectin family, which are present on the surface of endothelial cells (Phillips et al. 1990; Takada et al. 1993; Tozeren et al. 1995). However, the molecular basis of the ectopic expression of sLex antigen in colorectal cancer remains unclear. In fact, the mRNA levels of the fucosyltransferases (FucTs) and sialyltransferases directly involved in the biosynthesis of this antigen do not appear to correlate with
Fig. 1. Schematic representation of the short and the long β4GalNAcT-II transcripts. Both forms comprise 11 exons; exons 2–11 are common to both forms, whereas exon 1 is divergent. Exons 1S and 1L are 38 and 253 nt long, respectively, and both contain a translational start site. The two transcripts encode two polypeptides differing in the NH₂ termini. The protein-coding domain is in gray.

the level of sLex antigen expression (Kudo et al. 1998). The downregulation of β4GalNAcT-II might conceivably play a pivotal role in the ectopic expression of sLex antigens in colon cancer. In fact, the close proximity of the sugar residues on the common substrate α2,3-sialylated type 2 chain, on which β4GalNAcT-II and FucTs act, facilitates the competition between these two enzyme classes. This notion is supported by the observation that sLex fails to act as an acceptor for β4GalNAcT-II (Serafini-Cessi et al. 1995). Moreover, the tetrasaccharide Sia₂,3(GalNAc₁,4)Galβ₁,4GlcNAc, prepared in our laboratory by β4GalNAcT-II-mediated addition of GalNAc to 3'-sialyllactosamine (Malagolini et al. 1994), fails to act as a substrate for human colon cancer FucTs (F. Dall’Olio, unpublished observation).

In this work, we have studied the contribution of β4GalNAcT-II and α1,3-FucTs in the biosynthesis of the sLex antigen in human colon cancer cells. By transfecting the short and the long forms of β4GalNAc-II in the cell line LS174T, we have found that the expression of the short form is associated with higher levels of β4GalNAc-II enzyme activity. Both forms can efficiently inhibit sLex expression in LS174T cells. However, in colorectal cancer tissues, β4GalNAcT and sLex expression shows a paradoxically direct relation. This can be explained by the fact that in colon cancer tissue, the two antigens are carried by different glyco-proteins, that in the vast majority of cancer specimens sLex expression is a direct function of β4GalNAcT-II and α1,3-FucTs show a parallel regulation.

Results

Expression of the Sdα antigen by β4GalNAcT-II-transfected clones

We analyzed two clones transfected with the empty vector (Neo1 and Neo2), two clones transfected with the cDNA of the short form (S2 and S11), and two clones transfected with the long form (L20 and L21). The β4GalNAcT-II activity (Figure 2A, black bars) was undetectable inNeo clones and was expressed at a higher level in short-form transfectants than in the long-form transfectants. The β4GalNAcT-II mRNA, determined by northern blot analysis (Figure 2B) after normalization with the β-actin transcripts (Figure 2A, white bars), was undetectable in Neo clones; in long-form transfectants, it was expressed at almost 2-fold higher level compared with short-form transfectants. The level of Sdα antigen expression, determined by dot blot analysis (Figure 2C), was about the same in the four β4GalNAcT-II-transfected clones (Figure 2A, gray bars), regardless of the level of enzyme activity. Collectively, these data indicate that both the short and the long cDNA forms induce β4GalNAcT-II activity, whereas the former is more efficient than the latter and that the level of the expression of the Sdα antigen is relatively independent of the level of β4GalNAcT-II activity.

Inhibition of sLex expression by β4GalNAcT-II transfectants

Figure 3 shows that both forms of β4GalNAcT-II are able to reduce sLex expression, whether detected by FACS analysis (A) or dot blot analysis (B). On the basis of dot blot analysis, we calculated an approximately 75% reduction of sLex antigen expression in clones S2, S11, and L20 and a 35% reduction in clone L21. To rule out the possibility that the different level of sLex in the six clones was due to clonal differences in α1,3/4-FucT activities, we measured the level of FucT activity toward type 2 chains using a α2,3-sialylated acceptor (3'-sialyllactosamine) in the six clones and found it to be very similar (data not shown). Inhibition of sLex expression by β4GalNAcT-II was confirmed by immunofluorescence microscopy (Figure 4A), which showed the absence of anti-Sdα antibody reactivity in Neo1 cells and a granular, membrane-associated pattern of reactivity in S2 cells. As expected, on the basis of FACS and dot blot data, in S2 cells the expression of the sLex antigen on the cell membranes was markedly attenuated, although not completely abolished. Western blot analysis (Figure 4B) revealed that in these cells, the Sdα antigen was mainly expressed by high molecular weight glycoproteins with an approximate 290 kDa size, as well as by glycoproteins of approximately 190 kDa. The sLex antigen was mainly expressed in mock-transfected cells on 290 kDa glycoproteins, a molecular weight identical to that of those carrying the Sdα antigen, although not on 190 kDa glycoproteins.
Interestingly, in β4GalNAcT-II-expressing cells, on 290 kDa glycoproteins, the sLex antigen was replaced by the Sd α antigen, strongly suggesting that the two antigens can be carried by the same 290 kDa glycoprotein. Analysis of the presence of the two antigens in the extracellular medium (Figure 4C) revealed that both antigens are secreted actively and that the expression of β4GalNAcT-II also reduces the expression of the sLex antigen in soluble form. These data indicate that both the long and the short forms of β4GalNAcT-II are able to reduce sLex expression on membranes and secretions of LS174T cells by replacing the sLex antigen with the Sd α antigen on a specific class of high molecular weight glycoproteins. The level of inhibition did not appear to be proportional to the level of β4GalNAcT-II activity, provided that a given threshold of activity was reached.

Expression of Sdα antigen in normal colonic tissue
The distribution of the Sdα antigen in normal human colonic mucosa is shown in Figure 5. In Figure 5A, the longitudinal section of a gland with active secretion of Sda-positive material into the lumen is shown (arrows). As evident in Figure 5B, Sda reactivity is mainly associated with goblet cells, whereas Figure 5C shows very intense secretion in the gut lumen (arrows). These data indicate that in normal colon, Sdα reactivity is mainly associated with goblet cells and their secretion. Among enterocytes, strongest reactivity was displayed by those most differentiated, located in the upper portion of the gland.

Expression of β4GalNAcT-II in normal and cancer colonic specimens
The β4GalNAcT-II enzyme activity expressed by normal and cancer tissues from 10 patients is reported in Figure 6A. The patients were ordered according to a proximal/distal order of the anatomical location of the tumor (Table I). In normal colon, β4GalNAcT-II was expressed according to a decreasing proximal-to-distal gradient. The low level of enzyme activity detected in a few cases (patients 1, 8, 10) was probably related to an individually weak Sdα expression status in
these patients, whereas the β4GalNAcT-II activity expressed by cancer tissues was completely independent of the anatomical location of the tumor. With the exception of patient 10, who was also the only stage A patient examined, the activity in cancer tissues was much lower than that of normal mucosa. However, the extent of reduction was markedly different among specimens. In fact, the residual activity in carcinoma ranged from 76% in patient 9 to 4% in patient 5.

The expression of the two types of β4GalNAcT-II transcript was investigated in cancer tissues by real-time polymerase chain reaction (PCR) analysis (Table II). Both transcripts were expressed, although the short form appeared to be expressed at a higher level. Moreover, the expression of the two forms shows a parallel expression ($P < 0.0001$, according to the linear regression analysis), suggesting a coordinate regulation of their transcription.

**Relation between β4GalNAcT-II, Sda, and sLex expression in normal and cancer colon specimens**

The expression of Sda and sLex antigens in the normal and cancer colon specimens was detected by dot blot analysis (Figure 6B). Consistent with enzyme activity data (Figure 6A), patient 10 was the only one showing a higher Sda expression in the cancer with respect to normal tissue. However, the level of Sda antigen expression did not appear to be closely associated with that of enzyme activity, especially in normal tissues. According to data reported in the literature (Ogata et al. 1995; Mann et al. 1997), sLex antigen is present in normal colon, although its expression is masked by O-acetylation of sialic acids. In fact, we found that in native dot blots (non-O-deacetylated), sLex was expressed only by a few normal tissues (patients 8 and 9) at low levels, whereas in alkali-treated blots (O-deacetylated) the antigen became detectable. O-Deacetylation did not modify the reactivity with anti-Sda antibody (data not shown). The quantitative relation between Sda antigen and β4GalNAcT-II activity was not statistically significant in normal tissues (Figure 6C) but was significant ($P = 0.041$) in cancer tissues (Figure 6D). Consistent with the data of the in vitro study, we observed a tendency toward an inverse relation between β4GalNAcT-II activity and sLex expression in O-deacetylated normal samples ($P = 0.16$) (Figure 6E).

Unexpectedly, we observed a highly significant direct linear relation between the two parameters in cancer tissues ($P < 0.001$) (Figure 6F), which indicates that the higher the β4GalNAcT-II activity, the higher the sLex expression. To explain the lack of inhibition of sLex expression by β4GalNAcT-II in colon cancer tissues, we analyzed the glycoproteins expressing the two antigens by western blot analysis (Figure 6G). Unlike in the LS174T cell line, in colon cancer tissues the two antigens were carried by glycoproteins of different molecular weights. Namely, the Sda antigen was carried by glycoproteins with an approximate molecular weight of 340 kDa, whereas the sLex antigen was expressed by glycoproteins of 290 kDa, a similar size if not identical to those of the sLex-positive glycoproteins in LS174T cells. This finding explains the lack of inhibition of sLex expression by β4GalNAcT-II, although it does not explain the positive relation between the two parameters.

**α1,3/4-FucTs in colon cancer specimens**

To investigate the molecular bases of sLex expression in colonic tissues and of the direct relation between β4GalNAcT-II and sLex in colon cancer specimens, we measured the activity of the α1,3-FucTs involved in sLex biosynthesis in normal and cancer tissues, using α2,3-sialylated type 2 chain as acceptor (Figure 7A). The FucT activity in cancer tissues was higher than that in normal tissues ($P = 0.013$, according to the Student $t$ test for paired samples). The only exception was in patient 1, which was a highly undifferentiated cancer. The plot of the FucT activity versus the sLex antigen expression, detected by dot blot analysis of O-deacetylated samples (Figure 6B), does not reveal a significant positive relation in normal tissues (Figure 7B), whereas in cancer tissues (Figure 7C) there is an obvious direct linear relation for 8 patients out of 10; patients 3 and 8 behaved differently. The FucT--sLex relation for these eight patients was
highly significant \((r = 0.985; P < 0.0001)\). These data indicate that in the vast majority of the colon cancer cases, the expression of sLex antigen was a direct function of the level of \(\alpha_1,3\)-FucT activity toward \(\alpha_2,3\)-sialylated type 2 chains. In the majority of patients, there was a statistically significant positive linear relation between FucT activity toward \(\beta_4\)-galactosaminyltransferase II (\(\beta_4\)GalNAcT-II) activity \((r = 0.808; P = 0.015)\) (Figure 7D).

Different FucT activities, namely FucT-III, -IV, -V, -VI, and -VII, can contribute to sLex biosynthesis. A previous study (Kudo et al. 1998), using conventional reverse transcription–PCR (RT–PCR), reported the presence of FucT-III, -IV, and -VI transcripts, and not FucT-V and -VII in colon cancer tissues, whereas another study (Koike et al. 2004), using real-time PCR, reported an elevation of FucT-VII transcript in colon cancer tissues. To address the contribution of the different FucT genes to the FucT activity, we preliminarily...

### Table I. Clinical features of the patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor location</th>
<th>Stage a</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77</td>
<td>M</td>
<td>Cecum</td>
<td>D</td>
<td>T3N2M1</td>
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<tr>
<td>2</td>
<td>78</td>
<td>F</td>
<td>Cecum</td>
<td>C1</td>
<td>T3N2M0</td>
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<tr>
<td>3</td>
<td>54</td>
<td>M</td>
<td>Ascending colon</td>
<td>B2</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>M</td>
<td>Ascending colon</td>
<td>B2</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>F</td>
<td>Transverse colon</td>
<td>C1</td>
<td>T3N1M0</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>M</td>
<td>Transverse colon</td>
<td>B2</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>M</td>
<td>Descending colon</td>
<td>C1</td>
<td>T3N1M0</td>
</tr>
<tr>
<td>8</td>
<td>66</td>
<td>F</td>
<td>Sigma</td>
<td>B2</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>F</td>
<td>Rectum</td>
<td>B2</td>
<td>T3N0M0</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>M</td>
<td>Rectum</td>
<td>A</td>
<td>T3N0M0</td>
</tr>
</tbody>
</table>

\(a\)According to Astler–Coller-modified Duke classification of tumors.
analyzed the 10 colon cancer specimens by conventional RT–PCR analysis, finding no expression of FucT-V (data not shown). Then we quantitated by real-time PCR the level of expression of FucT-III, IV, -VI, and VII. The values, normalized to the constitutively expressed gene β-actin, are reported in Table II. In considering these data, it should be kept in mind that this type of analysis does not allow a direct comparison between the levels of expression of the different transcripts, but only of each transcript in different samples. These data indicate that FucT-III, -IV, -VI, and VII contribute to the total FucT activity toward 3′-sialylated acceptors in colon cancer tissues. Statistical analysis revealed a significant positive correlation between the transcript level of the shared region of β4GalNAcT-II and that of FucT-III (P = 0.015) and FucT-VII (P < 0.0001), thus providing a basis for the parallel expression of β4GalNAcT-II and FucT activities observed.

**Discussion**

In this work, we have shown that both the long and the short forms of β4GalNAcT-II are able to induce the synthesis of the Sda antigen and that the expression of the short form is associated with a higher level of enzyme activity. It is not clear at the moment whether this difference is due to a higher translational rate of the short-form transcript, or to a higher stability of the short-form protein, or to a higher catalytic efficiency of the short-form enzyme. Whatever the mechanisms, it is possible that through the differential expression of the two transcripts, the cell can achieve a fine regulation of the β4GalNAcT-II activity level. In previous works, we determined that the Km value for donor substrate was 75 μM for β4GalNAcT-II from human colon (Malagolini et al. 1989) and 66 μM for β4GalNAcT-II from human urine (Serafini-Cessi et al. 1988). Owing to the fact that the type of β4GalNAcT-II which is produced by the kidney and secreted with urine is unknown at the moment, it is not clear whether this small difference is due to the different tissue origin of the two enzymes or to other reasons.

As expected on the basis of the competition between β4GalNAcT-II and FucTs for α2,3-sialylated N-acetyllactosaminic units, the expression of β4GalNAcT-II inhibits that of sLex in LS174T cells. However, in partial contrast to a previous study showing a complete inhibition of sLex expression in colon and gastric cancer cells transfected with the long form of β4GalNAcT-II (Kawamura et al. 2005), in our model

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**Table II.** Expression level of β4GalNAcT-II and FucT transcripts determined by real-time PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>β4GalNAcT-II</th>
<th>FucT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Shared region</td>
<td>Short form</td>
</tr>
<tr>
<td>1</td>
<td>0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>2</td>
<td>3.01</td>
<td>5.61</td>
</tr>
<tr>
<td>3</td>
<td>8.16</td>
<td>7.13</td>
</tr>
<tr>
<td>4</td>
<td>0.69</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>7.44</td>
<td>3.25</td>
</tr>
<tr>
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<td>4.16</td>
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<td>7</td>
<td>26.29</td>
<td>15.10</td>
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<tr>
<td>8</td>
<td>1.27</td>
<td>1.11</td>
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<tr>
<td>9</td>
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<td>0.71</td>
</tr>
<tr>
<td>10</td>
<td>18.82</td>
<td>15.07</td>
</tr>
</tbody>
</table>

The values represent the level of glycosyltransferase transcripts normalized to the constitutively expressed gene β-actin, according to the equation 2^−ΔΔCT × 1000, as described in the Materials and methods section. The data are representative of three independent determinations performed in triplicate.

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**Fig. 7.** α1,3-FucT expression in colon specimens. (A) The FucT activity in the normal (gray bars) or cancer (black bars) tissue homogenates of the 10 patients (nmol/mg protein h) was measured using 3′-sialyllactosamine as acceptor. The data are the mean ± SD of three independent determinations. The activity is increased in the cancer tissues (P = 0.013), with the only exception of patient 1. (B) Scatter plot of the FucT activity versus sLex antigen expression in normal specimens; the two parmers do not show a significant relation. (C) The scatter plot of the FucT activity versus sLex antigen expression in cancer specimens reveals an obvious direct linear relation for eight patients (P < 0.0001), whereas two patients (patients 3 and 8, indicated) show a different behavior. (D) Scatter plot of the FucT activity versus the β4GalNAcT-II activity: with the exception of patient 3, the FucT activity parallels the β4 GalNAcT-II activity (P = 0.04).
system the degree of inhibition reached by β4GalNAcT-II expression did not exceed 80%, even though the level of β4GalNAcT-II expression in clones S2 or S11 was of the same order as that of normal colon. In addition, we have shown that β4GalNAcT-II expression induces an active secretion of Sda-positive glycoconjugates, inhibiting the secretion of sLex-active compounds. This observation is consistent with the data reporting the presence of the Sda antigen and the absence of sLex antigen on mucins secreted by normal colon (Capon et al. 2001).

In normal colon, β4GalNAcT-II is expressed at a highly variable level according to a proximal/distal gradient of the anatomical location, although it is also strongly dependent on individual variability. Histochemical detection has revealed that the Sda antigen is mainly associated with goblet cells and their secretions and with highly differentiated enterocytes located in the apical position of the gland. This is consistent with our previous data showing that in the Caco2 colon cancer cell line, enterocytic differentiation was associated with our previous data showing that in the Caco2 colon cancer cell line, enterocytic differentiation was associated with β4GalNAcT-II activity elevation (Malagolini et al. 1991) and reinforces the notion of a strict relation between β4GalNAcT-II and cell differentiation (Dall'Olio et al. 1990).

The striking direct, rather than inverse, relation that links β4GalNAcT-II and sLex in colon cancer specimens can be explained by the fact that, unlike in the LS174T cell line, in colon cancer tissues the glycoproteins carrying the Sda and sLex antigens are different, ruling out the possibility of a competition for the acceptor between the two types of glycoconjugate. Moreover, the total FucT activity toward α2,3-sialylated type 2 chains and β4GalNAcT-II activity show a parallel regulation. Thus, unlike in the LS174T cell line, in which the FucT level was similar in the six clones examined, in colon cancer tissues, the higher the β4GalNAcT-II, the higher the FucT activity. Owing to the fact that the acceptor glycoproteins of β4GalNAcT-II and FucTs are different, the higher sLex expression in specimens with higher β4GalNAcT-II is simply explained by a higher FucT level. The addition of fucose to type 2 chains can be mediated by at least five FucTs, namely FucT-III, -IV, -V, -VI, and -VII. Using competitive RT–PCR analysis, it was found (Kudo et al. 1998) that FucT-V and FucT-VII are expressed at a very low level or not at all in colonic tissues, whereas FucT-IV transcript was found to be upregulated in colon cancer tissues (Ito et al. 1997; Kudo et al. 1998), whereas no relation was found between FucT-IV overexpression and sLex expression in cancer tissues. Moreover, transfection experiments have shown that FucT-IV is the least efficient in sLex biosynthesis, being oriented toward the biosynthesis of Lex antigen (Kimura et al. 1997; Grabenhorst et al. 1998). In another study (Koike et al. 2004), the FucT-VII transcript was found to be elevated in colon cancer tissues by real-time PCR, whereas no relation with the relative sLex level was reported. In this study, by real-time PCR analysis, we failed to observe a significant correlation between the individual FucT transcripts and the level of FucT activity or the sLex level, suggesting that the total FucT activity in colon cancer tissues results from the contribution of several independently regulated genes. On the other hand, we found a significative correlation between the level of βGalNAcT-II transcript and those of both FucT-III and FucT-VII, thus providing a basis for the observed β4GalNAcT-II/Fuc-T correlation at the enzyme activity level.

This study, which reports the parallel measurement of the level of glycosyltransferase transcripts, of the cognate enzyme activity, and of the sugar antigen product in a series of clinical specimens, allows some general conclusions to be drawn on the quantitative relation between these parameters. We observed a good correlation between enzyme activity and sugar antigen products (β4GalNAcT-II/Sda and α1,3Fuc-T activity/sLex) in cancer tissues but not in normal tissues. On the other hand, the quantitative relation between glycosyltransferase transcripts and enzyme activity is usually poor. There are several reasons that can explain these apparent discrepancies. The lack of a strict enzyme–product relation can be explained by the fact that the biosynthesis of a terminal structure, such as the Sda or the sLex antigens, is the product of a complex series of enzymatic reactions, involving glycosyltransferases of several classes. Thus, it is conceivable that the level of expression of the antigen is not necessarily related only to the level of expression of the last enzyme of the series. The lack of a close quantitative relation between glycosyltransferase transcript and enzyme activity could be explained by different efficiencies of the translational machinery in different tumor tissues, but also the mechanisms affecting protein turnover or posttranslational modifications of the enzyme proteins could play a role in determining the steady state of enzyme molecules. These notions are supported by a previous study from our group (Dall'Olio et al. 2001) showing that in cells transfected with the cDNA of ST6Gal.I driven by a constitutive promoter, the ST6Gal.I activity is also not expressed at a constant level. This finding can be partially explained by the existence of posttranslational mechanisms of regulation.

The detection of the sLex antigen by dot blot analysis after O-deacetylation allows a previously unrecognized relation with glycosyltransferase expression for at least two reasons: first, the use of dot blot analysis permits the measurement of sLex antigen expression in a single experiment, regardless of the nature of the glycoconjugates carrying the molecule; secondly, the O-deacetylation of the blots is necessary to measure sLex antigen expression in those cases, including colon cancer, in which the presence of O-acetyl groups on sialic acid hides the presence of the antigen.

In conclusion, our study shows that β4GalNAcT-II has the potential to inhibit sLex biosynthesis by competing with FucTs, provided that the substrate glycoproteins are the same, as occurs in LS174T, and the FucTs are not upregulated. In human colon cancer tissues, these conditions do not appear to be satisfied.

Materials and methods

Construction of the β4GalNAcT-II transfectants

Five micrograms of total RNA from the human colon cancer cell line Caco2 were reverse-transcribed using a TaKaRa RT–PCR kit, version 2.1 (TaKaRa, Shouzo, Japan) according to the manufacturer’s instructions, using random 9mers as primers in a final volume of 20 μL. Two microliters of the cDNA reaction mixture was used for PCR amplification of the β4GalNAcT-II long and short forms, using forward primer L.16 (5’-CACCATGGGAGCGCTGGCTTC-3’) or L.19 (5’-CACCATGACTTCCGCGGCTCG-3’) for the
long and the short forms, respectively, and the common reverse primer R.10 (5'-CCAGTAACTGACCATTTCCC TTTC-3') with a preliminary denaturing step of 94°C, 1 min; then 35 cycles of the following program: denaturing 94°C, 1 min; annealing 60°C, 1 min; extension 72°C, 2 min; followed by a final extension of 5 min at 72°C. Each reaction contained, in a final volume of 50 μL, 2 μL of cDNA, 1× Taq polymerase buffer, 1.7 mM MgCl₂, 0.2 mM dNTPs, 250 nM each primer, and 0.5 U of PfTurbo DNA polymerase (Stratagene, La Jolla, CA). The gel-purified PCR products were cloned directly in the pC DNA3.1 Directional TOPO Expression vector (Invitrogen, Paisley, UK). The two forward primers used for PCR amplification contain the CACC sequence (in bold), as recommended by Invitrogen, which acts as the Kozak consensus sequence and allows insertion in the TOPO expression vector. The presence of the insert in proper orientation in four randomly chosen colonies was confirmed by sequencing. The cell line LS174T (a kind gift of Professor Vincenzo Macchia, Dipartimento di Biologia e Patologia Cellulare e Molecolare L. Califano, University of Naples, Naples, Italy) was routinely grown in Dulbecco’s Modified Eagle medium (GIBCO, Paisley, UK) containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Subconfluent cells were transfected by the calcium phosphate method with either construct or empty vector and subjected to selection in the presence of 0.4 μg/mL G418 (Sigma, St Louis, MO). Resistant clones were isolated with cloning cylinders, expanded, and screened for the expression of β4GalNAcT-II activity.

**β4GalNAcT-II and FucT activities**

For the determination of β4GalNAcT-II enzyme activity, cell pellets or tissues were homogenized in ice-cold water, and the protein concentration of the homogenates was measured by the Lowry method (Lowry et al. 1951). β4GalNAcT-II enzyme activity was measured in whole homogenates, essentially as described previously, using fetuin N-linked glycopeptide as an acceptor (Malagolini et al. 1989). Briefly, the assay mixture contained in a final volume of 25 μL, 80 mM Tri-HCl buffer, pH 7.5, 10 mM MnCl₂, 0.5% Triton X-100, UDP-[³H]GalNAc (ARC, St Louis, MO) at a specific activity of 550 dpm/μmol, 2 mM ATP, 250 μg of fetuin N-linked glycopeptide, prepared by pronase digestion of fetuin (Sigma) (Serafini-Cessi and Dall’Olio 1983) or fetuin asialo glycopeptide (prepared by desialylation of fetuin in 50 mM H₂SO₄, 80 °C, 2 h, followed by extensive dialysis) as acceptors and 10–20 μg of protein homogenates as the enzyme source. After 3 h at 37°C, the reaction was stopped by addition of 0.5 mL of phosphate-buffered saline (PBS; 20 mM Na/phosphate buffer, 130 mM NaCl), boiled for 2 min, centrifuged for 5 min in a minifuge at the maximum speed, and loaded on a Bio-Gel P-30 (Bio-Rad Laboratories, Hercules, CA) column (1.5 × 27 cm) equilibrated with PBS. Fractions coeluting with original fetuin glycopeptide were counted for radioactivity. Each specimen was analyzed in triplicate. FucT activities were measured in whole cell or tissue homogenates, essentially as described (de Vries et al. 1995; Borsig et al. 1999), with some modifications. Briefly, the assay mixture contained, in a final volume of 50 μL, 50 mM Na/cacodylate buffer, pH 6.5, 15 mM MnCl₂, 0.5% Triton X-100, 5 mM ATP, 100 μM unlabeled GDP–fucose (Sigma), 55 000 dpm GDP-[¹⁴C] fucose (925 Bq/μL, 10.8 GBq/mmol) (Amersham, Little Chalfont, UK), and 0.1 mM 3′–sialyllactosamine (Sigma). Reactions were incubated at 37°C for 3 h, then they were diluted with 200 μL of ice-cold water and loaded on 1 mL columns of Dowex 1X8 (formate form) equilibrated with water. The columns were washed with 2 mL of water. The effluent was collected in liquid scintillation vials and counted. The radioactivity measured in the absence of acceptor was subtracted.

**Northern blot analysis**

Ten micrograms of total RNA was electrophoresed on a formaldehyde denaturing gel. Blotting and hybridization were performed as described previously (Chiricolo et al. 2006).

**Western blot and dot blot analyses**

One hundred micrograms of cell or tissue homogenates were electrophoresed under reducing conditions on a 6% polyacrylamide gel (14.5 × 15.5 cm) and electrotransferred to a Hybond nitrocellulose membrane (Amersham). For dot blot analysis, different amounts of the homogenates used for enzyme assay or of conditioned media were applied to Hybond membrane with the use of a manifold. Blots were blocked by incubation for 1 h at room temperature with 2.5% blocking reagent (Roche, Milan, Italy) in PBS containing 0.1% Tween-20 (PBS-T). After three washings with PBS-T, blots were incubated for 1 h at room temperature with anti-sLex antibody, precipitated from the culture media of hybridoma CSLEX1 (ATCC HB85-80), diluted 1:300 or with anti-Sd⁺ KM694 antibody, kindly provided by Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan, diluted 1:1000. Blots were incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG or anti-IgM secondary antibodies (Sigma), diluted 1:3000 and washed as described earlier. The reaction was then developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer’s instructions and detected with an autoradiography film. Intensity of the dots was quantitated by the Kodak 1D image software. O-Deacetylation of the blots was obtained by treatment with 0.1 N NaOH for 20 min at room temperature (Murayama et al. 1997), followed by three washings in PBS-T, before treatment with blocking reagent.

**Immunofluorescence**

For flow cytometry analysis, cells were released by trypsin treatment and stained with anti-sLex antibodies diluted 1:300 in PBS–bovine serum albumin (BSA) for 20 min in ice. After a wash by centrifugation in PBS–BSA, cells were reacted with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma) diluted 1:300 with PBS–BSA for 20 min in ice. After a wash by centrifugation, cells were analyzed by FACS. For fluorescence microscopy, cells were seeded 48 h before staining on glass coverslips. Staining was performed with primary anti-Sd⁺ antibodies or anti-sLex antibodies, followed by secondary FITC-labeled goat anti-mouse IgM or anti-mouse IgG (Sigma), respectively. Details of the staining procedure have been reported elsewhere (Chiricolo et al. 2006).

**Surgical specimens**

Patients underwent surgery for colorectal cancer during 1991–1992 at the Bologna University Hospital. This study was
approved by the Senior Committee Board regulating noninterventional studies, comparable with the Institutional Prereview Board. The clinical features of the patients are reported in Table I. After a careful examination by the pathologist, a portion of normal and cancer tissues was dissected for biochemical studies, snap-frozen in liquid nitrogen and conserved at −80 °C. From these tissues, homogenates were prepared as described earlier, with the use of a potter homogenizer, and RNA was prepared with RNAzol B (Bio tex Laboratories, Houston, TX).

RT–PCR analysis
Preliminarily, all RNA preparations were subjected to DNase treatment with Turbo DNA-free (Ambion, Huntingdon, Cambridgeshire, UK) according to the manufacturer’s instructions. cDNAs were prepared as described earlier, using 5 μg of DNase-treated RNAs. For real-time PCR analysis, cDNA samples were diluted 20-fold with water and PCR amplified with Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the following primer with the primer shared region of Biosystems, Foster City, CA) with the following primer

BGSACACAGAGGCAGCTTC-3
GGGGCATCACCTCCACCTC-3

ATATCCCGATG-3

TTCCGAAC-3

pairs. Shared region of Biosystems, Foster City, CA) with the following primer

product size 143 nt; FucT-VII: FUT7L.4 (5-CA
GGGGCATCACCTCCACTC-3’, product size 158 nt; short form of B4GalNAcT II: GalNAcL.25 (5'-ATGACTTCCGGGC GGCTCGA-3’)/GalNAcR.34 (5'-TTGAAGGAAGATGC TTCCGAC-3’), product size 124 nt; long form of B4GalNAcT II: GalNAcL.24 (5'-CCACGTTGAGTGGCC TCT-3’)/GalNAcR.33 (5’-GGTCAGCGCTCTGA-GTCC-3’), product size 116 nt; FucT-III: FUT3L.4 (5’-ACCTAC AGGCTCGCTGAC-3’)/FUT3R.4 (5’-GTCGCTGAAT ATATCCGATG-3’), product size 113 nt; FucT-IV: FUT 4L.2 (5’-GGCTCTCAGGGAGGGCTCA-3’)/FUT4R.2 (5’-ATCCACCTCTCGGCAGTGT-3’), product size 114 nt; FucT-VI: FUT6L.3 (5’-TGGGAGACCACCGATTT-3’)/FUT6R.3 (5'-ACCACGCAGCAAGGAA-GG-3’), product size 143 nt; FucT-VII: FUT7L.4 (5’-CTCCTGGCT CCTGTGGCTGCT-3’)/FUT7R.4 (5’-AGGCTGGGTT GGCACGACTG-3’), product size 173 nt; β actin: ACTL.4 (5’-TGCCATGGATGTGGGCTCAAGAT-3’)/ACTR.4 (5’-CGT CCGATGTTGAGACAGTG-3’), product size 108 nt. To assure the ampiclon specificity of each primer set, the products were then subjected to a melting curve analysis. For the quantitation of the glycosyltransferase transcripts normalized to the constitutively expressed gene β actin, the cycle threshold (CT) for glycosyltransferases and β actin for each sample was calculated. A normalized target value was then derived by subtracting the amount of glycosyltransferase by that of β actin for each sample was calculated and by the equation 2-ΔΔCT × 1000.

References


FucT-III–FucT-VII, fucosyltransferases III–VII; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylgalactosamine; PBS, phosphate-buffered saline; PBS–BSA, PBS containing 1 mg/mL BSA; PBS–T, PBS–TWEEN; RT–PCR, reverse transcription–polymerase chain reaction; Sia, sialic acid; sLex, sialyl Lewis x; TBS, Tris-buffered saline; TBS–BSA, PBS containing 1 mg/mL BSA.

Conflict of interest statement
None declared.

Abbreviations

BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; FucT-III–FucT-VII, fucosyltransferases III–VII; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylgalactosamine; PBS, phosphate-buffered saline; PBS–BSA, PBS containing 1 mg/mL BSA; PBS–T, PBS–TWEEN; RT–PCR, reverse transcription–polymerase chain reaction; Sia, sialic acid; sLex, sialyl Lewis x; TBS, Tris-buffered saline; TBS–BSA, PBS containing 1 mg/mL BSA.

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