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Biological significance of cancer-associated sialyl-Tn antigen: Modulation of malignant phenotype in gastric carcinoma cells

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Abstract

The activation of an abnormal glycosylation pathway in cancer cells leads to the formation of the sialyl-Tn antigen, blocking regular carbohydrate chain elongation. Sialyl-Tn antigen is rarely expressed in normal tissues but is aberrantly expressed in a variety of carcinomas, where it constitutes a marker of poor prognosis. Although the clinical significance of sialyl-Tn is well characterized, a functional role for this glycan and its contribution to cancer progression remain to be elucidated. This study evaluates the capability of sialyl-Tn to modify processes like cell cycle, apoptosis, actin cytoskeleton dynamics, adhesion and motility on ECM components, cell–cell aggregation and invasion. *De-novo* expression of sialyl-Tn leads to major morphological and cell behavior alterations in gastric carcinoma cells which were reverted by specific antibody blockage. Sialyl-Tn antigen is able to modulate a malignant phenotype inducing a more aggressive cell behavior, such as decreased cell–cell aggregation and increased ECM adhesion, migration and invasion.

Keywords: Sialyl-Tn; Gastric carcinoma; Sialyltransferase; Malignant phenotype; Adhesion; Invasion

1. Introduction

Altered glycosylation is a common feature of cancer cells. Membrane-bound carbohydrates are key-factors of cellular recognition processes, with crucial relevance to the onset of metastasis [1,2]. The aberrant expression of sialylated structures at the surface of cancer cells has been widely reported

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and the extent of cell surface sialylation has been repeatedly correlated with the invasive potential of various cell lines [3–5]. The sialyl-Tn antigen (Neu5Acα2-6GalNAc-*O*-Ser/Thr), also known as STn and CD175s, is a simple mucin-type carbohydrate antigen which has attracted much attention because it is highly expressed in most gastric [6–8], colorectal [9], ovarian [10], breast [11,12] and pancreatic [13] carcinomas, whereas no expression is observed in the respective normal tissues. In addition, sialyl-Tn expression is associated with carcinoma aggressiveness and poor prognosis. Sialyl-Tn expression has been shown to be an independent indicator of poor prognosis in carcinomas and in sera of patients with gastric [14–16], colorectal [17,18] and ovarian cancer [19]. Sialyl-Tn is also aberrantly detected in premalignant lesions of the gastrointestinal tract, such as intestinal metaplasia [7], adenomatous polyps [17], chronic ulcerative colitis [18] and pancreatic intraepithelial neoplasias [13]. Furthermore, the expression of sialyl-Tn in carcinomas has been associated with the aberrant glycosylation of the MUC1 mucin, with both sialyl-Tn and MUC1 being used as target for cancer immunotherapy in preclinical and clinical studies [20–27].

The synthesis of the simple mucin-type sialyl-Tn antigen depends upon the activity of an α2,6-sialyltransferase that catalyzes the transfer of sialic acid from CMP-Neu5Ac to a GalNAc O-linked to serine or threonine residues of a polypeptide. Depending on the cell type and the tissue of origin the GalNAcα-*O*-Ser/Thr, also known as Tn antigen, is processed by different glycosyltransferases, adding monosaccharides in a sequential way in the Golgi apparatus lumen, forming a variety of *O*-glycans, such as the core structure Core1 (Galβ1-3GalNAcα-*O*-Thr/Ser), also known as T antigen, and subsequently Core2 (GlcNAcβ1-6[Galβ1-3]GalNAcα-*O*-Thr/Ser) which may be further processed forming elongated structures. A common feature in carcinoma cells is the activation of an alternative pathway corresponding to the early α2,6-sialylation of GalNAcα-*O*-Ser/Thr leading to the formation of the sialyl-Tn antigen. The sialyl-Tn structure cannot be further processed and therefore blocks the biosynthesis of any core structure and the posterior elongation [28]. Among the eight α2,6-sialyltransferases identified so far, only ST6GalNAc-I and ST6GalNAc-II have been shown to be able to sialylate *in vitro* the Tn antigen (GalNAcα-*O*-Ser/Thr) and the T antigen (Galβ1-3GalNAcα-*O*-Ser/Thr), thus leading to the formation of sialyl-Tn and sialyl-6T antigen (Galβ1-3[Neu5Acα2-6]GalNAcα-*O*-Ser/Thr), respectively [29–31]. We have previously shown that substrate affinity and availability determines the ST6GalNAc-I as the major sialyl-Tn synthase, supporting the hypothesis that the expression of the sialyl-Tn antigen in cancer cells is due to ST6GalNAc-I activity [32]. Recently, Sewell et al. have further confirmed this hypothesis by showing that sialyl-Tn expression in breast cancer is correlated with ST6GalNAc-I, and not with ST6GalNAc-II [33].

Whereas the clinical significance of sialyl-Tn and its biosynthetic pathway have been characterized, a functional role for this glycan in cancer cells remains to be elucidated. Little is known about the mechanisms by which this structure could induce cancer aggressiveness. Sialyl-Tn expression is thought to be implicated in the cell's interactions and recognition with the surrounding environment as its biosynthesis gives rise to negatively charged short carbohydrate chains. It induces further modification on the cell surface glycosylation profile due to the exposure or masking of underlying sugar structures [34,35]. These major glycosylation alterations induce or prevent the recognition by lectin like molecules, such as selectins [36], siglecs [37] and galectins [38], which may contribute to cancer development by modulating cell–cell and cell–matrix interactions. Invasion is a crucial step in the development of cancer since it is responsible for malignancy either through locoregional spread or through metastasis to distant organs. The cross talk between cancer cells and host elements modulates invasion-associated processes such as cell–cell adhesion, cell–matrix interaction, proteolysis, ectopic survival, growth and motility [39,40]. Additionally, previous studies have also shown that sialyl-Tn antigen plays a role in cancer cell recognition by the immune system, protecting metastatic cells from degradation in the blood stream [41].

In the present report, we examine the biological significance of sialyl-Tn expression in human gastric carcinoma cells stably transfected with either ST6GalNAc-I or ST6GalNAc-II and expressing mostly sialyl-Tn or sialyl-6T, respectively. Our aim is to evaluate whether sialyl-Tn expression can modulate *per se* a malignant phenotype in gastric carcinoma cells by interfering with processes related to carcinoma progression and aggressiveness, such as cell cycle, apoptosis, actin cytoskeleton dynamics, adhesion and motility on extracellular matrix

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components, homotypic cell–cell aggregation and invasion.

2. Materials and methods

2.1. Cells, antibodies and reagents

MST6-I, MST6-II and mock-transfected cell lines were previously established by ourselves by stable transfection of MKN45 gastric carcinoma cell line with ST6GalNAc-I, ST6GalNAc-II or empty vector, respectively [32]. Cells were grown in RPMI 1640 medium with Glutamax supplemented with 10% inactive fetal calf serum (FCS), 50 μg/ml gentamicin and 0.3 mg/ml G418 (Gibco BRL), at 37 °C in a humidified atmosphere of 5% CO_2. Dulbecco's modified Eagle medium (DMEM), trypsin/EDTA, were purchased from Gibco BRL. The monoclonal antibodies (MAbs) used for immunodetection of carbohydrate antigens, are listed in Table 1. Rabbit non-immune serum and FITC-conjugated rabbit anti-mouse immunoglobulin were from DakoCytomation. FITC-conjugated AfinityPure immunoglobulin anti-mouse IgG for flow cytometry was purchased from Jackson ImmunoResearch Laboratories. Neuraminidase from *Clostridium perfringens* type IV, trypan blue, propidium iodide (PI), FITC-phalloidin, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), collagen I and fibronectin were all from Sigma. The medium used to preserve fluorescence was Vectashield from the Vector Laboratories, Inc. The *in vitro* invasion assays were performed in BD Biocoat™ Matrigel™ invasion chamber from BD Biosciences. The matrigel basement membrane matrix contains laminin, collagen type IV, heparan sulfate proteoglycan, among other factors as described by BD Bioscience. 3,3'-Dihexyloxalocarbocyanine [$DiOC_6(3)$] was acquired from molecular probes.

2.2. Immunofluorescence microscopy

Cell lines were fixed in acetone for 5 min prior to immunofluorescence. Detection of antigen sialyl-6T was done using either the anti-T antibodies HH8 or HB-T (Table 1) after neuraminidase treatment. Neuraminidase treatment and immunofluorescence were performed as previously described [42].

A semi-quantitative approach was used to score the immunofluorescence labelling. Percentage of positive cells was scored as: <25%, 25–50%, 50–75% and >75%, based on the analysis of approximately 1×10^4 cells. Intensity of immunofluorescence labelling was graded as: −, negative; +, weakly positive; ++, moderately positive; +++, strongly positive. Results are based on three independent assays analyzed by two independent observers.

2.3. Flow cytometric analysis

Cells were trypsinized and washed twice with PBS. After being centrifuged at 1000 rpm for 10 min, cells were resuspended at a concentration of 2×10^6 cells/ml in PBS. Aliquot cells of 1 ml were fixed in 70% ethanol at 4 °C for 15 min. After washing with PBS, samples designed for neuraminidase treatment were incubated with the enzyme as described above. The incubation was carried out for 1 h at 37 °C and washed with cold PBS. Cells were incubated with MAb TKH2 or HH8 (Table 1) diluted 1:2 in PBS for 1 h on ice. Cells, once washed with PBS, were stained with FITC-conjugated AfinityPure immunoglobulin anti-mouse IgG diluted 1:50 in PBS and then subjected to FACScan in a Counter Epics® XL-MCL (Coulter).

2.4. Cell growth analysis

Cells were grown in 24-well culture plates at a density of 5×10^4 cells/well. At different time points (24, 48, 72 and 96 h), cells were trypsinized and aliquots of these cells

Table 1
Specificity and dilution of monoclonal antibodies

Specificity	Monoclonal antibody	Dilution	References
Tn (GalNAcαThr/Ser)	HB-Tn	1:60	DAKO
Tn (GalNAcαThr/Ser)	1E3	1:2	Clausen and Hakomori[a]
Sialyl-Tn (NeuAcα2-6GalNAcαThr/Ser)	HB-STn	1:100	DAKO
Sialyl-Tn (NeuAcα2-6GalNAcαThr/Ser)	TKH2	1:2	[62]
T (Galβ1-3GalNAcαThr/Ser) [b]	HB-T	1:100	DAKO
T (Galβ1-3GalNAcαThr/Ser)[b]	HH8	1:2	[63]
Le^a (Galβ1-3(Fucα1-4)GlcNAc-R)	CA3F4	1:5	[64]
Sialyl-Le^a (NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc-R)	HB-80	1:5	[65]
Le^b (Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-R)	BG6	1:150	Signet pathology systems
Le^x (Galβ1-4(Fucα1-3)GlcNAc-R)	SH1	1:5	[66]
Sialyl-Le^x (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R)	FH6	1:5	[67]
Le^y (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc-R)	AH6	1:5	[68]

[a] Clausen and Hakomori, unpublished.
[b] Sialyl-6T antigen was detected using anti-T antigen antibodies after neuraminidase treatment as described in Section 2.

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resuspended in PBS containing trypan blue 0.1%. Trypan blue negative viable cells were counted using a Neubauer chamber. Triplicate wells were analyzed for each time point.

2.5. Cell cycle analysis

Cells were grown previously in serum-free medium for 24 h to arrest the cells in G1 phase. Then cells were cultured in 20% serum-containing medium. Cells were collected 48 h later, washed twice in PBS and then permeabilized with ice-cold 70% ethanol for 1 h before being washed again. Cells were stained for 30 min at 37 °C with 50 μg/ml of propidium iodide (PI) and 100 μg/ml of RNase in PBS. Samples were filtered through 44 μm nylon mesh and labelled cells were immediately acquired in a FACScan Counter Epics® XL-MCL and DNA content (PI fluorescence) monitored.

2.6. Detection of apoptosis

For assessment of apoptosis, culture supernatants were collected and adherent cells were harvested by gentle trypsination. Cells and supernatants were combined and briefly centrifuged to allow sedimentation. To obtain a quantitative estimate of the percentage of cells undergoing apoptosis, cells were stained, during 30 min at 37 °C, with 400 μl of PBS containing 25 μl of 0.5 μg/ml $DiOC_6(3)$, a lipophilic cationic fluorochrome that is retained within the mitochondria under the influence of the inner mitochondrial potential gradient ($\Delta\Psi_m$). Used in conjunction with 100 μl of 50 μg/ml of PI, during 10 min at 4 °C, to gate dead cells allows resolution of apoptotic populations ($\Delta\Psi_m$ low/PI low). Fluorescence intensity of $DiOC_6(3)$ and PI were analyzed in a FACScan Counter Epics® XL-MCL.

2.7. Cell adhesion assay

Cell adhesion assays were performed in a 96-well plate coated with different extracellular matrix (ECM) proteins, collagen I (3.3 μg/well) or fibronectin (3.0 μg/well). Viable cells (1×10^4 cells/well) were introduced into the plates and allowed to adhere for 20 h in serum-free medium, at 37 °C. After several washings with PBS, cells attached into each well were incubated with 20 μl of MTT solution (5 mg/ml), at 37 °C for 5 h. After removing the medium, 200 μl of DMSO was added to each well and incubated at 37 °C for 5 min. The absorbance was measured at $\lambda = 540$ nm.

2.8. Slow aggregation assay

Slow aggregation assays were done as described [43]. Briefly, single cell-suspensions were seeded onto 96-well plates (2×10^4 cells/well) coated with 50 μl of semi-solid agar (0.66% w/v) freshly prepared in sterile Ringer's salt solution (NaCl 155 mM, 9.0580 g/L; $CaCl_2$ 2.3 mM, 0.2553 g/L; KCl 5.5 mM, 0.4100 g/L; pH 7.4), in the absence or presence of HB-STn, an anti-sialyl-Tn antibody, and HB-T, an anti-T antibody. After 24, 48 and 72 h cell aggregation was evaluated under an inverted microscope, with a magnification of 40×.

2.9. Cell migration assay

Plates were coated with either collagen I (300 μg/ml) or fibronectin (10 μg/ml), or uncoated. Cells were allowed to attach to a restricted area of the bottom of each well in a 24-well plate placed under a 15° slope. After 24 h, culture medium was replaced with fresh medium, slope was eliminated and cell culture edge was plotted. At 48 h cell migration was evaluated by counting the number of cells which had reached a defined distance from the edge of the cell culture.

2.10. Actin immunofluorescence

Glass coverslips were placed in a 24-well plate and coated with either collagen I (300 μg/ml) or fibronectin (10 μg/ml) overnight at 4 °C and then blocked with 2% BSA/PBS. Cells were serum-starved overnight and then seeded at a density of 5×10^4 cells in a 24-well plate, and allowed to adhere for 24 h. After washing two times with PBS, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 5 min. Cells were then stained with FITC-conjugated phalloidin and with DAPI, 30 min in the dark. All staining procedures were carried out at room temperature. Cells were washed with PBS and then coverslips were mounted upside-down on microscope slides with Vectashield medium.

2.11. Invasion assay

Invasion assays were performed in a BD Biocoat™ Matrigel™ invasion chamber, with an 8-μm diameter pore size membrane and a thin layer of matrigel, in a 24-well plate. Membranes were re-hydrated in serum-free DMEM medium for 2 h at 37 °C before the experiment. After detachment of subconfluent cells with trypsin/EDTA, cells were suspended in culture medium supplemented with 5% inactivated FBS, counted and seeded into the upper side of the matrigel-coated chamber at a density of 2×10^6 cells/well. Culture medium containing 20% inactivated FBS was added to the lower part of the chamber as chemoattractant. After 48 h at 37 °C, non-invading cells on the upper side of the membranes were completely removed and cells that had invaded the underside of the membranes were fixed with cold methanol for 5 min and stained with DAPI at RT for 20 min. The membranes were washed with PBS, carefully removed from the inserts

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and mounted on microscope slides with Vectashield medium for fluorescence. The stained cells which had invaded the underside of the membranes were counted in a fluorescence microscope. Results are presented as means ± SD for each sample. Invasion levels are expressed as a *ratio* of the results obtained with the mock-transfected control cell line.

2.12. Cell lysis, immunoprecipitation and blotting

MKN45*wt* and MST6-I cells were washed twice with ice-cold PBS (pH 7.45) and lysed with ice-cold nondenaturating RIPA buffer (0.1 M PMSF, 0.2 M NaOVa, Protease Inhibitor Cocktail (Roche)). Cells were scrapped off and the suspension was kept 30 min on ice. Total cell lysates were cleared by microcentrifugation (15 min at 16,000*g*, 4 °C) and by incubation with protein G-Sepharose beads (Sigma) for 30 min at 4 °C.

For immunoprecipitation, protein G-Sepharose beads were incubated with MAb TKH2 (anti-sialyl-Tn; 2 μg) or MAb HMFG1 (anti-MUC1; 200 μl) [44] for 2 h at 4 °C under rotation. Beads were then washed twice with RIPA buffer. Three milligram of cleared cell lysates was incubated with previously prepared MAb TKH2 or MAb HMFG1 conjugated beads for 2 h at 4 °C under rotation. Beads were then washed twice with washing buffer (1 M Tris–HCl, pH 7.4, 0.25 M EDTA, 5 M NaCl, 0.1% Triton-X100, 0.003 M NaN_3). Bound proteins were eluted by boiling in sample buffer (125 mM Tris–HCl, pH 6.8, 4% (v/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol) for 10 min. Supernatants were subjected to 4–10% gradient SDS–PAGE electrophoresis and transferred onto an Immobilon-P polyvinylidene di-fluoride (PVDF) membrane in 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 20% methanol at 50 mA for 1 h. Membranes were blocked with 0.02% (v/v) Tween 20 in PBS containing 5% non-fat skim milk for 1 h at RT. Membranes were then incubated overnight at 4 °C with MAb TKH2 (6 μg) or HFMG1 diluted 1:5 in blocking buffer. After washing three times with PBS–Tween, membranes were incubated for 1 h with peroxidase-labeled anti-mouse IgG diluted 1:1000 in blocking buffer. After washing, the bound antibodies were revealed by chemiluminescence using the ECL Kit (BIO-RAD).

3. Results

3.1. Expression of carbohydrate structures in MST6-I and MST6-II cells

MST6-I, MST6-II and mock-transfected cell lines, previously established by stable transfection of MKN45 gastric cell line with ST6GalNAc-I, ST6GalNAc-II or empty vector, respectively [32], were characterized for carbohydrate antigens expression by immunofluorescence (Table 2) and FACScan analysis (Fig. 1). MST6-I showed high levels of expression of sialyl-Tn in more than 85% of the cells as shown in Fig. 1B. ST6GalNAc-I also induced low levels of expression of sialyl-6T in less than 20% of the cells (Fig. 1E). On the contrary, MST6-II showed high levels of expression of sialyl-6T in 65% of the cells (Fig. 1F) and also induced low levels of expression of sialyl-Tn, in 15% of the cells (Fig. 1C). Antigen Tn was expressed in similar levels both in mock-transfected cell line and MST6-II. Lower expression of Tn antigen was observed in MST6-I. Antigen T was not observed in the MST6-I, MST6-II and mock-transfected cell lines. Expression of terminal carbohydrate antigens, such as the Lewis antigens Le^a, Le^b, Le^x, Le^y, sialyl- Le^a and sialyl-Le^x, were similar among cell lines MST6-I, MST6-II

Table 2
Expression of carbohydrate antigens in MKN45 cells transfected with ST6GalNAc-I and ST6GalNAc-II and mock-transfected

	Mock-transfected		MST6-I		MST6-II	
	Intensity[a]/ localization[b]	Percentage of positive cells	Intensity/ localization	Percentage of positive cells	Intensity/ localization	Percentage of positive cells
Tn	++/D,M	50–75	+/D,M	25–50	++/D,M	50–75
Sialyl-Tn	–	NA[c]	+++/M,PN	>75	+/M,PN	<25
T	–	NA	–	NA	–	NA
Sialyl-6T	–	NA	+/M,D	<25	+++/M,PN	50–75
Le^a	+++/D,M	<25	+++/D,M	<25	+++/D,M	<25
Sialyl-Le^a	+/PN,D	<25	+/PN,D	Rare	+/PN,D	Rare
Le^b	+/M,D	<25	+/M,D	<25	+/M,D	<25
Le^x	+++/D,M	25–50	+++/D,M	25–50	+++/D,M	25–50
Sialyl-Le^x	+/D,M	Rare	+/D,M	Rare	+/D,M	Rare
Le^y	+/D	Rare	+/D	Rare	+/D	Rare

[a] Intensity grading: –, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.
[b] PN, perinuclear staining; D, diffuse cytoplasmic staining; M, membrane staining.
[c] NA, not applicable.

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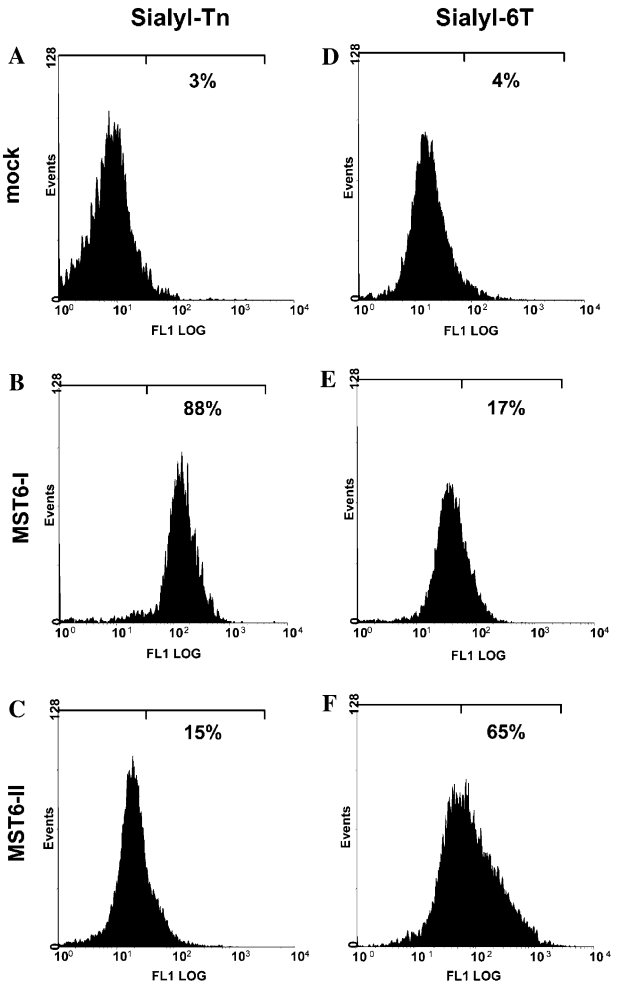

Fig. 1. Flow cytometry analysis of the expression of sialyl-Tn and sialyl-6T in MST6-I, MST6-II and mock-transfected cell lines. Detection of sialyl-Tn was done using MAb TKH2 (A–C). Detection of sialyl-6T was done using the anti-T MAb HH8 after neuraminidase treatment (D–F). MST6-I and MST6-II showed 88% and 15% of sialyl-Tn positive cells, respectively. In contrast, MST6-I and MST6-II showed 17% and 65% of sialyl-6T positive cells, respectively.

and mock-transfected (Table 2). In all experiments mock-transfected cell line showed the same results as MKN45*wt* (data not shown).

3.2. MST6-I and MST6-II cells show lower proliferation rates

We analyzed whether MST6-I and MST6-II showed different growth rates when compared to mock-transfected cell line. Growth curves of MST6-I and MST6-II cells showed a significant time-dependent growth inhibition at 72 and 96 h when compared to mock-transfected cell line, with $P < 0.05$ and $P < 0.01$, respectively (Fig. 2A).

In order to analyse, the basis for the lower proliferation ability of MST6-I and MST6-II we analyzed both cell cycle progression (Fig. 2B) and percentage of apoptotic cells (Fig. 2C) by flow cytometry. Cell populations were synchronized in G0/G1 by serum starvation and released into the cell cycle by addition of FBS. No significant differences in the cell cycle progression of MST6-I and MST6-II cells were observed when compared to mock-transfected cells (Fig. 2B). Further evaluation of the percentage of apoptotic cells by double staining with $DiOC_6(3)$ and PI showed an increase in the percentage of apoptotic cells in MST6-I and MST6-II, 18% and 24%, respectively, when compared to mock-transfected cell line (6%; Fig. 2C). These observations suggest that the decreased cell growth may be due to an increase in the percentage of apoptotic cells and not to a G1 cell cycle delay.

3.3. MST6-I and MST6-II cells show reduced homotypic cell–cell aggregation

In the slow aggregation assay, mock-transfected cells, which have no sialyl-Tn, aggregated forming compact cell clusters (Fig. 3A). The addition of HB-STn to the

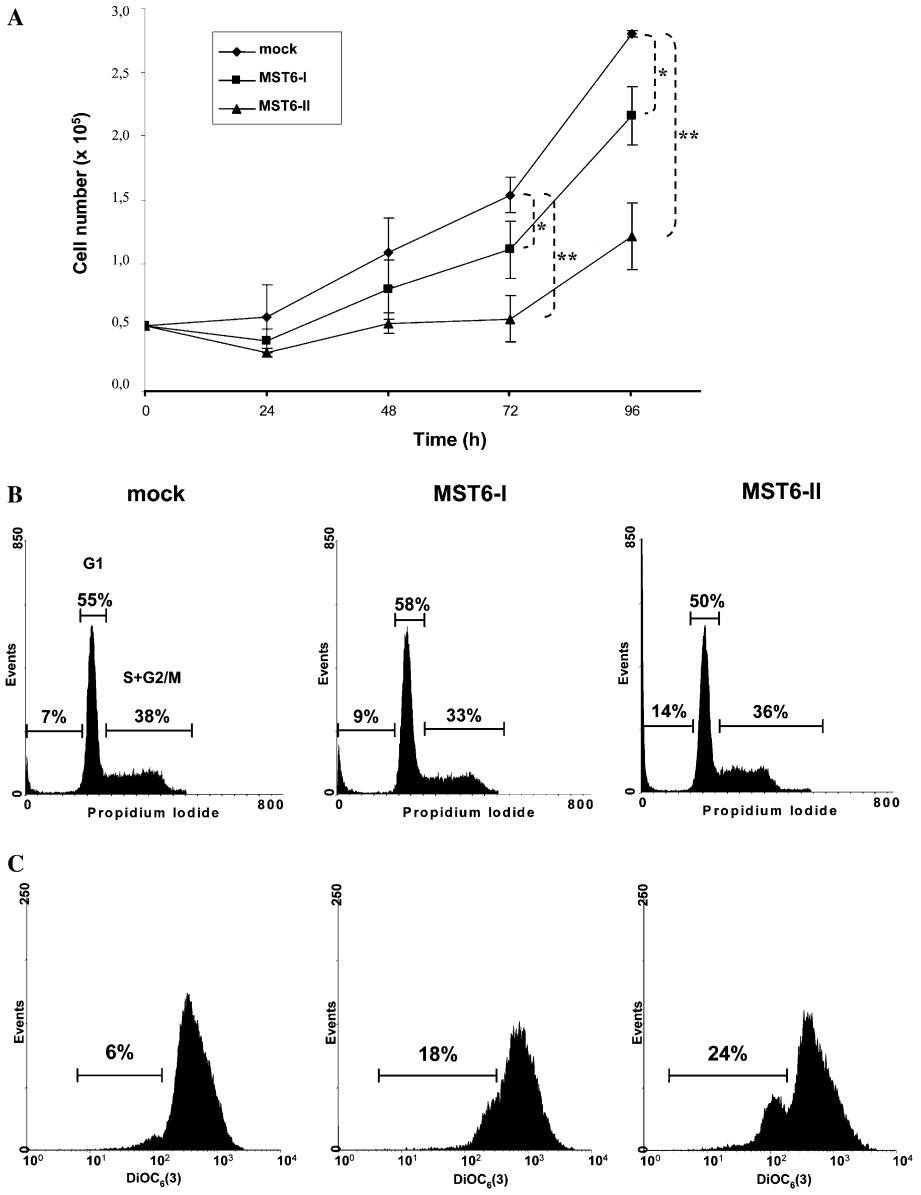

Fig. 2. Growth rate of MST6-I and MST6-II is impaired and is due to an increase in the percentage of apoptotic cells rather than by G1 cell cycle inhibition. (A) MST6-I and MST6-II cells show a decreased growth rate when compared to mock-transfected cells. Data points represent the means ± SD of four independent experiments, each carried out in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$; Student's t test. (B) Flow cytometric histograms of PI staining of synchronized cell populations, to evaluate cell cycle progression by DNA content. Histograms show the percentage of sub-G0 cells, cells into the G1 and S + G2/M phases. (C) Flow cytometric histograms of $DiOC_6(3)$ staining of MST6-I, MST6-II and mock-transfected cells. Cells were also stained with PI, in conjugation with $DiOC_6(3)$ to gate dead cells, allowing resolution of apoptotic populations ($DiOC_6(3)$ low). Data are representative of three independent experiments.

medium induced no alterations to the aggregation phenotype (Fig. 3B). On the contrary, sialyl-Tn expressing MST6-I cells, showed a markedly reduced homotypic cell–cell aggregation, with most cells remaining solitary (Fig. 3D). The addition of HB-STn to the medium reverted the single cell phenotype of MST6-I with the formation of cell clusters similar to mock-transfected cells (Fig. 3E). MST6-II, which has low levels of sialyl-Tn, showed cluster formation with loose aggregates (Fig. 3G). Blockage with HB-STn MAb also reverted the phenotype of MST6-II cells to aggregation levels similar to mock-transfected cell line (Fig. 3H). In the presence of a control HB-T antibody none of the cells showed phenotype alterations.

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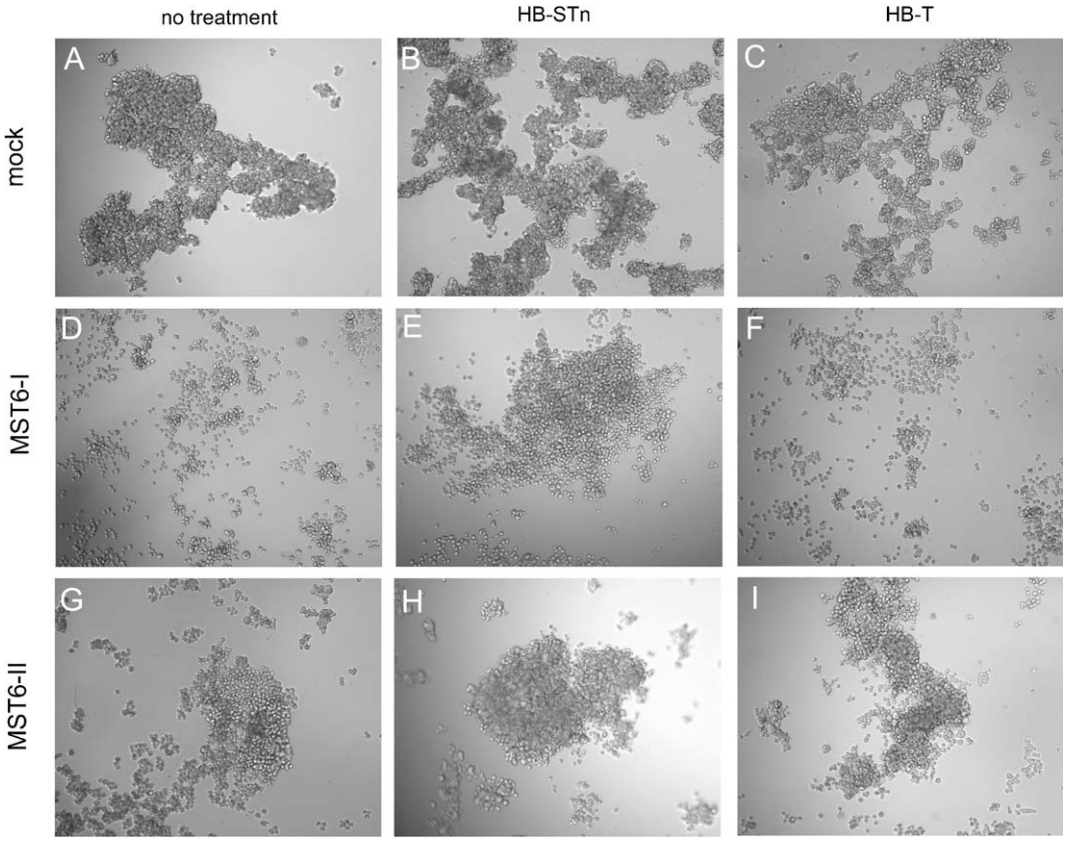

Fig. 3. Slow aggregation assay of MST6-I, MST6-II and mock-transfected cell line, with or without specific MAb incubation. (A–C) Mock-transfected cells aggregated forming compact cell clusters, and blockage of sialyl-Tn exerted no effect; (D–F) MST6-I cells showed a markedly reduced aggregation, with most cells remaining solitary, but blockage of sialyl-Tn reverted the phenotype; (G–I) MST6-II cells show cluster formation but with some loose aggregates and blockage of sialyl-Tn also reverted its phenotype. HB-T MAb was used as control and exerted no effect on the cell lines.

3.4. High adhesion and migration of MST6-I cells in ECM proteins coated surfaces

We investigated whether overexpression of sialyl-Tn or sialyl-6T affected the ability of MST6-I and MST6-II cells to adhere and to move on culture plates coated with collagen I or fibronectin, or uncoated. Using the MTT assay, significant differences in adhesion capability were observed in MST6-I cells when plated on these extracellular matrix (ECM) components. MST6-I cells showed a significantly increased adhesion to collagen I ($P < 0.02$) and to fibronectin ($P < 0.05$), when compared to mock-transfected cells (Fig. 4A). The migration potential of MST6-I cells expressing sialyl-Tn showed a significant increase on fibronectin ($P < 0.02$), and on collagen I ($P < 0.02$), when compared to mock-transfected cells (Fig. 4B). MST6-II cells also showed an increase, although not so evident, in adhesion and migration properties on ECM components when compared to those of mock-transfected cells. All cell lines showed lower adhesion and migration on uncoated plates. The increase in motility of MST6-I cells was associated to major cytoskeletal alterations: MST6-I cells are larger and more flattened, forming actin based membrane specializations, such as lamellipodia and filopodia (Fig. 4C). On the other hand, mock-transfected cells presented a roundish cell shape and lacked the actin extensions. MST6-II cells also showed lamellipodia and filopodia extensions.

3.5. High invasion potential of MST6-I cells mediated by sialyl-Tn

MST6-I cells expressing sialyl-Tn showed a 2.5-fold increased invasion capacity when compared to mock-transfected cells (Fig. 5A and B) ($P < 0.005$). MST6-II cells showed no alteration in invasion capability. Incubation with the blocking MAb anti-sialyl-Tn reverted the invasiveness behavior of MST6-I cells close to the levels observed in the mock-transfected cells. The addition of a control antibody (HB-T) to the medium did not modify the original invasion capability of MST6-I cells as well as the behavior of MST6-II and mock-transfected cells (Fig. 5A).

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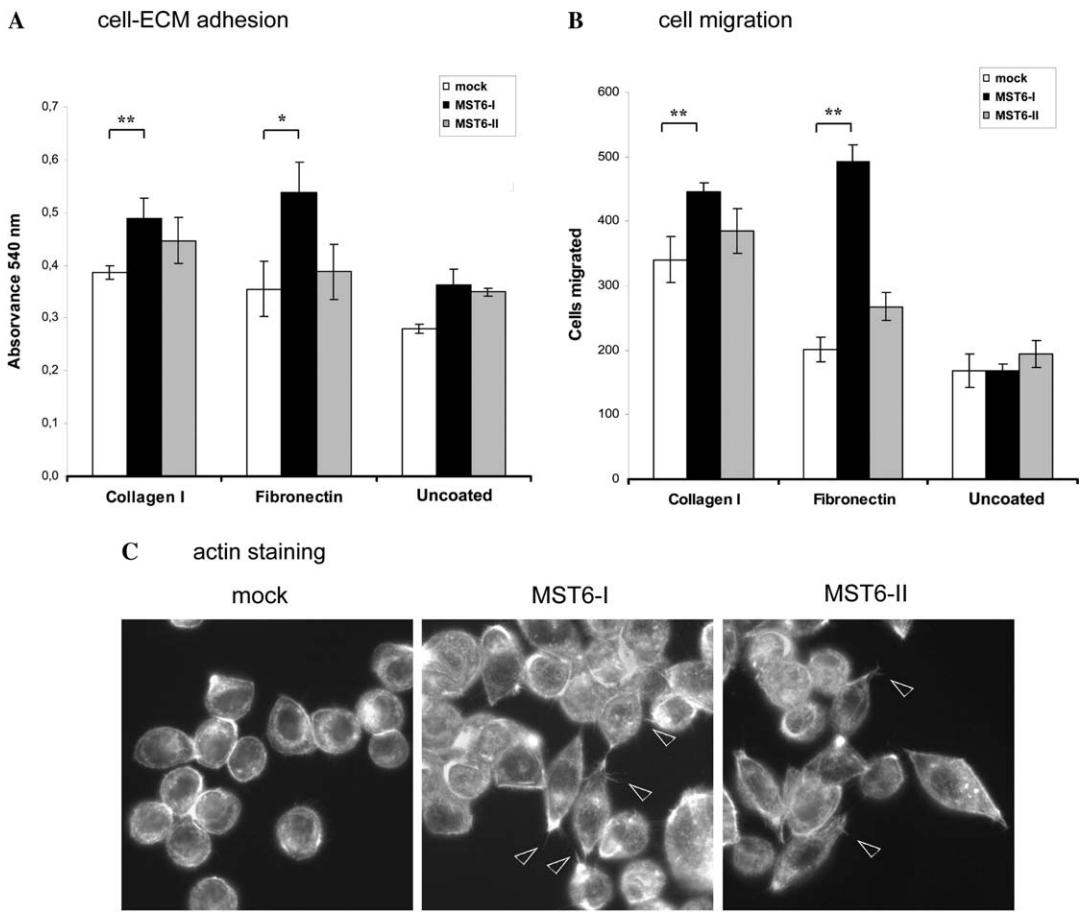

Fig. 4. Adhesion and migration of MST6-I, MST6-II and mock-transfected cells on basal membrane components. (A) Cell adhesion to extracellular matrix (ECM) coated microwells was evaluated using the MTT assay and represented as absorbance values (540 nm), in three independent experiments in triplicate; (B) cell migration on ECM coated wells was evaluated after 48 h, in three independent experiments in triplicate. Uncoated wells were used as control. $^{*}P < 0.05$ and $^{**}P < 0.02$, compared to mock-transfected cells; Student's t test. (C) Representative photomicrographs of stained cells with FITC-phalloidin, showing the actin cytoskeleton of mock-transfected, MST6-I and MST6-II cells. The *arrowheads* specify the presence of filopodia.

3.6. MUC1, a carrier of sialyl-Tn in MST6-I cells

Total protein extracts from MST6-I cells and parental cell line MKN45*wt* were submitted to Western blotting and developed with anti-sialyl-Tn MAb TKH2. As shown in Fig. 6A, no sialyl-Tn was detected on protein extracts from MKN45*wt* cells. In MST6-I cells, four bands were detected in the range 100–250 kDa and one band with a molecular weight higher than 250 kDa, suggesting that this band may correspond to mucins. In order to study which glycoproteins carry sialyl-Tn antigen, and considering the high molecular weight bands, protein extracts from MST6-I and MKN45*wt* cells were immunoprecipitated for MUC1 followed by Western blotting and detection for sialyl-Tn and MUC1. Both cell lines showed a similar pattern of MUC1 glycoforms. MUC1 bearing sialyl-Tn antigen was detected only in MST6-I cells, observed only in the highest molecular weight glycoform (Fig. 6B).

4. Discussion

The aberrant expression of sialyl-Tn observed in various types of cancers is associated with aggressiveness of the disease and with unfavorable patients prognosis. Expression of some glycan structures in cancer cells have been shown to have functional relevance in the process of cell adhesion, invasion and metastasis. These observations, taken together with the fact that metastization is a multiple-step process, raised the question of whether sialyl-Tn is involved in any of the processes associated with malignancy, or in all of them. It is the cross talk between cancer cells and host elements that modulates invasion-associated activities like cell–cell adhesion, cell–matrix interaction, tumor cell growth and motility [39,40].

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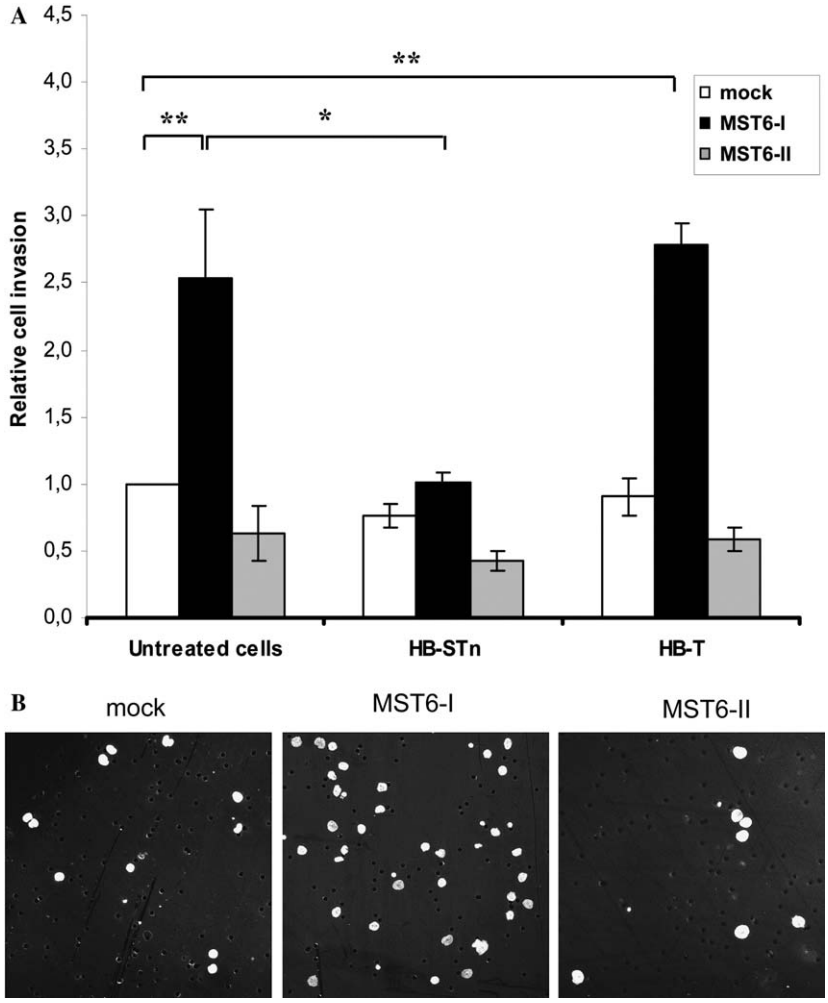

Fig. 5. Invasion ability of MST6-I, MST6-II and mock-transfected cells. performed in a BD Biocoat™ Matrigel™ invasion chamber. (A) Invasion levels of MST6-I and MST6-II cells after 48 h. For MAb inhibition experiments cells were incubated with anti-sialyl-Tn MAb HB-STn or with HB-T, a control MAb. Data have been normalized by the invasion levels of mock-transfected cells and represents the mean ± SD ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.005$; Student's t test. (B) Representative photomicrographs for each transfectant, which had migrated through the membrane.

The present study confirms previous observations that the expression of ST6GalNAc-I generates high levels of sialyl-Tn and low levels of sialyl-6T, whereas the expression of ST6GalNAc-II induces high levels of sialyl-6T and low levels of sialyl-Tn [32]. Our results showed that MST6-I cells expressing sialyl-Tn had a markedly reduced homotypic cell–cell aggregation, with most cells remaining solitary, in contrast to mock-transfected cells, which have no sialyl-Tn, and aggregated forming compact cell clusters. MST6-II cells, which have low levels of sialyl-Tn, showed intermediate levels of cell–cell aggregation, with the formation of cell clusters with loose aggregates. The addition of anti-sialyl-Tn MAb HB-STn to the medium, blocked the exposure of the antigen at the cell surface, inhibiting its putative interaction with neighbor cells, and reverted the phenotype of MST6-I and MST6-II cells, with the formation of compact cell clusters similar to mock-transfected cells.

Oncogenic transformation is often associated with changes in organization of the cytoskeleton that can influence cell migration, adhesion and invasiveness. We observed that MST6-I and MST6-II cells displayed distinct morphologic characteristics when compared to mock-transfected cells, exhibiting the formation of lamellipodia and filopodia as evidenced by phalloidin staining of the actin

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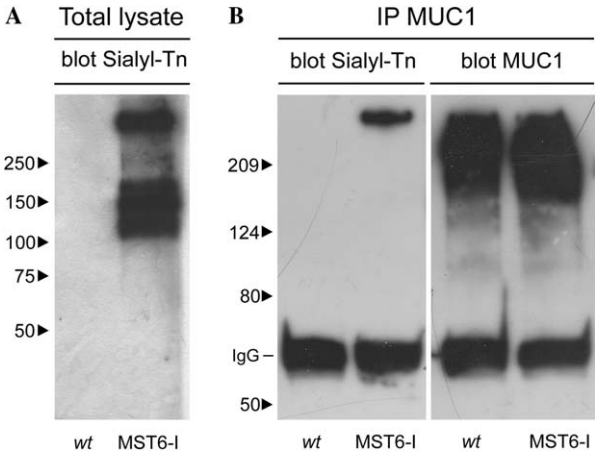

Fig. 6. Analysis of *O*-glycosylated proteins carrying sialyl-Tn antigen. (A) Total cell extracts of wild type MKN45 and MST6-I cells were subjected to an SDS–PAGE on 4–20% gradient polyacrylamide gels and western blotting with anti-sialyl-Tn MAb TKH2. (B) Immunoprecipitated proteins with anti-MUC1 MAb were subjected to an SDS–PAGE on 4–10% gradient polyacrylamide gels and Western blotting with either anti-sialyl-Tn MAb TKH2 or anti-MUC1 MAb HMFG1.

filaments (Fig. 4C). These cells are also larger and more flattened, therefore contacting the extracellular matrix (ECM) at more sites. Actin cytoskeleton plays an important role in cell locomotion via the extension of these protrusions that initiate ECM recognition and binding [45,46].

Concomitant to these morphological alterations significant changes in adhesion and migration of cells were found. MST6-I cells showed an increased adhesion and migration capabilities on components of the ECM, both on fibronectin and collagen I. MST6-II cells also showed an increase, although not so evident, in adhesion and motility properties on ECM components when compared to that of mock-transfected cells. Integrin α2β1 is the cell surface receptor for collagen I. [47] and α5β1 integrin is the main cell surface receptor for fibronectin [48]. Previous studies on mouse B16 melanoma cells were able to show that sialylation of β1-integrins is associated with the binding activity to fibronectin [49]. Since the binding to fibronectin and collagen I was altered in our experiments, it is likely that the β1-chain is affected by expression of sialyl-Tn. Recent studies confirmed this hypothesis by demonstrating that murine carcinoma cells transfected with ST6GalNAc-I expressed sialyl-Tn on β1 integrin [50].

We further evaluated the capability of cell invasion in matrigel chamber assays. Invasion is a crucial step in the development of cancer since it is responsible for malignancy either through locoregional spread or through metastasis to distant organs. MST6-I cells showed a 2.5-fold increase in invasion capacity when compared to mock-transfected cells. The role of the sialyl-Tn in augmenting the invasion capacity of the cell line was confirmed by incubation of the cells with the blocking MAb directed to the sialyl-Tn antigen, which completely reverted the invasion phenotype. These results confirm that the increased invasion capability of MST6-I is mediated by sialyl-Tn.

The mechanisms underlying this phenotypic shift may be mediated by the *O*-glycosylated proteins that bear the sialyl-Tn antigen. Western blot analysis using an anti-sialyl-Tn monoclonal antibody shows that sialyl-Tn is carried by at least five high molecular weight (>100 kDa) *O*-glycoproteins. We observed by immunoprecipitation studies that one of the carriers is MUC1 (Fig. 6B). MUC1 is a membrane-associated mucin expressed in different tissues that displays altered *O*-glycosylation patterns in carcinomas [51–54]. In addition, MUC1 is a carrier of the sialyl-Tn antigen in colon (HT29 and $CaCO_2$) and pancreatic carcinoma cell lines [55]. Our observation that MUC1 is also a carrier of sialyl-Tn in the MST6-I cells suggests that the reduced cell–cell adhesion and invasive properties described previously for MUC1 [56–58] may be related to its *O*-glycosylation pattern. Recent studies have shown similar results in breast cancer cell lines transfected with hST6GalNAc-I, identifying MUC1 and β1-integrins as carriers of the sialyl-Tn structure [50,59]. It is likely that the former molecules use the sialyl-Tn in the process of invasion by adhering to one or more components of the matrigel basement membrane matrix, which contains laminin, collagen type IV and heparan sulfate proteoglycan, among other factors.

In order to exclude the hypothesis that the increased motility and invasion were due to increased cell proliferation rates we analyzed the cell growth. We observed decreased proliferation rates in both MST6-I and MST6-II cells when compared to the mock-transfected cells, which is in agreement with previous studies showing an impaired proliferation in cells transfected with ST6GalNAc-I [50,59,60]. This growth inhibition appeared not to be due to any alteration in the cell cycle progression, but rather to an increase in the number of apoptotic cells (Fig. 2B and C).

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It is noteworthy that, besides the *de novo* expression of sialyl-Tn and sialyl-6T in MST6-I and MST6-II cells, none of the terminal carbohydrate antigens analyzed, such as Lewis antigens Le^a, Le^b, Le^x, Le^y, sialyl-Le^a and sialyl-Le^x, showed significant alterations (Table 2). These observations exclude any influence of these structures in the differences observed in cell behavior. In addition, blocking of sialyl-Tn by incubation with a MAb completely reverted the aggregation and invasion phenotype, confirming this antigen as the responsible structure for the alterations observed. Furthermore, MST6-II cells, which express low levels of sialyl-Tn, presented similar but less intense alterations in processes like aggregation, adhesion, motility and invasion than MST6-I cells, behaving like an intermediate positive clone for sialyl-Tn expression. The low levels of sialyl-Tn in these cells, rather than the high levels of sialyl-6T, correlated with its moderate increase in aggressive behavior. Overall, these results suggest that it is the expression of sialyl-Tn, and not any other structure, that is contributing to the alterations observed and can induce by itself the common features of malignant cell behavior.

In carcinomas, it is likely that the expression of sialyl-Tn facilitates the release of single cells from the primary tumor by reducing homotypic aggregation, for instance, by inhibiting the interaction of galectins with terminal galactose residues [61]. Single cells expressing sialyl-Tn are then more able to migrate and invade the underlying mucosa and eventually reach lymph or blood vessels. On the other hand, specific adhesive properties are mandatory for successful extravasation of metastatic cells and invasion of the target tissue. Therefore, cells which are more able to interact with basal membrane substances such as fibronectin and collagen I have an advantage. Future studies using animal models addressing the invasion and metastization phenotype of the gastric carcinoma cell lines will further elucidate the process of carcinoma cell invasion and metastization *in vivo*.

In conclusion, our results show that sialyl-Tn antigen plays a major role in the phenotype of carcinoma cells, profoundly altering various processes related to malignancy. The expression of sialyl-Tn is able to modulate *per se* a malignant phenotype, inducing a more aggressive cell behavior such as decreased cell–cell aggregation and increased ECM adhesion, migration and invasiveness. These findings show that sialyl-Tn antigen plays a crucial role in the development of a malignant phenotype and strongly suggest its involvement in gastric carcinoma progression.

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