Suppression of β1,3galactosyltransferase β3Gal-T5 in cancer cells reduces sialyl-Lewis a and enhances poly N-acetyllactosamines and sialyl-Lewis x on O-glycans

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We investigated the role of β3Gal-T5, a member of the β1,3galactosyltransferase (β1,3Gal-T) family, in cancer-associated glycosylation, focusing on the expression of sialyl-Lewis a (sLeα) and sialyl-Lewis x (sLeβ) antigens. A clone permanently expressing an antisense fragment of β3Gal-T5 was obtained from the human pancreas adenocarcinoma cell line BxPC3 and characterized. Both β1,3Gal-T activity and sLeβ expression are dramatically impaired in the clone. Analysis of the oligosaccharides synthesized in cells metabolically labelled with tritiated galactose shows that a relevant amount of radioactivity is associated to large O-glycans. Endo-β-galactosidase mostly releases NeuAcα2-3Galβ1-3(Fucβ1-4)GlcNAcβ1-3Gal and NeuAcα2-3Galβ1-3(Fucβ1-4)GlcNAcβ1-3Gal from such O-glycans of BxPC3 membranes, but GlcNAcβ1-3Gal and type 2 chain oligosaccharides, including NeuAcα2-3Galβ1-4(Fucβ1-3)GlcNAcβ1-3Gal, from those of the antisense clone.

Furthermore, BxPC3 cells secrete sLeα in the culture media but not sLeβ, while antisense clone secretes mostly sLeβ, and accumulation of both antigens is prevented by benzyl-x-GalNAc. These data indicate that β3Gal-T5 suppression turns synthesis of type 1 chain O-glycans to poly N-acetyllactosamine elongation and termination by sLeβ. In other cell lines and clones, β3Gal-T5 transcript, β1,3Gal-T activity, and sLeβ antigen are also correlated, but quantitatively the relative expression ratios are very different from cell type to cell type. We suggest that β3Gal-T5 plays a relevant role in gastrointestinal and pancreatic tissues counteracting the glycosylation pattern associated to malignancy, and is necessary for the synthesis and secretion of CA19.9 antigen, whose expression still depends on multiple interacting factors.

Keywords: galactosyltransferase; gastrointestinal cancer; Lewis antigen; O-glycan; poly N-acetyllactosamine.

Aberrant glycosylation of glycoproteins and glycolipids is one of many molecular changes that accompany malignant transformation [1]. Perhaps the best known glycosylation change inducing malignancy is enhanced β1,6GlcNAc branching of N-glycans, leading to poly N-acetyllactosamine sequences frequently terminated by the sialyl-Lewis x (sLeβ) antigenic determinant [2]. GnT-V activity is mostly responsible for this as shown by several pieces of evidence obtained in vitro [3,4], and more recently in vivo [5].

Moreover, several studies indicated that O-glycan biosynthesis is also abnormal in cancer cells [6]. It has been shown that sLeα and poly N-acetyllactosamines are associated with increased malignancy of lung and colorectal cancers [7,8], and occur in core 2 and extended core 1 O-glycans in various cells [9,10]. On the other hand, the role of type 1 chain oligosaccharides in cancer-associated glycosylation is unclear. Although type 1 chain structures occur on all glycoconjugate classes, and CA19.9 antigen – that is the sLeα epitope carried by a mucin backbone [11] – has been used as a tumour marker in clinical practice for several years, little is know about their biosynthesis and differential expression in cancer. β1,3Gal-T activity was found to be reduced in colon cancer with respect to the normal mucosa [12], and in the CACO-2 cell model of intestinal differentiation β1,3Gal-T activity [13] and type 1 chain structures [14] were reported to increase with the differentiation process. β3Gal-T5 is the member of the β3Gal-T gene family that was proposed to be responsible for β1,3Gal-T activity and type 1 chain synthesis in epithelial cells of the digestive tract [15]. In a previous paper [16] we reported that β3Gal-T5 efficiently adds β1,3Gal residues to GlcNAcβ1-3Galβ1-4GlcNAcβ1-R branched chains of N-glycans, leading to Leβ and sLeα synthesis, and preventing poly N-acetyllactosamine extension and sLeβ expression. We also found that the β3Gal-T5 transcript is downregulated in colon...
but it has not yet been demonstrated in vivo if it works on O-glycans that are assumed to be largely expressed in epithelial cells and to be the more relevant carriers of sLe^a epitope in CA19.9 mucin. As no other member of the β3Gal-T gene family known at present is expressed in epithelial cells and able to synthesize type 1 chain oligosaccharides, the very low levels of β3Gal-T5 transcript detectable in colon cancer specimens pose the question of whether relevant amounts of type 1 chain O-glycans are formed in cancer cells.

To address these issues, we tried to study the effect of β3Gal-T5 suppression in the human pancreatic adenocarcinoma cell line BxPC3 that expresses low levels of β3Gal-T5 transcript but well detectable amounts of β1,3Gal-T activity and sLe^a, that is presumably carried by O-glycans and even secreted into the culture medium. To this purpose we transfected the cells with a β3Gal-T5 cDNA fragment placed in the antisense orientation under the control of a strong promoter, and isolated a recombinant clone that stably expresses high levels of the antisense transcript. We then measured the β1,3Gal-T activity present in the antisense clone, as well as the Lewis antigens expressed on the cell surface or secreted in the culture medium. We also studied the radioactive sugar chains synthesized in parental BxPC3 cells and in the recombinant antisense clone upon metabolic labelling with triitated Gal, with emphasis on O-glycans and poly N-acetyllactosamines. We also compared the amount of β3Gal-T5 transcript and β1,3Gal-T activity with the levels of sLe^a expressed in other cell lines and clones.

**Experimental procedures**

**Cell cultures and treatments**

COLO-205, HCT-15, CACO-2, HT-29, SW-1116 (from human colon adenocarcinomas), and MKN-45 (from human gastric cancer) cells were cultured as described previously [16,18]. Human pancreatic adenocarcinoma cells BxPC3 (ATCC CRL-1687) and Panc-1 (ATCC CRL-1469) were cultured in Dulbecco’s modified Eagle’s medium containing 10% foetal bovine serum, 100 U/mL penicillin, 1.0 mg/mL streptomycin and 2 mM t-Glu. For treating BxPC3 cells and clones with drugs affecting glycosylation, 1 x 10^5 cells were plated in 12-well plates, incubated for 30 h with high medium that was replaced with medium containing 1.0 μg/mL swainsonine (Sigma) or 2 mM benzyl-α-GalNAc (Sigma). After growing for 60 h in the presence of drugs, media were collected again. Media obtained before and after treatment were centrifuged at 3000 g for 10 min and the clean supernatants were used for dot-bLOTS.

Cultured cells were harvested, centrifuged, aliquoted, and freshly processed for flow cytometry as reported [16], or homogenated for RNA extraction or enzyme assay, according to the procedures described [18].

Preparation of pSV2Neo, pcDNA1/Fuc-TIII, and pCDM8/β3Gal-T5 was as reported [16]. Antisense plasmid pEFNeo/ASβ3Gal-T5 was constructed by cloning a fragment of β3Gal-T5 cDNA in the antisense orientation in the vector pEFNeo, a generous gift of N. Hiraoka (Aiki Cancer Center, Nagoya, Japan). Vector relevant features include the strong human elongation factor-1α promoter [19], the linker sequence containing a 358-bp stuffer between two nonpalindromic BstXI sites, and the simian virus 40 (SV40) polyadenylation signals. cDNA was obtained from COLO-205 total RNA and amplified by PCR with a commercially available ‘high fidelity’ Taq polymerase (LA Taq, Takara) as reported [16], using specific primers as follows. Upper strand primer: 5'-GGCTCTAGACCCAGCTCTCAGCTTGCATAGGCC-3', having a 4-base filler, an XbaI restriction site (underlined), and a 27-base sequence corresponding to nucleotides –192 to –160 from the start ATG codon in the β3Gal-T5 gene. Lower strand primer: 5'-GGCAAGCTTGATATGGTGTCGCTGCTTGC-3', having a 4-base filler, a HindIII site (underlined), and a 27-base sequence corresponding to nucleotides 334–360 in the coding region of the gene. PCR reactions were incubated as follows: 94 °C for 3.5 min followed by 25 cycles of 1.5 min at 94 °C (melting) and 3.5 min at 72 °C (annealing plus extension), and a final extension step at 72 °C for 8 min. The amplified DNA was digested with XbaI and HindIII, for other purposes, or blunt-ended, ligated to BstXI adaptors, and cloned into the corresponding sites of pEFneo, using the procedure described [20]. Direct DNA sequencing of the construct obtained, performed by the dideoxynucleotide chain-termination method using an automated procedure, indicated that the sequence of the construct obtained, pEFneo/ASβ3Gal-T5, was identical to that expected.

**Construction of cell clones**

HCT-15 expressing β3Gal-T5, MKN-45 expressing Fuc-TIII, and BxPC3 expressing antisense β3Gal-T5 construct, were obtained by the calcium phosphate transfection method [21], using a modification of the procedure [16]. The DNA mixture contained 1.5 μg EcoRI-linearized pSV2Neo and 20 μg ScaI-linearized pcDNA1/Fuc-TIII, or 1.5 μg EcoRI-linearized pSV2Neo and 20 μg ScaI-linearized pCDM8/β3Gal-T5, or 1.5 μg EcoRI-linearized pSV2Neo and 20 μg Tth111I-linearized pEFNeo/ASβ3Gal-T5, respectively. Upon selection with 0.4 mg/mL G418, colonies were collected using cloning cylinders and grown in 48-well plates. G418-resistant HCT-15 and MKN-45 colonies were stained with anti-sLe^a Ig, analysed by fluorescence microscopy on tissue culture slides, and subcloned [16]. G418-resistant BxPC3 clones were screened by competitive RT/PCR. Total RNA was extracted from colonies and reverse transcribed, and cDNA submitted to PCR amplification with human β-actin primers, for normalization [16,20], or with primers specific to the antisense construct. Single colonies expressing a constant level of sLe^a, named HCT-15-T5 and MKN-45-T5, or of antisense β3Gal-T5 construct, named T5AS, were selected and used for further characterization and experiments.

**Metabolic labelling and carbohydrate analysis**

BxPC3 cells and T5AS clone (4.0 x 10^6 cells) were plated in 25-mm² flasks containing 0.2 mCi [3H]Gal (Amersham...
Pharmacia Biotech) in 4.0 mL culture medium and incubated for 40 h under regular conditions. Labelled cells were harvested, resuspended in phosphate-buffered saline at a density of $4 \times 10^7$ cellsmL$^{-1}$, and processed according to published procedures [9,16,22], with some modifications. Total lysates were obtained by boiling 10 min in phosphate-buffered saline containing 0.5% SDS and 1.0% 2-mercaptoethanol, and spinning at 12 000 r.p.m. for 10 min. The clean supernatants were made 1% for Nonidet P40 and 50 mM for sodium phosphate buffer pH 7.5, and treated with N-glycanase (New England Biolabs P0704), 50 000 NEB Umg$^{-1}$ cell lysate protein, for 20 h at 37 °C. Lysate protein was 0.8 mgmL$^{-1}$. Reaction mixtures were passed through a Sephadex G-50 column (0.7 × 50 cm) equilibrated and eluted with water at a flow rate of 0.11 mLmin$^{-1}$, 3 min per fraction. Material collected with the inclusion volume of the column was lyophilized and passed through a Bio-Gel P-4 column (0.7 × 50 cm) equilibrated and eluted with water at a flow rate of 0.10 mLmin$^{-1}$, 5 min per fraction, and the high molecular mass substances, collected with the exclusion volume, lyophilized and referred to as the N-glycans. Material collected with the exclusion volume of the Sephadex G-50 column was lyophilized and submitted to β-elimination, incubating 40 h at 45 °C in 50 mM NaOH containing 0.5 m sodium borohydride. Unreacted NaBH$_4$ was inactivated with an excess of glacial acetic acid, and the solution neutralized with NaOH and buffered with 0.1 m ammonium bicarbonate. Total reactions were passed through a Bio-Gel P-4 column (1.0 × 50 cm), equilibrated and eluted with water at a flow rate of 0.24 mLmin$^{-1}$, 5 min per fraction. Radioactive material collected with the inclusion volume of this column was referred to as the small O-glycans, while the material collected in the flow-through of the column was lyophilized and passed through a Sephadex G-50 column (0.7 × 50 cm) equilibrated and eluted as above. Radioactivity collected with the inclusion volume, referred to as the large O-glycans, was lyophilized, resuspended with water at a concentration of 10 000 cpmμL$^{-1}$, and submitted to endo-β-galactosidase digestion using the enzyme from Bacteroides fragilis (Sigma E6773), 0.4 μμL$^{-1}$, for 20 h at 37 °C. The reaction mixture was diluted with water and applied to a QAE-Sephadex column to separate neutral and charged sugars, according to a reported procedure [22]. Material collected in the flow-through of the column was lyophilized and submitted to a Bio-Gel P-4 column (0.7 × 100 cm), eluted with water at a flow rate of 0.06 mLmin$^{-1}$, 6.5 min per fraction. The obtained peaks were collected, lyophilized, treated with glycohydrolases, and submitted to Bio-Gel P-2 chromatography for characterization [16]. β1,3-galactosidase (New England Biolabs P0726), α1,3/4-fucosidase (Sigma F-3023), β-N-acetylgalactosaminidase (New England Biolabs P0721), and β1,4-galactosidase (Sigma G-0413) digestions were performed on radioactive oligosaccharides, 400–1000 c.p.m.μL$^{-1}$, according to the manufacturer’s recommendations.

Analytical procedures

For transcript quantification, competitive RT/PCR was performed essentially as reported previously [16,20]. First-strand cDNA was prepared for samples and controls in the presence or absence of the reverse transcriptase, respectively, and reactions incubated under the conditions reported [20]. cDNA was amplified (25 μL reaction volume) in the presence of 10 fg (glycosyltransferases) or 100 fg (antisense construct) of the correct competitor for 35 cycles, or in the presence of 10 pg competitor (β-actin) for 25 cycles, under the conditions reported [16]. No amplification was detected when the control reactions were used as template. Human β-actin and βGal-T5 competitors and oligonucleotide primers were those already described [16]. For β3Gal-T5 antisense construct, the competitor was prepared digesting pEFneo/ASβ3Gal-T5 plasmid with Pmel and Bsp1407I, blunting the ends, removing the 235-bp fragment, and self-re-ligating the truncated plasmid. The following primers were used: upper strand primer, 5′-CCCTCACCACCTCTCTTTCCCCCAC-3′, corresponding to nucleotides 262–237 of the reverse strand of the β3Gal-T5 coding sequence; lower strand primer, 5′-CAGGTTCACGGGGAGTGTGCGGAG-3′, corresponding to nucleotides 31–8 of the reverse strand of the SV40 polyadenylation signal sequence of pEFneo vector.

β1,3Gal-T activity was determined in the reported reaction mixture [16], using 0.6 mM GlcNAc as acceptor, in the presence of cell homogenates at protein concentrations of 0.5–4.0 mgmL$^{-1}$. Incubations were performed at 37 °C for 60 min. At the end of incubation, reaction products were assayed by Dowex chromatography and characterized according to previously reported protocols [18]. In all cases the reaction product was found to be a disaccharide sensitive to β1,3galactosidase, as expected. In fact, GlcNAc is not used as acceptor by β1,4galactosyltransferases under the reported assay conditions [18,20]. $K_m$ values were determined as reported [18].

For dot-blots, 50-μL aliquots of the culture media were applied to the blotting membrane by vacuum aspiration. Serial dilution of samples were performed in preliminary experiments to set the amounts needed for detection. Membranes were washed, blocked, stained with primary and peroxidase-labelled secondary antibodies, and visualized by enhanced chemoluminescence as reported for Western blotting [23]. Monoclonal anti-CEA, anti-sLea, but not Lea, and anti-sLex (from hybridoma 1116-NS-19–9), and anti-sLex (from hybridoma CSLEX1) Igs were as reported [16,20]. $K_m$ values were determined as reported [23].

Results

Construction and characterization of a BxPC3 clone expressing an antisense β3Gal-T5 fragment

To study the role of β3Gal-T5, we permanently suppressed the expression in a cell line by an antisense approach. We chose BxPC3 cells for transfection as they express low levels of the transcript (0.2 fgpg$^{-1}$ β-actin) but still well detectable amounts of β1,3Gal-T activity (16.0 nmol transferred Gal-mg protein$^{-1}$h$^{-1}$) and sLea, but not Lea,

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Le<sup>b</sup> or sLe<sup>a</sup>. Moreover, sLe<sup>a</sup> expression in these cells is affected by benzyl-α-GalNAc but not by swainsonine. These facts were expected to make the experiment technically feasible, and the high β<sub>1,3</sub>Gal-T activity/β<sub>3</sub>Gal-T transcript ratio to provide clear-cut results. Cells were transfected with a linearized plasmid containing a 553-bp fragment of β<sub>3</sub>Gal-T cDNA, that includes the initial 360 bp of the coding sequence and 192 bp of the 5′ untranslated region of the gene, placed in the antisense orientation under the control of the elongation factor-1α promoter, and followed by SV40 polyadenylation signals (Fig. 1). This scheme basically follows the one used successfully by Hiraiwa et al. for suppressing fucosyltransferase FucT-VII in lymphoid cells [24]. A cassette for G418 resistance was cotransfected for selection of recombinant clones. To quantify the levels of the antisense construct expressed in transfected cells, and provided a 515-bp amplification fragment detected as β<sub>3</sub>Gal-T antisense construct target in Fig. 2A.

To understand better the consequences of β<sub>3</sub>Gal-T activity in BxPC3 cells (β<sub>1,3</sub>Gal-T activity in BxPC3 cells (β<sub>1,3</sub>Gal-T activity in BxPC3 cells) and characterized the clone, named T5AS, retains a low expression of β<sub>3</sub>Gal-T-5 transcript as in the parental cell line (Fig. 2A). This indicates that antisense-mediated mechanism of gene suppression does not involve transcript synthesis in this case, as already reported [24]. On the other hand, β<sub>1,3</sub>Gal-T activity is dramatically reduced and became faintly detectable in the clone (Fig. 2B). Moreover, the T5AS clone expresses much less sLe<sup>a</sup> on the cell surface than BxPC3 cells (Fig. 2C). These data indicate that β<sub>3</sub>Gal-T-5 is the gene responsible for β<sub>1,3</sub>Gal-T activity and sLe<sup>a</sup> antigen synthesis in these cells. In addition, T5AS clone became weakly positive to sLe<sup>a</sup>, that instead is undetectable in BxPC3 cells, and remains negative to Le<sup>a</sup>, faintly positive to Le<sup>c</sup>, and moderately positive to SNA, as are the original BxPC3 cells (Fig. 2C). A relevant amount of sLe<sup>c</sup> is also found in the culture medium, where sLe<sup>α</sup>, that is secreted by BxPC3 cells, is almost undetectable.

**Characterization of sugar chains synthesized in the antisense clone**

To understand better the consequences of β<sub>3</sub>Gal-T-5 suppression on cell glycosylation, we characterized the main oligosaccharide chains synthesized by such cells. To this aim, the antisense clone and parental BxPC3 were metabolically radiolabelled with tritiated Gal, and the distribution of radioactivity studied as outlined in Fig. 3. Table 1 shows that Gal is incorporated into high molecular mass substances attached to the cell membranes, without relevant differences between parental cells and antisense clone. The amount of radioactivity released by N-glycanase is moderate in both cases, while the bulk of incorporated...
Radioactivity is sensitive to β-elimination providing two fractions: small O-glycans, recovered in the included volume of the Bio-Gel P4 column, and large O-glycans, collected with the excluded volume of the Bio-Gel P4 and the included volume of the Sephadex G-50 column (Fig. 4B and C). Small O-glycans are present in similar amounts in BxPC3 and the T5AS clone (Table 1), and to be mostly constituted by sialylated or neutral disaccharides. They probably represent core 1 O-glycans that are not potential substrates of β3Gal-T5 and were not studied further. Large O-glycans are found in relevant amounts in both cells. Their size was confirmed by Bio-Gel P-4 chromatography performed in 0.1 M acetic acid that shows that they move between N-glycans and small oligosaccharides (Fig. 4D). Large O-glycans are sensitive to endo-β-galactosidase treatment, providing neutral (unbound to QAE-Sephadex) and acid (bound to QAE-Sephadex) oligosaccharides (Table 1). Neutral oligosaccharides released by endo-β-galactosidase from BxPC3 large O-glycans contain a minimal amount of radioactivity and were not analysed further, whereas those released from T5AS clone mostly show a disaccharide peak and a smaller trisaccharide peak (Fig. 4, lower). The disaccharide is sensitive to β-hexosaminidase, giving rise to radioactive Gal, and identified as GlcNAcβ1-3Gal. The trisaccharide was mostly sensitive to β1,4galactosidase, giving rise to a disaccharide and a monosaccharide, and is thus identified as Galβ1-4GlcNAcβ1-3Gal. The acid fraction of endo-β-galactosidase sensitive large O-glycans from BxPC3 cells, upon specific removal of α2,3 sialyl residues, contains mostly a tetrasaccharide and a trisaccharide, and an oligosaccharide peak close to but separated from the void volume (Fig. 4, lower). The trisaccharide is sensitive to both β1,3- and β1,4galactosidases, giving rise to a disaccharide and a monosaccharide, and is thus identified as a mixture of Galβ1-3GlcNAcβ1-3Gal and Galβ1-4GlcNAcβ1-3Gal. The tetrasaccharide is sensitive to β1,3,4 fucosidase giving rise to a trisaccharide that provides equal amounts of radioactive disaccharide and monosaccharide upon β1,3galactosidase treatment, and is thus identified as Galβ1-3[Fucα1-4]GlcNAcβ1-3Gal. The acid fraction of endo-β-galactosidase sensitive O-glycans from the antisense clone, upon removal of α2,3 sialyl residues, contains mostly a trisaccharide, a small shoulder corresponding to a tetrasaccharide, and the oligosaccharides peak separated from the void volume as well. The trisaccharide was mostly sensitive to β1,4galactosidase, giving rise to a disaccharide and a monosaccharide, and is thus identified as Galβ1-4GlcNAcβ1-3Gal, while the tetrasaccharide was sensitive to β1,3,4 fucosidase, giving rise to a trisaccharide. The latter was sensitive to both β1,4- and β1,3galactosidases, giving rise to a disaccharide and a monosaccharide, and was thus identified as a mixture of Galβ1-4[Fucα1-3]GlcNAcβ1-3Gal and Galβ1-3[Fucα1-4]GlcNAcβ1-3Gal. The calculated amounts of each oligosaccharide are summarized in Table 2. These data indicate that the repression of β3Gal-T5 reduces the synthesis of type 1 chain carbohydrates, including sLeα, and enhances that of poly N-acetylactosamines and sLeα on O-glycans. We were unable to characterize the peak separated from the void volume, but we believe that it may represent the reducing end of the sugar chain remaining after endo-β-galactosidase digestion.

Fig. 3. Scheme of sugar chain purification. The scheme outlines the procedure followed for preparing different sugar fractions from metabolically radiolabelled cells. The main fractions obtained are in boldface, and the more relevant treatments are italicized. The corresponding qualitative results are presented in Fig. 4, and the quantitative data in Table 1.

Table 1. Radioactivity distribution in BxPC3 cells and T5AS clone metabolically radiolabelled with [3H]Gal. Values are expressed as c.p.m. × 10⁶·mg⁻¹·cell protein.

<table>
<thead>
<tr>
<th></th>
<th>BxPC3 (%)</th>
<th>T5AS (%)</th>
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<tbody>
<tr>
<td>Total cell incorporation</td>
<td>7.40 (100)</td>
<td>7.23 (100)</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>5.92 (80.0)</td>
<td>5.66 (78.2)</td>
</tr>
<tr>
<td>N-glycans</td>
<td>0.85 (11.4)</td>
<td>0.74 (10.2)</td>
</tr>
<tr>
<td>O-glycans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>2.30 (31.1)</td>
<td>2.41 (33.3)</td>
</tr>
<tr>
<td>Large</td>
<td>2.24 (30.4)</td>
<td>2.53 (35.1)</td>
</tr>
<tr>
<td>Upon endo-β-galactosidase</td>
<td></td>
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</tr>
<tr>
<td>Unbound to QAE-Sephadex</td>
<td>0.38 (5.1)</td>
<td>0.57 (7.8)</td>
</tr>
<tr>
<td>Bound to QAE-Sephadex/</td>
<td>1.12 (15.1)</td>
<td>0.91 (12.5)</td>
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Fig. 4. Characterization of radioactive oligosaccharides formed in metabolically radiolabelled cells. The main radioactive oligosaccharides formed in BxPC3 cells (■ in lower part, and A, B, and C of upper part) and T5AS clone (□ in lower part, and A, B, and C of upper part) metabolically radiolabelled with [3H]Gal were characterized. Upper part: cell lysates were treated with N-glycanase and passed through a Sephadex G-50 column (A) and the material collected with the flow-through of the column (horizontal bar) was submitted to β-elimination. Upon β-elimination the material was passed through a Bio-Gel P-4 column (B), and the material collected with the excluded volume of the column (horizontal bar) was passed again through a Sephadex G-50 column (C). Material included in this last column (horizontal bar) was submitted to α elim-ination. Upon α-elimination (D) N-glycans (□), obtained by Bio-Gel P-4 purification of the included volume of the column in (A), large O-glycans (■), obtained as the included volume of the column in (C), and small O-glycans (○), obtained as the included volume of the column in (B), were analysed by a Bio-Gel P-4 column equilibrated and eluted with 0.1 M acetic acid. The profiles obtained with the radioactive fractions prepared from BxPC3 cells are presented, those obtained with fractions from T5AS clone were identical. Lower part: large O-glycans were treated with endo-β-galactosidase and passed through a QAE-Sephadex column. Radioactivity not bound to QAE-Sephadex was lyophilized and applied directly to a long Bio-Gel P4 column (neutral fraction), while radioactivity bound to QAE-Sephadex and eluted with NaCl was desalted, treated with α2,3 sialidase, and then applied to the column (acid fraction). Column calibration is shown at the top.

If so, it is interesting to note that the O-glycans carrying Lewis antigens in BxPC3 appear to be very complex structures comparable in size to those recently reported in other cells [25].

**Secretion of Lewis antigens in the antisense clone**

To assess the effect of β3Gal-T5 repression on the sugar chains of molecules secreted in the culture media, BxPC3 cells and the antisense clone were cultured and the media analysed by dot-blot after adding drugs affecting glycosylation. To obtain comparable data, preliminary experiments were performed in order to normalize the amount of media to be blotted. To this purpose we used CEA as a reference, as it is secreted by the cells, and stained the blots with anti-CEA Ig. Fig. 5 shows the results obtained by staining blots prepared using such amounts of culture media with anti-sLea and anti-sLex Ig’s, respectively. BxPC3 cells secrete sLea in the media but not sLex, while T5AS clone secretes mostly sLea. Accumulation of both antigens is prevented by benzyl-β-D-GalNAc, an inhibitor of O-glycosylation, while it is not affected by swainsonine, an inhibitor of N-glycosylation. These results confirm that β3Gal-T5 is responsible even for sLea secreted by the cells, and that O-glycans carried by secreted molecules are modified upon β3Gal-T5 repression in a similar manner as those carried by membrane-bound molecules.

**β1,3Gal-T activity, β3Gal-T5 transcript levels, and sLea expression in cancer cell lines and recombinant clones**

We also measured the levels of β3Gal-T5 transcript and β1,3Gal-T activity in different cancer cell lines and clones, and compared them with the amount of sLea antigen expressed on the cell surface. We found that cells expressing high levels of transcript, such as COLO-205, SW-1116 or recombinant HCT-15-T5, express high levels of enzyme activity; cells expressing lower levels of transcript, such as Caco-2, HT-29, or BxPC3, express lower β1,3Gal-T activity levels; while cells not expressing the transcript at all, such as HCT-15 or Panc-1, have no measurable enzyme activity (Fig. 6). Surprisingly, the range of β1,3Gal-T activity/β3Gal-T5 transcript ratio is very broad. The highest value is found in BxPC3 cells, while it is 16-fold lower in the HCT-15-T5 clone. To verify that the enzyme activities measured are due to β3Gal-T5 only, we determined the enzyme kinetics from representative cells, and found that the β1,3Gal-T activities detected are kinetically identical to those of genuine β3Gal-T5. Altogether these data suggest that β3Gal-T5 repression on the sugar chains of molecules secreted in the culture media, BxPC3 cells and the antisense clone were cultured and the media analysed by dot-blot after adding drugs affecting glycosylation. To obtain comparable data, preliminary experiments were performed in order to normalize the amount of media to be blotted. To this purpose we used CEA as a reference, as it is secreted by the cells, and stained the blots with anti-CEA Ig. Fig. 5 shows the results obtained by staining blots prepared using such amounts of culture media with anti-sLea and anti-sLex Ig’s, respectively. BxPC3 cells secrete sLea in the media but not sLex, while T5AS clone secretes mostly sLea. Accumulation of both antigens is prevented by benzyl-β-D-GalNAc, an inhibitor of O-glycosylation, while it is not affected by swainsonine, an inhibitor of N-glycosylation. These results confirm that β3Gal-T5 is responsible even for sLea secreted by the cells, and that O-glycans carried by secreted molecules are modified upon β3Gal-T5 repression in a similar manner as those carried by membrane-bound molecules.

**β1,3Gal-T activity, β3Gal-T5 transcript levels, and sLea expression in cancer cell lines and recombinant clones**

We also measured the levels of β3Gal-T5 transcript and β1,3Gal-T activity in different cancer cell lines and clones, and compared them with the amount of sLea antigen expressed on the cell surface. We found that cells expressing high levels of transcript, such as COLO-205, SW-1116 or recombinant HCT-15-T5, express high levels of enzyme activity; cells expressing lower levels of transcript, such as Caco-2, HT-29, or BxPC3, express lower β1,3Gal-T activity levels; while cells not expressing the transcript at all, such as HCT-15 or Panc-1, have no measurable enzyme activity (Fig. 6). Surprisingly, the range of β1,3Gal-T activity/β3Gal-T5 transcript ratio is very broad. The highest value is found in BxPC3 cells, while it is 16-fold lower in the HCT-15-T5 clone. To verify that the enzyme activities measured are due to β3Gal-T5 only, we determined the enzyme kinetics from representative cells, and found that the β1,3Gal-T activities detected are kinetically identical to those of genuine β3Gal-T5. Altogether these data suggest that β3Gal-T5 repression on the sugar chains of molecules secreted in the culture media, BxPC3 cells and the antisense clone were cultured and the media analysed by dot-blot after adding drugs affecting glycosylation. To obtain comparable data, preliminary experiments were performed in order to normalize the amount of media to be blotted. To this purpose we used CEA as a reference, as it is secreted by the cells, and stained the blots with anti-CEA Ig. Fig. 5 shows the results obtained by staining blots prepared using such amounts of culture media with anti-sLea and anti-sLex Ig’s, respectively. BxPC3 cells secrete sLea in the media but not sLex, while T5AS clone secretes mostly sLea. Accumulation of both antigens is prevented by benzyl-β-D-GalNAc, an inhibitor of O-glycosylation, while it is not affected by swainsonine, an inhibitor of N-glycosylation. These results confirm that β3Gal-T5 is responsible even for sLea secreted by the cells, and that O-glycans carried by secreted molecules are modified upon β3Gal-T5 repression in a similar manner as those carried by membrane-bound molecules.
Discussion

We have found that β3Gal-T5 is responsible for sLe^a antigen synthesized on O-glycans expressed on or secreted by an epithelial cell line, whereas antisense-mediated suppression of the enzyme turns synthesis of O-glycans to poly N-acetyllactosamine elongation and termination by sLex. Taken together with our previous data on β3Gal-T5 downregulation in colon cancer and N-glycansynthesis [16], the results suggest that β3Gal-T5 may play a protective role in gastrointestinal and pancreatic cells, counteracting the glycosylation pattern associated to malignancy.

We found in fact that NeuAc^a2-3Gal^b1-3[Fuc^a1-4]GlcNAc^b1-3Gal and NeuAc^a2-3Gal^b1-3GlcNAc^b1-3Gal are the main oligosaccharides released by endo-β-galactosidase treatment of large O-glycans in BxPC3 cells, while in the clone where β3Gal-T5 is suppressed they are mostly replaced by poly N-acetyllactosamine units differently substituted by siaic acid and fucose. The levels of α1,3 fucosylation and sLe^a expression were rather low in this case, probably because BxPC3 cells express Fuc-TIII but almost no pure α1,3fucosyltransferase [27], including Fuc-TVII that is not expressed in any cell line used in the present study [27–29].

However, moderate amounts of sLe^a were recently proved to be the most efficient in promoting metastatic spread [30]. These data match the finding that CEA synthesized by normal mucosa has abundant N-linked type 1 chains due to β3Gal-T5 activity, and that are replaced by poly N-acetyllactosamine in cancer where the enzyme is downregulated [16,31]. Altogether they suggest that β3Gal-T5 synthesizes type 1 chains that do prevent poly N-acetyllactosamine elongation and sLex synthesis on both N- and O-glycans. Due to the involvement of such structures in malignancy, β3Gal-T5 regulation may play an important role in colon cancer, as the residual expression level potentially contributes to prevention of the malignant phenotype.

Synthesis and expression of sLe^a is a relevant issue per se, as it is the epitope of the CA19.9 antigen, sometimes found to be elevated in the serum of patients with various abdominal illnesses [32] including cancers of the digestive tract [33–35]. Moreover, it is an E-selectin ligand [36] and may be involved in the metastatic spread of cancer cells, as suggested for other selectin ligands [37]. Previous data indicate that β3Gal-T5 is the enzyme candidate for synthesis of sLe^a [15–18], but the finding that sLe^a is strongly expressed in normal mucosa

Table 2. Main oligosaccharides released from BxPC3 cells and T5AS clone by endo-β-galactosidase treatment of metabolically labelled O-glycans. Values are expressed as c.p.m. × 10^3 mg^-1 cell protein.

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<td>3.1</td>
<td>12</td>
<td>1.4</td>
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Fig. 5. Secretion of Lewis antigens in the culture medium of BxPC3 cells and T5AS clone. Cells were grown under regular conditions for 30 h before treatment, then the tissue culture media were collected and replaced with fresh regular media (controls), or containing 1.0 μg mL^-1 swainsonine or 2 mM benzyl-α-GalNAc. Media were collected again 60 h after treatment. Aliquots of collected media, normalized with respect to the amount of secreted CEA, were blotted and stained with primary anti-sLe^a or anti-sLe^x Igs followed by peroxidase-labelled secondary antibody.

Fig. 6. Expression of β3Gal-T5 and sLe^a in different cells. Different cell lines and clones were cultured, harvested, and analysed as follows. β3Gal-T5 transcript (filled bars) was quantified by competitive RT/PCR starting from RNA extracted from aliquots of the cell pellets, and β3Gal-T5 activity (empty bars) was determined by in vitro assay using homogenates prepared from a second aliquot of the cell pellet. sLe^a antigen expressed on the cell surface (grey bars) was determined by immunostaining and flow cytometry performed on a fresh aliquot of the cell pellet. Results are expressed as relative values, 100% corresponds to 18 fg μg^-1 β-actin for transcripts, to 190 ng of transferred Gal μg^-1 homogenate protein h^-1 for enzyme activity, and to 50 arbitrary units for fluorescence.

Table 2. Main oligosaccharides released from BxPC3 cells and T5AS clone by endo-β-galactosidase treatment of metabolically labelled O-glycans. Values are expressed as c.p.m. × 10^3 mg^-1 cell protein.
makes this open to question. Here we found evidence that β3Gal-T5 is actually necessary for sLeα synthesis on O-glycans in gastrointestinal and pancreatic cells. In fact, in BxPC3 cells antisense suppression of the gene dramatically reduces β1,3Gal-T activity as well sLeα antigen expression and secretion. Moreover, only cell lines expressing β3Gal-T5 express the antigen, and cells not expressing are forced to do by cDNA transfection. On the other hand, sLeα synthesis and secretion appear to depend on multiple molecular or enzymatic mechanisms. We speculate they may include several interacting factors such as the nature and availability of substrates, including nucleotide sugars [38], the presence of other cooperative or competing enzymes [39], as well their sub-Golgi localization [40]. Our working hypothesis is that the biological role of β3Gal-T5 includes, but is not restricted to, sLeα synthesis, that probably requires several concurrent factors in vivo. Phylogenetic observations agree with this concept. In fact, while α1,4 fucosylation and thus sLeα synthesis are recent evolutionary acquisitions belonging to humans and some primates [41], β3Gal-T5 is present in other mammals such as mice [42], rats (GenBank accession XM221525), and very probably pigs [43]. While this manuscript was being completed, Ishiki et al. reported that β3Gal-T5 is transcriptionally regulated by homeoproteins specific to the intestinal mucosa [44]. They also found that some of these homeoproteins, as well as β3Gal-T5, are upregulated during CACO-2 cell differentiation and downregulated in colon cancer, but that β3Gal-T5 protein is not correlated with the amount of CA19.9 in cancer tissues. Such results elegantly show that type 1 chain carbohydrates are products of β3Gal-T5 activity as a part of the specific phenotype of the normal intestinal mucosa. Taken together with our previous [16] and present findings, and with those on CACO-2 differentiation [13,14], they corroborate the hypothesis that β3GalT-5 and type 1 chain carbohydrates are ‘markers’ of normal glycosylation in epithelia of the digestive tract. In this context, the use of CA19.9 antigen as a tumour marker appears paradoxical, since it is a product of β3Gal-T5 activity on type 1 chain O-glycans. We believe that further studies are needed to elucidate the metabolic origin of CA19.9 circulating in patients and to confirm the actual ability of gastrointestinal and pancreatic cancers to synthesize and secrete large amounts of sLeα.

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