ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity

S. Julien1, E. Adriaenssens3, K. Ottenberg4, A. Furlan3, G. Courtand5 A.-S. Vercoüter-Edouart2, F.-G. Hanisch4, P. Delannoy1,2, and X. Le Bourhis3

1Unite de Glycobiologie Structurale et Fonctionnelle, UMR CNRS n° 8576, GDR CNRS n° 2590, Universite des Sciences et Technologies de Lille, F-59655 Villeneuve d’Ascq, France; 2Laboratoire de Biologie du Developpement, INSERM ERI8, URPEA 1033, Universite des Sciences et Technologies de Lille, F-59655 Villeneuve d’Ascq, France; 3Centre de Biochemistry and Center for Molecular Medicine Cologne, University of Cologne, Joseph-Stelzmann-Street 52, D-50931 Cologne, Germany; and 4Centre Commun de Mesures Imagerie Cellulaire, Universite des Sciences et Technologies de Lille, F-59655 Villeneuve d’Ascq, France

Glycobiology vol. 16 no. 1 pp. 54–64, 2006
doi:10.1093/glycob/cwj033
Advance Access publication on August 31, 2005

Key words: breast cancer/cell adhesion/sialyl-Tn/ST6GalNAc I/tumour growth

Introduction

Overexpression of sialylated antigens at the surface of cancer cells has been widely reported (Dabelsteen, 1996; Kim and Varki, 1997). Among these antigens, sialyl-Tn antigen (STn) (Neu5Acα2-6GalNAcα1-0-Ser/Thr) is an O-linked disaccharide structure abnormally expressed in several epithelial cancers (i.e., gastric, pancreatic, colorectal, ovarian, and breast cancers) (Julien and Delannoy, 2003). STn is often associated with lymph node involvement, distant metastasis, and a decreased survival of patients in gastric (Imada et al., 1999; Takano et al., 2000; Nakagoe et al., 2002), colorectal (Nakagoe et al., 2001), and breast cancers (Schmitt et al., 1995; Soares et al., 1996). Although STn expression is usually associated with poor prognosis, nothing is known about the effective incidence of the antigen expression on the biological properties of cancer cells.

The biosynthesis of O-glycans is a posttranslational process that occurs in the Golgi apparatus requiring the sequential action of several membrane-bound glycosyltransferases. The first step is the transfer of an N-acetylgalactosamine (GalNAc) residue onto a serine or a threonine residue of the protein backbone. Subsequent addition of galactose (Gal) and/or N-acetylglucosamine (GlcNAc) by specific glycosyltransferases leads to the formation of the common O-glycan Core structures. These Cores can be further elongated and terminated by the transfer of sialic acid or fucose residues. Alternatively, a premature sialylation of GalNAc or Core 1 may prevent the elongation of the glycans (Figure 1). Because many of the oligosaccharide structures that occur in this biosynthetic pathway may serve as acceptor substrates for a variety of glycosyltransferases, the relative activities and the competitive specificities of these enzymes is thought to rule the sequence of the newly synthesized glycans. For example, relative activities of the UDP-GlcNAc:R-Galβ1-3GalNAc (GlcNAc to GalNAc) β1,6-GlcNAc transferase (EC 2.4.1.102) (C2GnT1) and ST3Gal-I glycosyltransferases determine the prevalence of Core 1 structure in breast cancer cell lines (Figure 1) (Dalziel et al., 2001).

The sialyltransferase CMP-Neu5Ac: R-GalNAcα1-O-Ser/Thr α2,6-sialyltransferase (EC 2.4.99.3) (ST6GalNAc I) is able to sialylate the Thomsen-nouveau antigen (Tn antigen) (GalNAcα1-0-Ser/Thr) carried by asialo-ovine submaxillary mucin (Ikehara et al., 1999). Moreover, STn positive clones exhibit an increased tumour growth in severe combined immunodeficiency (SCID) mice. These observations suggest that modification of the O-glycosylation pattern induced by ST6GalNAc I expression are sufficient to enhance the tumourigenicity of MDA-MB-231 breast cancer cells.

© The Author 2005. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oupjournals.org
Sialyl-Tn expression enhances breast cancer cell tumorigenicity

O-glycosylation modifications on tumour cell growth both in vitro and in vivo.

Results

STn antigen is detected on various O-glycosylproteins in STn+ clones

To investigate the proteins carrying STn in MDA-MB-231 cells, total cell lysates from both a mock transfectant (STn−) and two ST6GalNAc I expressing clones (STn+A and STn+B) were analyzed by western blot using the anti-STn HB-STn1 monoclonal antibody (mAb). As shown in Figure 2A, there was no immunostaining in mock transfected control cells, whereas several stained bands ranging from 90 to >200 kDa were detected in both MDA-MB-231 STn+ clones. MUC1 and CD44 were immunoprecipitated from total cell lysates using LICR-LON-M8 and HCAM-DF1485 mAbs, respectively. Immunoprecipitates were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and western blot using the HB-STn1 mAb. As shown in Figure 2B, MUC1 immunoprecipitated from STn+ cells stained with the HB-STn1 mAb, whereas there was no staining in the STn− cells. This indicates that ST6GalNAc I expression induces the expression of STn on MUC1 in MDA-MB-231 cells. Similarly, after CD44 immunoprecipitation with the HCAM-DF1485 mAb, a 100 kDa STn positive band was revealed in the lanes corresponding to STn+ clones, indicating that CD44 was also substituted by STn.

Glyco-profiling of a recombinant secreted MUC1–MFP6 fusion protein expressed in MDA-MB-231 STn+ cells

To determine the O-glycan profiles and relative abundance of STn in MUC1 tandem repeats, we have induced the expression of a recombinant secreted MUC1–MFP6 in the MDA-MB-231 STn+ A clone, as previously described (Muller and Hanisch, 2002). The MUC1–MFP6 fusion protein was purified from conditioned medium and the O-glycan pool was released by hydrazinolysis and analyzed by normal phase high performance liquid chromatography (HPLC) (Muller and Hanisch, 2002). As summarized in Table I, the expression of ST6GalNAc I converted 22% of total O-glycans to STn, whereas this antigen was not expressed in ST6GalNAc I negative cells. In parallel, Core 1 and Core 2 related structures were decreased by 23%, confirming that STn is expressed instead of preexisting more extended structures. This result also clearly indicates that overexpressed ST6GalNAc I competes with the UDP-Gal: GalNAc β1,3-galactosyltransferase (EC 2.4.1.122) (Core1 βGalT), which is the key enzyme in the biosynthesis of Core 1 and Core 2 related structures (Figure 1). Furthermore, we observed an increase of sialyl-6T (from 5.4 to 15.7%) and a concomitant decrease of sialyl-3T (from 27.6 to 17.2%) in STn+ cells. Finally, because of ST6GalNAc I expression, we observed an enrichment in sialic acid content of the glycan moiety, as shown by the increased Neu5Ac/GalNAc ratio in STn+ cells compared with control cells (Table I).

Glyco-profiling of a recombinant secreted MUC1–MFP6 fusion protein expressed in MDA-MB-231 STn+ cells

Structural characterization of O-linked glycans by mass spectrometric sequencing and linkage analysis

The monosaccharide composition of permethylated glycan alditols was deduced from the sodiated molecular ions for N-acetylneuraminic acid (NeuAc), deoxyhexose (dHex), hexose (Hex), and N-acetylatedamine (HexNAc) (Table II). The compositional data were in agreement with the major species profiled by HPLC analysis (see above and Table I). Sequence information was obtained by collision-induced dissociation experiments in electrospray ionization mass spectrometry (ESI–MS) and registration of MS/MS spectra, which
revealed prominent fragment ions of the Y, Z and B, C series (Domon and Costello, 1988) for sodiated molecular ions (Table II). The ion at \( m/z \) 895 was a mixture of two isomeric trisaccharide alditols indicated by the respective Y\(_2\) and Z\(_1\) ions (NeuAc-Hex-HexNAc-ol) and by the Y\(_{1\alpha}\) and Z\(_{1\alpha}\) ions (NeuAc-(Hex)-HexNAc-ol). Another structural isomery was revealed for the \( M + Na \) ion at \( m/z \) 1344, which corresponded to two isomeric pentasaccharide alditols. Sequence assignments were corroborated by complementary MS/MS spectra registered for the corresponding

---

Table I. Glyco-profiling of MUC1-MFP6 protein expressed in MDA-MB-231 STn\(^+\) cells

<table>
<thead>
<tr>
<th>Structures</th>
<th>Control cells (Müller 2002)</th>
<th>STn(^+) A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn</td>
<td>GalNAc-O-R</td>
<td>-</td>
</tr>
<tr>
<td>STn</td>
<td>GalNAc-O-R</td>
<td>trace 22.2</td>
</tr>
<tr>
<td>Neu5Ac2-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Tn related</td>
<td></td>
<td>22.2</td>
</tr>
<tr>
<td>T (Core 1)</td>
<td>Galβ1-3GalNAc-O-R</td>
<td>5.9</td>
</tr>
<tr>
<td>sialyl-6T</td>
<td>Galβ1-3GalNAc-O-R</td>
<td>5.4</td>
</tr>
<tr>
<td>Neu5Ac2-6</td>
<td></td>
<td>15.7</td>
</tr>
<tr>
<td>sialyl-3T</td>
<td>Neu5Ac2-3Galβ1-3GalNAc-O-R</td>
<td>27.6</td>
</tr>
<tr>
<td>disialyl-T</td>
<td>Neu5Ac2-3Galβ1-3GalNAc-O-H</td>
<td>9.4</td>
</tr>
<tr>
<td>Neu5Ac2-6</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Total Core 1 related</td>
<td></td>
<td>48.3</td>
</tr>
<tr>
<td>Core 2</td>
<td>Galβ1-3GalNAc-O-R</td>
<td>15.1</td>
</tr>
<tr>
<td>Neu5Ac2-3Galβ1-4GlcNAcβ1-6</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>Monosialyl-Core 2</td>
<td></td>
<td>24.2</td>
</tr>
<tr>
<td>Disialyl-Core 2</td>
<td>Neu5Ac2-3Galβ1-3GalNAc-O-H</td>
<td>12.2</td>
</tr>
<tr>
<td>Neu5Ac2-3Galβ1-4GlcNAcβ1-6</td>
<td></td>
<td>18.7</td>
</tr>
<tr>
<td>Total Core 2 related</td>
<td></td>
<td>51.5</td>
</tr>
<tr>
<td>Ratio Core 1/Core 2</td>
<td>0.94</td>
<td>1.10</td>
</tr>
<tr>
<td>Ratio Neu5Ac/GalNAc</td>
<td>1.00</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Table II. Electrospray mass spectroscopy in the MS/MS mode of permethylated glycan alditols

<table>
<thead>
<tr>
<th>Molecular ions</th>
<th>Fragment ions (( m/z ))</th>
<th>Assigned structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M + Na ) ([M + H] )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>691</td>
<td>B(_2)(398), Y(_1)(316)</td>
<td>NeuAc-HexNAc-ol</td>
</tr>
<tr>
<td>895</td>
<td>B(_2)(398), Z(_1)(298), Y(_2)(520)</td>
<td>NeuAc-Hex-HexNAc-ol</td>
</tr>
<tr>
<td>895</td>
<td>B(<em>2)(398), Y(</em>{1\alpha})(520), Z(_{1\alpha})(659)</td>
<td>Hex-(NeuAc)-HexNAc-ol</td>
</tr>
<tr>
<td>983</td>
<td>C(<em>1)(259), B(<em>2)(480), Y(</em>{1\beta})(520), Z(</em>{1\beta})(747)</td>
<td>Hex-(Hex-HexNAc-)-HexNAc-ol</td>
</tr>
<tr>
<td>1256</td>
<td>B(<em>2)(398), Z(</em>{1\alpha})(659), Y(<em>{1\beta})(881), Y(</em>{1\alpha})(1108), Z(_{1\beta})(1108)</td>
<td>NeuAc-Hex-(NeuAc)-HexNAc-ol</td>
</tr>
<tr>
<td>1344</td>
<td>C(<em>1)(259), B(<em>1)(398), Z(</em>{1\beta})(472), Y(</em>{1\alpha})(32(733)</td>
<td>NeuAc-Hex-(Hex-HexNAc-)-HexNAc-ol</td>
</tr>
<tr>
<td>1706</td>
<td>B(_2)(398), B(<em>3)(484), C(<em>2)(620), Z(</em>{1\alpha})(1108), Y(</em>{1\beta})(735(506)</td>
<td>NeuAc-Hex-(NeuAc-Hex-HexNAc-)-HexNAc-ol</td>
</tr>
</tbody>
</table>
Influence of STn expression on cell growth and cell mobility

In standard monolayer culture conditions, there was no significant difference in growth curves of the STn+ clones compared to STn− cells after 6 days of culture (Figure 3A).

In contrast, analysis of cell motility using Transwell system showed a 1.6-fold increase of migrating cells for STn+ clones compared with control cells (Figure 3B). In parallel, video analysis also showed that the distance covered by moving cells during 10 h of monitoring is 1.6-fold greater for STn+ than for STn− cells (Figure 3C). These two different approaches both demonstrate that STn+ cells exhibit a significantly increased mobility compared with control cells.

STn expression and GalNAcα-O-bn treatment both decrease cell adhesion

As shown in Figure 4C, STn− cells were similarly adherent to either uncoated and type I collagen or fibronectin-coated wells. For the two STn+ clones, cell adhesion was significantly decreased in all the conditions. To clarify the role of O-glycans shortening in the decrease of the cell adhesion, we have subjected the cells to 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BGN) treatment. It has been previously demonstrated that BGN is converted into the disaccharide Galβ1-3GalNAcα-O-bn by the Core-1 β1,3-galactosyltransferase; but this conversion does not impair the β1,3-galactosylation of GalNAc O-linked to the proteins expressed by the cells. However, the soluble disaccharide Galβ1-3GalNAcα-O-bn has been proven to behave as a strong competitive inhibitor of the elongation of the mucin Galβ1-3GalNActα sequences by N-acetylgalcosaminyltransferases, sialyltransferases, and fucosyltransferases (Huet et al., 1998).

Table III. Linkage analysis of O-glycans by GC-MS identification of partially methylated alditol acetates

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Fragment ions (m/z)</th>
<th>Structural assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.8 –</td>
<td>118, 131, 162, 175</td>
<td>Terminal fucose</td>
</tr>
<tr>
<td>11.8 +</td>
<td>118, 129, 145, 161, 205</td>
<td>Terminal galactose</td>
</tr>
<tr>
<td>12.9 +</td>
<td>161, 190, 234</td>
<td>2-linked galactose</td>
</tr>
<tr>
<td>13.4 –</td>
<td>118, 189, 234, 305</td>
<td>3,6-linked galactose</td>
</tr>
<tr>
<td>13.6 (+)</td>
<td>130, 133, 246, 258, 290</td>
<td>3-linked GalNAc-ol</td>
</tr>
<tr>
<td>14.2 +</td>
<td>130, 174, 218, 290</td>
<td>6-linked GalNAc-ol</td>
</tr>
<tr>
<td>15.2 +</td>
<td>130, 246, 276, 290, 318</td>
<td>3,6-linked GalNAc-ol</td>
</tr>
<tr>
<td>15.5 +</td>
<td>159, 203, 233</td>
<td>4-linked GlcNAc</td>
</tr>
<tr>
<td>16.0 –</td>
<td>159, 161, 274, 318</td>
<td>3-linked GlcNAc</td>
</tr>
<tr>
<td>16.3 –</td>
<td>159, 244, 300, 346</td>
<td>3,4-linked GlcNAc</td>
</tr>
</tbody>
</table>

(+), trace amounts; –, absence of the respective compounds to exclude specific structural elements in the O-linked glycans; +, presence of the respective compounds to exclude specific structural elements in the O-linked glycans. *2-linked galactose and 3-linked galactose coelute in one chromatographic peak but can be discriminated on the basis of specific fragment ions.

protonated molecular ions, which yield preferentially B ion series (data not shown).

Linkage analysis by gas chromatography (GC)–MS identification of partially methylated alditol acetates via specific fragment ions (Table III) revealed the presence of terminal Gal (2,3,4,6-tetra-O-methyl-1,5-di-O-acetylgalactitol), 3-linked Gal (2,4,6-tri-O-methyl-1,3,5-tri-O-acetylgalactitol), traces of 3-linked GalNAc-ol (4,6-di-O-methyl-1,3,5-tri-O-acetyl-N-acetylgalactosaminol), 6-linked GalNAc-ol (major 3,4-di-O-methyl-1,5,6-tri-O-acetyl-N-acetylgalactosaminol), 3,6-linked GalNAc-ol (major 4-mono-O-methyl-1,3,5,6-tetra-O-acetyl-N-acetylgalactosaminol), and 4-linked GlcNAc (3,6-di-O-methyl-1,4,5-tri-O-acetyl-N-acetylgalactosaminol). According to these data, the major ion at m/z 691 (Table II), corresponding to the disaccharide alditol NeuAc-HexNAc-ol, is identified as NeuAc2-6GalNAc-ol and confirms the de novo formation of STn in cotransfected MDA-MB-231 cells. No terminal fucose or 2-linked Gal was registered indicating absence of blood-group H structures. The same holds true for branched polylactosamines (absence of 3,6-linked Gal), type 1 lactosamines (absence of 3-linked GlcNAc), and Lewis-type glycans (absence of 3,4-linked GlcNAc).

Fig. 3. Cell growth and migration analysis. (A) Growth of monolayers. Means of fluorescence readings in 10 wells per clones and dates. a.u., arbitrary units. (B) Transmembrane cell migration. Number of migrating cells counted in eight fields per membrane, three membranes counted per condition. Histogram is representative of three independent experiments. (C) Video cell tracking. Means of the length of cellular movements (10 cells per clone). STn−, control cells; STn+ A and B, stable ST6GalNAc I transfectants. Differences in migration were statistically tested using the Student t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Fig. 4. BGN treatment effect and cell adhesion analysis. (A) Flow cytometric analysis. The presence of T antigen (Galβ1-3GalNAcα-O-Ser/Thr) was revealed using peanut agglutinin (PNA), and the presence of STn (Neu5Acα2-6GalNAcα1-O-Ser/Thr) was revealed using HB-STn1 mAb. Filled peaks, negative controls (secondary antibodies only); thin lines, cells cultured in standard condition (EMEM 10% FCS); thick lines, BGN-treated cells (2 mM, 48 h). (B) PNA labelling of total cell lysate. Total cell lysates (100 μg of proteins) from cells treated or not by BGN were subjected to SDS–PAGE. The desialylation was performed using 50 mU/mL of sialidase from *Clostridium perfringens*. O-Glycosylproteins were labelled with digoxigenin-conjugated PNA and revealed by nitroblue tetrazolium (NBT)/X-phosphate staining. (C) Cell adhesion assay. Means of the fluorescence measured from adherent cells on various extracellular matrix components (10 wells per condition). Fluorescence is given in arbitrary units (a.u.). This histogram is representative of two independent experiments. Differences in adhesion were statistically tested using the Student *t*-test: ***, *p* < 0.001. STn−, control cells; STn+ A and B, stable ST6GalNAc I transfectants.
Sialyl-Tn expression enhances breast cancer cell tumourigenicity

2000; Zanetta et al., 2000; Gouyer et al., 2001). Thus, when it is incorporated by cells, BGN mainly acts as an inhibitor of either the elongation or sialylation of the Galβ1-3GalNAcα sequence (T antigen) of mucins (Figure 1). The efficiency of BGN treatment on our models was verified by subjecting both treated and untreated STn− and STn+ cells to peanut agglutinin (PNA) labelling, using either flow cytometry (Figure 4A) or western blot (Figure 4B).

As shown in Figure 4A (panel 1), BGN treatment dramatically increased the PNA reactivity of STn− control cells. In the STn+ clones, PNA binding was almost undetectable before BGN treatment (Figure 4A, panels 2 and 3). Although the BGN treatment increased the PNA reactivity of both STn− clones, the PNA labelling still remained significantly lower (at least 10-fold) in these cells than in STn− ones. In parallel, it appears that the BGN treatment had no effect on the STn expression level (Figure 4A, panels 4–6). These observations were confirmed by western blot analysis, using digoxigenin-labelled PNA to reveal the T antigen on O-glycoproteins. As shown in Figure 4B (left panel), the T antigen was weakly expressed in control cells (lane 1) and was almost undetectable in STn+ clones (lanes 2–3). However, after BGN treatment, the PNA binding was markedly increased for both control and STn+ cells (lanes 4–6), although the PNA staining remained weaker for STn+ clones. In addition, this experiment revealed that O-glycosylation changes occurred on several glycoproteins ranging from 70 to >200 kDa. Furthermore, to unmask sialylated Galβ1-3GalNAcα-O-Ser/Thr disaccharides, a twin membrane was desialylated before incubation with PNA. As shown in Figure 4B (right panel), the sialidase treatment strongly increases PNA binding, showing that the T antigen is abundantly sialylated in both STn− control cells (lane 1 vs. 7) and in STn+ clones (lanes 2 and 3 vs. 8 and 9). However, the sialidase treatment does not increase the PNA staining in BGN-treated control cells (lane 4 vs. 10), whereas it induces a slight increase of PNA staining of the glycoproteins in BGN-treated STn+ cells (lanes 5 and 6 vs. 11 and 12). This suggests that BGN treatment prevents almost all the sialylation of T antigen in STn− control cells although part of the sialylation in STn− BGN-treated cells remains effective.

BGN-treated cells were then subjected to cell adhesion assays, as described above. As shown in Figure 4C, STn− control cells became less adherent in all the conditions after BGN treatment. By contrast, cell adhesion in STn+ clones was not affected by BGN treatment. Interestingly, cell adhesion of the BGN-treated STn− control clone became similar to that of the STn+ clones.

In vivo development of STn+ tumours

After subcutaneous injection of MDA-MB-231 in SCID mice, occurrence and growth of tumours were regularly monitored. One group was injected with STn− cells (mice designated M1 to M7), whereas the two other groups were injected with cells from either the MDA-MB-231 STn− A or B clone (designated A1 to A7 and B1 to B7, respectively). As shown in Figure 5A, MDA-MB-231 STn− cells were more likely to form tumours (6/7 mice for clone A and 5/7 mice for clone B) than control cells (4/7 mice). However, the main observation was that STn− tumours grew faster than STn+ tumours: 40 days after injection, the average sizes of tumours developed by mice from the A and B groups were 0.17 and 0.26 cm³, respectively, whereas they were only 0.01 cm³ for the M group.

Immunohistochemical analysis of STn expression in tumour sections

STn expression was investigated in serial sections from all tumours, using the HB-STn1 mAb. Representative pictures of the observed staining are shown in Figure 5B. All of the control tumours were found to be STn negative. By contrast, all the tumours from the A and B groups were found to be STn positive, although the percentage of positive cells strongly varied according to the individual tumours (5–50%). Furthermore, STn antigen was sometimes more strongly detected at the periphery of the tumour (Figure 5B[b]) or in deeper areas which were in contact with mice tissues invading the tumour (Figure 5B[c]). STn expression seemed also to be related to the tumour mass density, because the antigen was more often detected in loose areas containing sparse cells weakly interacting together (Figure 5B[d]) compared with more dense areas (Figure 5B[e]).

Discussion

Overexpression of STn occurs in almost 40% of the breast cancers (Julien and Delannoy, 2003) and is correlated with a decreased overall survival of the patients (Miles et al., 1994; Kinney et al., 1997; Imai et al., 2001; Leivonen et al., 2001). Although STn expression seems to be related to cancer development, very little is known about its direct involvement in cellular mechanisms that may increase the aggressiveness of the tumour. To clarify the actual incidence of STn expression on biological properties of breast cancer cells, we have undertaken the characterization of MDA-MB-231 clones stably expressing ST6GalNAc I, the STn synthase (Julien et al., 2001), with regard to their O-glycosylation pattern and biological behaviour both in culture and in vivo.

STn is an O-linked antigen carried by mucins or by glycoproteins containing mucin-like domains. In particular, STn has previously been reported to be associated with MUC1 in pancreatic, colonic, and breast cancer cells (Burdick et al., 1997; Julien et al., 2005), with CD44 in colonic cancer cells (Singh et al., 2001) and with the integrin β1 subunit in mouse mammary cancer cells (Clement et al., 2004). Here we show that both MUC1 and CD44 bear the STn in MDA-MB-231 ST6GalNAc I transfectants, designated these proteins as common STn carriers. In contrast, we never succeeded in revealing the STn on immunoprecipitated integrin β1 subunit from human breast cancer cell lines (data not shown). Furthermore, the pattern of STn carrying glycoproteins that we describe in this article is different from the pattern we previously described in STn+ T47-D (Julien et al., 2005). Some of these proteins seem to be commonly expressed in both cell lines (i.e., MUC1), whereas others are cell line specific (i.e., CD44); however, most of them remain unidentified. It seems that multiple combinations of STn-carrying glycoproteins might be variously
expressed by the cells depending on species, cell type, or even the cell line.

We have investigated the effect of ST6GalNAc I expression on the O-glycosylation pattern in STn+ MDA-MB-231 cells, using the MUC1–MFP6 recombinant protein as a probe. This six tandem-repeated secreted MUC1–MFP6 protein, which is reported to be O-glycosylated in a similar manner to the endogenous MUC1 (Muller and Hanisch, 2002). Here, we show that MUC1–MFP6 secreted by STn+ clones is over-sialylated compared with MUC1 produced by STn− control cells (Table I). About 22% of the O-glycosylation sites of MUC1–MFP6 produced by STn+ cells were substituted by STn. Furthermore, STn expression is associated with a decrease of both Core 1 and Core 2 biosynthesis, leading to a general shortening of the O-glycan chains (Table I). This lowering of Core 1 expression is confirmed by the decreased PNA reactivity of the transfected cells (Figure 4). MS analysis also reveals that ST6GalNAc I expression modifies the sialylation pattern of the remaining Core 1, decreasing α3-sialylation and increasing α6-sialylation. Indeed, ST6GalNAc I is also able to sialylate the Galβ1-3GalNAcα1-O-Ser/Thr structure (Ikehara et al., 1999), leading to sialyl-6T expression. This reveals that there is an enzymatic competition between ST6GalNAc I and CMP-Neu5Ac: Galβ1-3GalNAc α2,3-sialyltransferase I (EC2.4.99.4) (ST3Gal I) for the use of the Core 1 structure as acceptor substrate (Figure 1). However, since ST6GalNAc I is not active on BGN derivatives (Ikehara et al., 1999; Marcos et al., 2004), this sialyl-6T synthesis cannot be blocked by BGN treatment. In agreement, sialidase treatment increases the PNA reactivity of the glycoproteins expressed by the BGN-treated STn+ cells (Figure 4B, lanes 5 and 6 vs. 11 and 12), thus confirming the presence of more sialyl-6T structures in STn+ cells than in STn− control cells. Interestingly, it has been previously demonstrated that an increased expression of sialyl-6T may contribute to the decrease of Core 2 related structures in human colorectal carcinomas (Schneider et al., 2001). This competition might also contribute to the Core 2 expression decrease observed in our model. It appears therefore that ST6GalNAc I expression not only induces the appearance of STn, but also modifies the whole O-glycosylation pattern of several glycoproteins expressed by the cancer cells.

Concomitant to these O-glycosylation modifications, STn+ clones exhibit a decreased cell adhesion and an increased migration. Because ST6GalNAc I expression induces a shortening of the O-glycans, we have investigated the effect of BGN treatment on cell adhesion. This treatment
drastically decreases the expression of Core 2-related structures leading to an equivalent O-glycan shortening (Huet et al., 2000; Zanetta et al., 2000; Gouver et al., 2001). Interestingly, BGN-treated cells developed a similarly low adherence as the STn+ cells when compared with the control cells, indicating that the glycan chain lengths might modulate the adhesion of the STn+ cells. Taken together, these experiments suggest that O-glycosylation regulation may act as an independent modulator of the cellular behaviour through modified O-glycoproteins functions. Indeed, it has been described that over-sialylation enhances the anti-adhesive properties of MUC1 in both normal and tumour cells (Ligtenberg et al., 1992; Wesseling et al., 1996). In addition, it has been reported that shortening of the O-glycans carried by CD44 increases the CD44-mediated adhesion of colonic cancer cells to hyaluronic acid (Dasgupta et al., 1996), potentially regulating their adhesion and migration (Naor et al., 1997; Herrera-Gayol and Jothy, 1999). Because both MUC1 and CD44, expressed at the cell surface, exhibit a modified O-glycosylation pattern in MDA-MB-231 transfectants, these proteins might be involved in the phenotype changes we report. However, STn appears to be carried by several other glycoproteins, some of them either more highly expressed or more densely glycosylated than MUC1 or CD44 (Figure 2). These unknown STn bearing glycoproteins remain to be identified to determine whether or not they may be involved in the molecular mechanisms responsible of adhesion and migration changes.

Decreased cell adhesion and increased cell migration denote an aggressive phenotype in tumour cells. In addition, in vivo experiment reveals that STn expression enhances the tumourigenicity of MDA-MB-231 cells in SCID mice. This enhancement suggests that the modifications of the O-glycosylation pattern may provide some advantage to the cancer cells growing in the complex cellular environment of the host tissues. Although the precise nature of this advantage remains to be determined, previous studies have argued to suggest that STn might modulate the interactions with immune cells. For example, it has been demonstrated that STn+ mucins may inhibit the cytotoxicity of natural killer (NK) cells (Ogata et al., 2003). Moreover, the presence of anti-STn-mAb/STn-bearing-protein immunocomplexes can enhance the vascular endothelial invasiveness was previously suggested for STn+ ovarian cancers (Davidson et al., 2000). Finally, we show that STn expression enhances tumourigenicity in SCID mice, suggesting that the deregulation of O-glycans biosynthesis has a direct impact on tumour development. We propose therefore, that MDA-MB-231 STn+ clones constitute a strong cellular tool to further investigate the molecular mechanisms that enhance the STn positive breast cancer aggressiveness.

Materials and methods

Cell culture

Breast cancer cell line MDA-MB-231 was obtained from the American Type Cell Culture Collection. Cells were routinely grown in Earles’ Minimum Essential Medium (Bio Whittaker, Rockland, ME) supplemented with 10% fetal calf serum (FCS, Life Technologies, Bethesda, MD), 100 μg·mL−1 penicillin, 100 U·mL−1 streptomycin, and 45 μg·mL−1 gentamycin. STn+ clones A and B arose from stable transfected cultures (Julien et al., 2001). Serial dilutions were performed in 96-well microplates to obtain single cells developing isolated clones. STn− cells used as control cells were MDA-MB-231 mock-transfected with the Prc-CMV empty plasmid. For all experiments, cells were detached from culture flasks using ethylenediaminetetraacetic acid (EDTA) (2 mM) in sterile phosphate-buffered saline (PBS).

Electrophoresis and western blotting

Cells from confluent 100 mm diameter dishes were lysed with PBS containing 1% Triton X-100, 1% NP-40, 300 μg·mL−1 phenylmethylsulphonylfluoride, 10 μg·mL−1 leupeptin and 10 μg·mL−1 aprotinin. One hundred micrograms of protein were loaded on a 4–16% gradient SDS–PAGE under reducing conditions. Electroblotting onto nitrocellulose membranes (Biotrace NT; Gelman Science, Ann Arbor, MI) was in accordance with standard procedures (Vaessen et al., 1981). For STn blotting, the membrane was treated with fat-free milk (5% in Tris buffer saline [TBS]) and incubated with the HBSTn1 mAb (Dako, Glostrup, Denmark), diluted 1/500 in TBS (5% in Tris buffer saline) and 10 μg of CaCl2, for 4 hours at 37 °C before incubation with the digoxigenin-labelled Fab fragments (Santa-Cruz, Santa Cruz, CA) 1/2500 in TBS Tween 0.05% for 90 min. For PNA blotting, the membrane was treated with 2% polyvinylpyrrolidone in TBS. If necessary, desialylation was performed by treatment of the membrane with 50 μM−1 of sialidase from Clostridium perfringens in 50 mM citrate buffer pH 6.0, 0.9% NaCl, 0.1% CaCl2, for 4 hours at 37°C before incubation with the digoxigenin-labelled lectin. Membranes (desialylated and nondesialylated) were incubated with PNA-dig (Peanut Agglutinin digoxigenin labelled) (2 μg·mL−1 in TBS Tween 0.05%, for 90 min). The nitrocellulose membranes were then incubated for 1 h with antidigoxigenin alkaline phosphatase-labelled Fab fragments (1 μg·mL−1 in TBS Tween 0.05% for 90 min). After washing, labelled glycosylproteins were revealed by nitroblue tetrazolium (NBT)/X-phosphate staining.

Immunoprecipitation

Total cell lysate protein (1 mg) was incubated in nonde-naturing conditions (overnight, 4°C) with either 5 μg of
the anti-CD44 mAb (HCAM-DF1485, Santa-Cruz) or 50 μL of culture supernatant containing anti-MUC1 mAb (LICR-LON-M8) (McIlhinney et al., 1985). Cell lysates were then incubated with 250 μL of magnetic beads coupled to a secondary antibody (Dynabeads M450 sheep anti-mouse Ig, Dynal, Oslo, Norway) for 4 h, at 4°C. Immunoprecipitated proteins were collected using the Dynal MPC-S magnet and subjected to SDS–PAGE, as described above.

O-glycan profiling

Expression of MFP6 plasmid. The MFP6 plasmid (pCEP-PU derived plasmid) encodes a fusion protein containing six MUC1 tandem repeats, a signal peptide for secretion and a Myc tag (Muller and Hanisch, 2002). The MDA-MB-231 STn+s clone A was transfected using lipofectamine reagent plus lipotransfectant (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were treated with 1 μg·mL⁻¹ puromycin during 3 days. Resistant transfectants were then expanded and cultured in the presence of 0.1 μg·mL⁻¹ puromycin to keep selective pressure on the vector.

Isolation and purification of MUC1 constructs from culture supernatants. As previously described, supernatants from confluent cell cultures were collected and proteins were separated by immobilized metal chelate affinity chromatography (Muller and Hanisch, 2002). Anti-myc positive fractions were subjected to further purification by HPLC on a reversed-phase C8-column (Vydac 214TP3410, MZ Analy-sentechnik, Mainz, Germany) (Muller and Hanisch, 2002).

Liberation, fluorescence labelling, and analysis of O-linked glycans. Glycans were liberated by hydrazinolysis, labelled with 1 M 2-aminobenzamide in acetic acid and 2 M glycans. Liberation, fluorescence labelling, and analysis of O-linked glycans were subjected to further purification by HPLC on a reversed-phase C8-column (Vydac 214TP3410, MZ Analy-sentechnik, Mainz, Germany) (Muller and Hanisch, 2002).

Mass spectrometric sequencing of permethylated glycan alditols

ESI MS data were acquired on a Q-Tof 2 quadrupole-time of flight mass spectrometer (Waters, Eschborn, Germany) equipped with a Z spray source. ESI(QToF) MS was performed in the positive ion mode using previously described conditions (Morelle et al., 2004). The permethylated glycans were dissolved in 80% methanol containing 1% acetic acid before loading 3 μL into a nanospray capillary coated with a thin layer of gold/palladium, tip inner diameter 2 μm (Proxen, Odense, Denmark). A potential of 800 V was applied to a nanoflow tip. The drying gas used was nitrogen and the collision gas was argon, with the collision gas pressure maintained at 0.5 bar. The cone voltage was set at 50 V. Collision energies varied in accordance with the type of molecular ion (M + Na, 50–75 V; M + H, 15–30 V).

Chemical derivatization for GC–MS and ESI–MS/MS

Permethylation was performed using the sodium hydroxide procedure of Ciucanu and Kerek as modified by Anumula (Anumula and Taylor, 1992). Partially methylated alditol acetates were prepared by hydrolysis of permethylated glycans with 2M TFA (Fluka, Taufkirchen, Germany) for 2 h at 121°C, by reduction with 10 mg/mL sodium borodeuteride (Sigma, St. Louis, MO) in 2 M aqueous ammonium hydroxide at room temperature for 2 h and acetylation with acetic anhydride (Fluka) at 100°C for 1 h (Albersheim et al., 1967). The partially methylated alditol acetates were extracted with chloroform-water, dried, and analyzed as a dichloromethane solution by GC–MS on a Fison MD800 (Thermo Electron, Dreieich, Germany) using a 15 m RTX5-SILMS column from Restek (Bad Homburg, Germany) and a temperature gradient from 60 to 100°C (40°C/min) followed by 100–280°C (10°C/min).

Monolayer growth assay. Cells (5 × 10³) were seeded in 96-well black plates (Costar, Acton, MA) and cultured in 10% FCS-containing medium. Cell growth was determined every day. Cells were washed once with PBS, fixed with cold methanol (20 min, −20°C) and stained with Hoechst 33528 (Sigma). The fluorescence of stained nuclei was measured using a microplate fluorescence reader (FLx800–Bio-Tek Instrument, Inc., Winooski, VT).

Cell migration analysis

Transwell system analysis. Cells (2 × 10⁴) were seeded in Transwell 12-well plates (Costar) and incubated for 18 h in 10% FCS-containing medium. Cells were fixed and stained with Hoechst 33528 (Sigma). The stained migrating cells, which had crossed the porous membrane, were then counted under fluorescence microscopy.

Video analysis. Low-density cell cultures (5 × 10⁵ cells in 25 cm² flasks) were recorded by a camera under standard culture conditions (10% FCS-containing medium, 37°C) over a 10-h period. The migration distance of cells was measured picture-by-picture, using homemade software developed with visual basic (Microsoft) and using Mil 7.5 algorithms (Matrox, Dorval, Canada).

BGN treatment protocol. Cells (1 × 10⁶) were seeded in 100 mm diameter dishes and cultured for 24 h in standard conditions. Cells were then treated with 2 mM BGN (Sigma) for 48 h with medium replacement every day. After treatment, cells were collected and used for further experiments.

Flow cytometric analysis. For PNA labelling, cells (6 × 10⁶) were incubated with digoxigenin-labelled PNA (Roche, Basel, Switzerland), 20 μg·mL⁻¹ in TBS, for 1 h on ice, washed twice in TBS, and incubated with FITC-conjugated antidigoxigenin secondary antibody (Dako), 1/100 in TBS, for 30 min on ice. For STn labelling, cells (6 × 10⁶) were incubated with the HB-STn1 mAb, 1/50 in PBS, 1% BSA, for 1 h on ice, washed twice with PBS 1% BSA, and incubated with FITC-conjugated anti-mouse whole IgG (Sigma), 1/62 in PBS 1% BSA, for 30 min on ice. Cells were then subjected to flow
cytometric analysis using a FACS calibur instrument (Becton Dickinson, San Jose, CA).

**Cell adhesion assay.** Cells (2 × 10^6) were seeded in 96-well black plates (Costar) coated with type I collagen (15 μg/cm²) or fibronectin (5 μg/cm²). After 18 h of incubation in 10% FCS-containing medium, wells were washed three times with PBS and the remaining adherent cells were fixed and stained with Hoechst 33528 (Sigma). The fluorescence of stained nuclei was measured using a microplate fluorescence reader (FLx800–Bio-Tek Instrument, Inc.).

**Tumour growth in SCID mouse xenograft model.** Six-week-old female SCID mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimatized for at least 2 weeks. Mice were maintained under a 12-h light/dark cycle (lights on from 6:00 A.M. to 6:00 P.M.) at a temperature of 20–22°C. Food and water were available ad libitum. Mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Cellular suspensions (in PBS) were injected subcutaneously into mice flanks (2 × 10^6 cells/flank) of 8-week-old SCID mice. The tumour volume was regularly monitored by measuring the length (l) and width (w) and calculating the volume (V = π/6 × l × w × [l + w]/2).

**Immunochemical detection of STn antigen.** Thick tumours sections (8 μm) were deparaffinized in xylene, rehydrated in ethanol series, incubated in a methanol, H2O, H2O2 (70:27:3 vol/vol/vol) solution (30 min), blocked with 1% BSA, 0.05% Tween TBS buffer (30 min), and then exposed to the HB-STn1 mAb (Dako) diluted 1/20 at 4°C (overnight). After washing, the sections were incubated with a donkey anti-mouse antibody conjugated to peroxidase (Jackson, West Grove, PA) diluted 1/500 at 4°C. Sections were washed again in PBS and developed using diaminobenzidine (DAB, Sigma). Counter-coloration was performed using Evans Blue.

**Acknowledgments**

We thank Johann Antol and Isabelle Pollet-Lefèvre for their excellent technical assistance. This work was supported by the University of Sciences and Technologies of Lille and the Association pour la Recherche sur le Cancer (Grant no. 5469). Sylvain Julien had a fellowship of the Gênonope de Lille.

**Abbreviations**

BGN, 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; ESI, electrospray ionization; Gal, galactose; GaNAc, N-acetylgalactosamine; GC, gas chromatography; GlcNAc, N-acetylglucosamine; HexNAc, N-acetyhexosamine; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MS, mass spectrometry; NeuAc, N-acetylneuraminic acid; PBS, phosphate-buffered saline; PNA, peanut agglutinin; SCID, severe combined immunodeficiency; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, ST6GalNAc I, CMP-Neu5Ac: R-GalNAcα1-O-Ser/Thr α2,6-sialyltransferase (EC 2.4.99.3); STn, sialyl-Tn antigen; TBS, Tris buffer saline.

**References**


Imada, T., Rino, Y., Hatori, S., Takahashi, M., Amano, T., Kondo, J., and Suda, T. (1999) Sialyl Tn antigen expression is associated with the


