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The influence of N- and O-glycosylation inhibitors on the glycosylation profile of cellular membrane proteins and adhesive properties of carcinoma cell lines

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Abstract. The effects of N- and O-glycosylation inhibitors on the expression of membrane proteins (MUC1 and some integrins) were evaluated in human endometrial (Ishikawa) and breast (MCF-7) cancer cells. Subconfluent cells were treated with 1-3 mg% concentration of tunicamycin and 2-10 mM of benzyl-N-acetyl-α-galactosaminide for 1-2 days, and used for flow cytometry, immunohistochemical staining, adhesion test and Western blotting. Benzyl-N-acetyl-α-galactosaminide inhibits MUC1 expression on the surface of breast more than endometrial cancer cells. Tunicamycin reduces MUC1 concentration on the cellular surface more than benzyl-glycoside, and greatly reduces glycosylation of glycoproteins, causing an increase in cell adhesion in both types of cancer cells. The expression of α2ß1 integrins on the surface of these cells was weak and decreased after treatment with inhibitors. Two different glycoforms of MUC1 proteins in endometrial cells and three in breast cancer cells were expressed and their molecular weights were reduced after treatment with glycosylation inhibitors. It was confirmed with lectin detection of carbohydrate epitopes (Tn and T) in MUC1 proteins. These observations show that glycosylation inhibitors altered the N- and O-glycan patterns in a sufficient manner, and positively modified the biological features of cancer cells.

Introduction

Glycoproteins with atypical O-glycans and N-glycans have been found on the cell surface, in secretions and in epithelial cancer cells. The structures of O-linked carbohydrate greatly contribute to the phenotype and biology of cancer tissue. Studies on the regulation of glycosylation mechanism may help in understanding and the control of the abnormal biology of tumor cells (1).

The functional role of carbohydrates has been studied by treating cultured cells with glycosyltransferase or glycosidase inhibitors. Tunicamycin is frequently used to inhibit N-glycosylation and indirectly O-glycosylation when the initial N-glycosylation is a precondition for O-glycosylation. Tunicamycin is a drug blocking the transfer of GlcNAc-P from UDP-GlcNAc to dolichol phosphate (2); it inhibits N-glycan processing and causes accumulation of improperly folded proteins inside the cells. Tunicamycin also inhibits intracellular protein transport and reduces glycoprotein incorporation to the cellular membrane (3).

Typical O-glycosylation inhibitors are less known. Benzyl-N-acetyl-α-galactosaminide (GalNAc-O-bn) is a synthetic analogue of N-acetylgalactosamine and inhibits elongation of O-glycans. The inhibition is competitive and instead of monosaccharide transfer the GalNAc bound to serine or threonine, the elongation of O-glycan chain occurs on GalNAc-O-bn molecules. Treatment of HT-29 mucus-secreting cells (HT-29 MTX) with GalNAc-O-bn reduces biosynthesis of secretory mucins, which are highly O-glycosylated glycoproteins (4,5). GalNAc-O-bn treatment also inhibits the apical targeting of several membrane brush-border glycoproteins, for instance membrane glycoprotein - MUC1 mucin, in differentially polarized HT-29 cells. The remaining glycoproteins are stored in the cytoplasm (6,7).

It is known that MUC1 mucin expression is considerably increased in carcinoma cells (8,9). Intensity of MUC1 incorporation to the cellular membrane can change adhesive properties of these cells and MUC1 shedding to body fluids. This phenomenon is important for cell to cell and cell to extracellular matrix adhesion, which decreases in carcinomatous tissues (10,11). We investigated how the glycosylation inhibitors change the glycosylation profile of the membrane proteins and what is the influence on adhesive properties of cancer cells in breast cancer and endometrial carcinoma cell lines.

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Materials and methods

Materials. GalNAcα-O-bn, tunicamycin, collagen I, SDS, *Clostridium perfingens* neuraminidase, protease inhibitor cocktail, trypan blue and culture media were purchased from Sigma Chemicals; monoclonal anti-MUC1 antibody: MAbs 4058 were from Chemicon International, and HMFG1 were from Immunotech, France. Monoclonal anti-MUC1 antibody conjugated with fluorescein isothiocyanate (HMPV-FITC) and IgG1-FITC isotype control were purchased from BD Pharmingen; monoclonal anti-integrin α2ß1 antibody: P1E6 was from Dako; Immobilon-P membrane was from Millipore (USA); culture flasks (25 cm^2) were from Sarstedt (USA); Labtek chamber slide 4-well were from Nunc; Cytofix to chamber slides was from Merck; Peanut, *Arachis hypogaea* agglutinin (PNA-HRP) and *Vicia villosa* agglutinin (VVA-A_4-HRP) were from LectinoTest, Danylo Halytsky State Medical University in Lvov. All other chemicals were of analytical grade purity from commercial sources.

Cell culture. Endometrial epithelial carcinoma cells - Ishikawa line and breast cancer MCF-7 cell line (purchased from the American Type Culture Collection, Rockwille, MD) were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin at 37˚C in 5% CO_2 and 95% air incubator. After reaching 80% of confluence cells were treated with one of the following: GalNAc-O-bn at concentration of 2, 5 and 10 mM, and tunicamycin at the concentrations of 1, 2, 3 mg% for 1-2 days before cell collecting. Inhibitors were dissolved in culture medium to a final concentration. In all the examined concentrations of inhibitors, >95% of cells showed viability as assessed by trypan blue assay. Two individual cultures were conducted for control and each drug concentration in three independent experiments.

Preparation of cell lysates. After reaching confluence, the cells were washed with phosphate-buffered saline (PBS), harvested by scraping and centrifuged (4,000 rpm, 10 min). Then the cells were homogenized in an ice bath using sonification (3 times for 15 sec; 50 Hz) in 50 mM Tris/HCl pH 7.5, containing 1 mM EDTA and protease inhibitor cocktail. The homogenate was incubated in an ice bath for 1 h, centrifuged at 16,000 rpm for 30 min, and supernatant was used as lysates for further determination.

SDS-PAGE and Western blotting. Equal amounts of cellular protein (30-50 μg) were submitted to electrophoresis on 7.5% SDS-polyacrylamide gels, according to the Laemmli method (12). After electrophoresis, the proteins were electro-transferred onto PVDF membranes (Immobilon-P) in a buffer consisting of 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.4, for 2 h at constant voltage (100 V) with cooling. Filters were blocked with 3% skim milk overnight, then incubated with anti-MUC1 MAb or with lectins peroxidase-labelled, for 2 h. To detection of MUC1, membranes were washed and incubated with anti-mouse IgG antibody conjugated with peroxidase. 4-Chloro-1-naphtol/H_2O_2 was used as specific reagent to peroxidase (13).

Flow cytometric analysis. To determine MUC1 expression on the cell membranes, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human MUC1 monoclonal antibody and mouse IgG1-FITC antibody as a negative control was used, according to the method described in our earlier study (14). Flow cytometric analysis was performed by Coulter Epics XL.

Immunohistochemistry. Cells grown on chamber slides were fixed in Cytofix, and then washed twice in PBS buffer, pH 7.4. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 5 min. After washing with Tris/HCl buffer solution, pH 7.4, slides were incubated with primary anti-bodies anti-MUC1 (HMFG1) and α2ß1 integrin. For detection the Dako Cytomation LSAB+ System HRP kit was used according to the instructions of the manufacturer, employing the streptavidin-biotin technique. All sections of slides were examined independently by two investigators under a standard light microscope using x200 and x400 magnification.

Cell adhesion assay. Adhesion assay (14) was performed with control or glycosylation inhibitor-treated cells. Culture plates (96-well), coated with 100 μg/ml collagen I, were blocked for 90 min at 37˚C with 1% heat-denatured BSA. Cells for adhesion assay were scraped off, washed in serum-free medium and re-seeded at a density of $5x10^4$ cells/well on collagen. Cells were allowed to adhere for 60 min at 37˚C. Non-adherent cells were removed by washing 3 times with PBS. Adherent cells were stained with 0.1% crystal violet for 30 min, washed in tap water and air-dried. Stained cells were lyzed overnight in 0.1% Triton X-100 to release the dye. The absorbance at 620 nm determined using ELISA-reader was proportional to cell numbers. Each data point was calculated from two separate experiments performed in quadruplicate and was expressed as the mean ± standard deviation (SD). Nonspecific cell adhesion as measured on BSA-coated wells was subtracted.

Assay of influence of MUC1 on cell adhesion. To test the effect of MUC1 on cell adhesion to collagen I, a part of cell suspension was preincubated with anti-MUC1 MAb 4058 (1:40 diluted) for 30 min at 37˚C, and added to each collagen coated well in quadruplicate. Further procedure was the same as described above for the adhesion test. Results were expressed as a percentage of OD value of cells with antibody in comparison to OD of the cells without antibody.

Statistical analysis. Data are presented as means ± SD. Statistical analysis was performed using Student's t-test to determine the significance of differences between means. A $p<0.05$ was considered statistically significant.

Results

We studied the effect of glycosylation inhibitors (benzyl-N-acetyl-α-D-galactosaminide and tunicamycin) on the expression, surface exposure of membrane glycoproteins, and cell adhesion in cancer lines: endometrial (Ishikawa) and breast (MCF-7).

Effect of glycosylation inhibitors on surface expression of membrane glycoproteins in endometrial cancer cells (A) and breast cancer cells (B).[a]

A, Endometrial cancer cells

GalNAc-O-bn concentration [mM]	Detection of MUC1	Detection of α2ß1 integrin	Tunicamycin concentration [mg%]	Detection of MUC1	Detection of α2ß1 integrin
0	++/+++ (87%)	+ (32%)	0	+++ (90%)	+/- (33%)
2	++/+++ (83%)	+ (21%)	1	+++ (87%)	+/- (31%)
5	++/+++ (82%)	+ (12%)	2	++ (68%)	+/- (11%)
10	++ (78%)	+ (11%)	3	++ (8%)	+/- (5%)

B, Breast cancer cells

GalNAc-O-bn concentration [mM]	Detection of MUC1	Detection of α2ß1 integrin	Tunicamycin concentration [mg%]	Detection of MUC1	Detection of α2ß1 integrin
0	+++/++ (83%)	+ (26%)	0	++ (83%)	+ (26%)
2	++ (60%)	+ (21%)	1	++ (21%)	+ (15%)
5	++/+ (46%)	+ (12%)	2	++/+ (13%)	+/- (8%)
10	+/++ (35%)	+/- (8%)	3	+/++ (9%)	+/- (5%)

[a]Optical intensity (scale: +, low; ++, moderate; +++, high; +/-, less than low) and percentage of these cells with positive immunostaining antigens after a 2-day exposure to glycosylation inhibitors.

Effects of glycosylation inhibitors on membrane localization of glycoproteins (MUC1 and α2ß1 integrins). The effect of inhibitors on the exposure of membrane glycoproteins on the cell surface was tested by immunohistochemical staining. To evaluate the expression of membrane glycoproteins, dependent on the treatment with inhibitors, we used monoclonal antibodies to core protein of MUC1 and to α2ß1 integrin.

Although only approximate results can be obtained with the technique used (Table I), some general conclusions can be presented. GalNAc-O-bn exerts only a slight effect of MUC1 exposure on the surface of endometrial cells, but about three-fold reduction was observed for α2ß1 integrin incorporation into the cellular membrane. Inhibitory effect of tunicamycin was considerably greater, after treatment with the highest concentration of inhibitor. Ten-fold reduction for MUC1 incorporation and six-fold for the integrins was observed (Table IA).

In breast cancer cells, the inhibitory effect of the benzyl-glycoside was higher, reaching two-fold reduction towards MUC1 incorporation into the cell membrane, similar to the endometrial level of inhibition for the integrins. Inhibition with tunicamycin was also high; reducing nine-fold the MUC1 level and five-fold the integrin on the cell surface, in comparison to control cells (Table IB).

Flow cytometry analysis of MUC1 expression on the surface of endometrial and breast cancer cells. Flow cytometry is a more reliable method to investigate the influence of inhibitors on MUC1 content in the cellular membrane. This method confirms the higher inhibitory effect of tunicamycin on MUC1 protein incorporation into the cancer cell membrane: 65% of inhibition was found in endometrial cells and 40% in breast cancer cells. Similar to the data in Table I, in the presence of GalNAc-O-bn, the inhibition was lower, reaching 36% of the inhibition in the endometrial cells and 25% in the breast cancer cells (Fig. 1A and B).

Effect of glycosylation inhibitors on cell adhesion to ECM. In the adhesion test, we studied the influence of inhibitors on cell

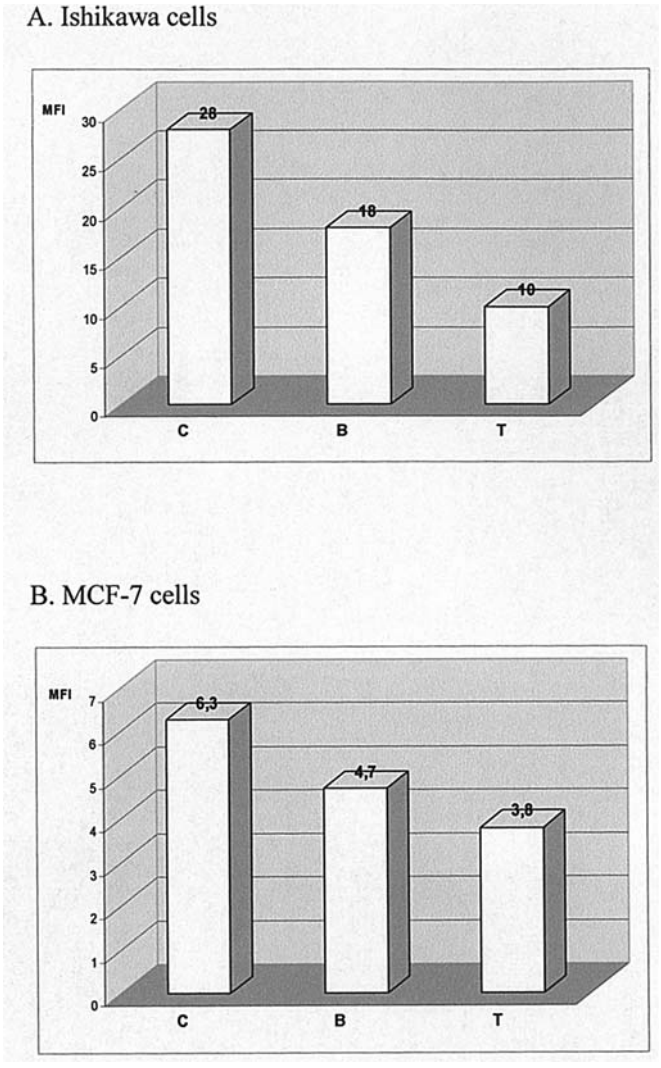

Figure 1. Flow cytometry analysis (mean fluorescence intensity, MFI) of MUC1 expression on the surface of Ishikawa (A) and MCF7 (B) cells. C, control, untreated cells; B, 2 mM GalNAc-O-bn; T, 3 mg% tunicamycin, cells were 2-day treated with inhibitors. Average values of MFI in 3 independent experiments are shown.

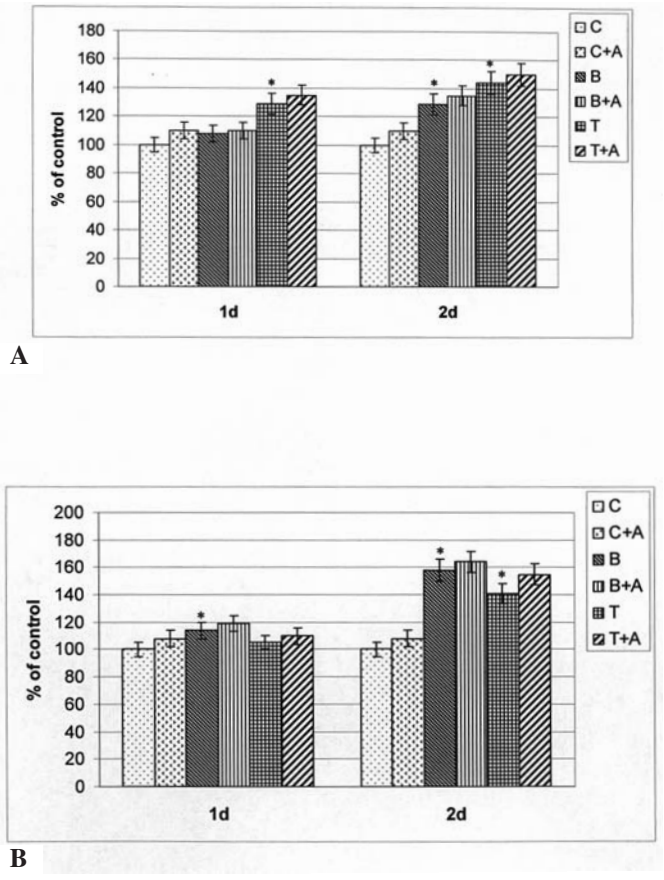

Figure 2. Effect of glycosylation inhibitors on binding of endometrial (A) and breast (B) cancer cells to type I collagen. The cells were cultured in standard medium supplemented with 2 mM GalNAc-bn (B) or tunicamycin (30 μg/ml, T) for 1-2 days. Control cells (C) were cultured in the same conditions without inhibitor. Some inhibitor-treated cells ($2x10^5$ cells) were incubated before the adhesion test, with anti-MUC1 antibody in 37˚C for 1 h (B+A, T+A). The cells were added to collagen-coated wells ($5x10^4$ cells/well) in quadruplicate for control and each drug concentration. A binding of the control cells (without drug) was accepted as 100%, and binding of the treated cells was expressed as a percentage of this value. Data are shown as means ± SD of two independent experiments. *$p<0.05$ was compared to the respective value from control group.

binding to collagen type I. We observed an increase in cell adhesion, increasing with the time of treatment. Results of a two-day treatment of Ishikawa cells with GalNAc-O-bn and tunicamycin, demonstrated a 29 and 44% increase in the adhesion level, respectively (Fig. 2A). For the breast cancer cells, the percentage of adhesion values to collagen (Fig. 2B) was increased, most (58%) in GalNAc-O-bn and 41% in tunicamycin-treated cells. The possible function of MUC1 mucin in the interaction of tumour cells with collagen was tested after incubation of these cells with anti-MUC1 antibody. In both cell types there was a slight blocking effect of MUC1 antibody on the adhesion level (Fig. 2A and B: bars B+A and T+A). Incubation of MCF-7 cells with anti-MUC1 antibody caused an insignificant increase in the adhesion level (5 and 14%).

Western blotting detection of MUC1 and sugar antigens in cells treated with glycosylation inhibitors. Detection of MUC1 antigens in cellular lysates was performed by electroimmunoblotting with anti-MUC1 antibody (MAb 4058, specific to tandem core of MUC1 protein). As shown in Fig. 3, in the presence of inhibitors, cancer endometrial cells synthesized two MUC1 glycoforms, with different molecular masses: one exceeding 205 kDa and the other 116 kDa, with a little change in band position with increasing concentration of glycosylation inhibitors (Fig. 3). Staining intensity of carbohydrate residues (Tn and T antigens) is reduced, especially for the cells treated with 2 mM GalNAc-O-bn (Fig. 4; L3) and for both concentrations of tunicamycin (Fig. 4; L4 and L5).

Detection of MUC1 antigens in the lysates of breast cancer cells (MCF-7), with anti-MUC1 antibody (to protein core), shows three bands; the first on top of the gel, the second below standard band of 216 kDa, and the weakly stained third, near 116 kDa. In the cells treated with glycosylation inhibitors, the intensity of staining in protein bands diminished and molecular weight of these proteins was reduced, with higher concentration of inhibitors, especially of tunicamycin (Fig. 5). Detection of Tn (or STn antigens after treatment with neuraminidase) by *Vicia villosa* lectin showed their presence

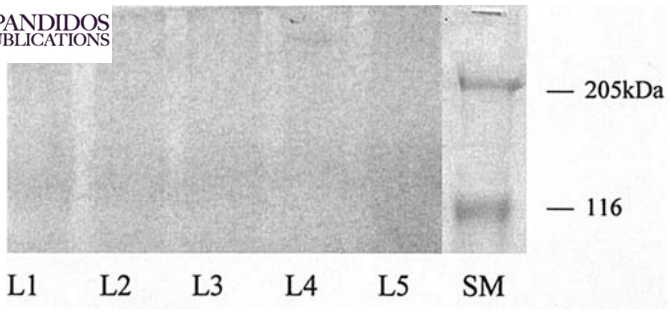

Figure 3. Detection of MUC1 antigens in lysates of endometrial cancer cells (Ishikawa line). L1, control cells (not treated with inhibitors); L2, 1 mM GalNAc-O-bn; L3, 2 mM GalNAc-O-bn; L4, 5 mg% tunicamycin; L5, 10 mg% tunicamycin, cells were 2-day-treated with inhibitors. SM, protein standards with high molecular mass.

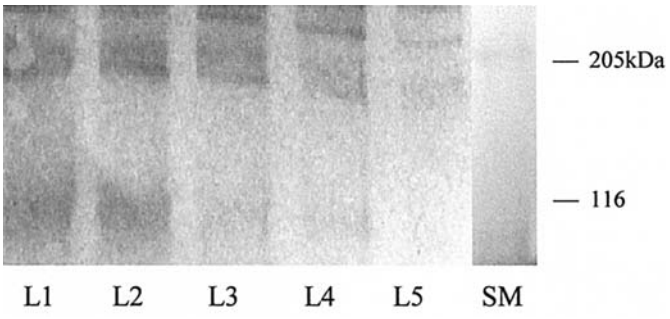

Figure 5. Detection of MUC1 antigens in lysates of breast cancer cells (MCF-7). L1, control cells (not treated with inhibitors); L2, 1 mM GalNAc-O-bn; L3, 2 mM GalNAc-O-bn; L4, 5 mg% tunicamycin; L5, 10 mg% tunicamycin, cells were 2-day-treated with inhibitors. SM, protein standards with high molecular mass.

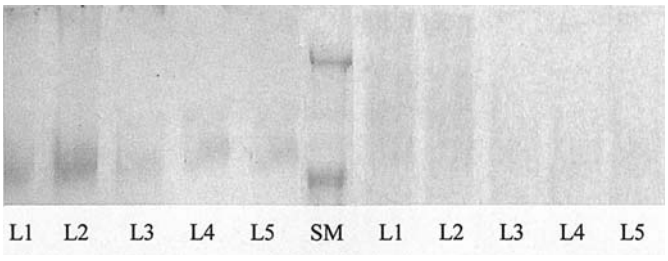

Figure 4. Detection of Tn (left) and T (right) antigens on intracellular proteins of endometrial cancer cells (with *Vicia villosa* and PNA lectins). L1, control cells (not treated with inhibitors); L2, 1 mM GalNAc-O-bn, L3, 2 mM GalNAc-O-bn; L4, 5 mg% tunicamycin; L5, 10 mg% tunicamycin, cells were 2-day-treated with inhibitors. SM, protein standards with high molecular mass.

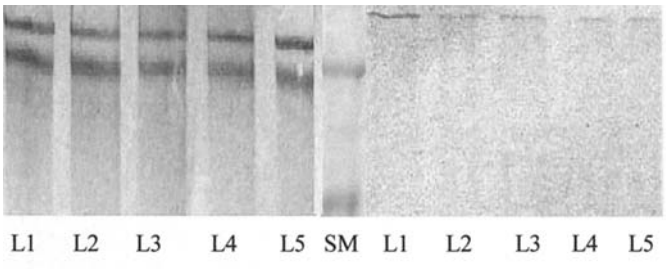

Figure 6. Detection of Tn (left) and T (right) antigens on intracellular proteins of breast cancer cells (with *Vicia villosa* and PNA lectins). L1, control cells (not treated with inhibitors); L2, 1 mM GalNAc-O-bn; L3, 2 mM GalNAc-O-bn; L4, 5 mg% tunicamycin; L5, 10 mg% tunicamycin, cells were 2-day-treated with inhibitors. SM, protein standards with high molecular mass.

on two proteins, of molecular weight near 205 kDa (Fig. 6, left). The figure also demonstrates the reduction of molecular mass of synthesized glycoproteins, after treatment with inhibitors. Detection of T antigens on the MUC1 molecules was positive only on high molecular proteins, located on top of the gels (Fig. 6, right), with the intensity dependent upon inhibitor concentration.

Discussion

The carbohydrate structures are of particular importance on the cellular surface, where they mediate fundamental processes underlying cell-cell, cell-matrix interactions, and cell proliferation. They may also act as immunodeterminants or mask underlying antigenic epitopes. It is well know that carcinogenesis and metastasis are associated with altered structure and expression of oligosaccharides on surface glycoproteins and aberrant glycosylation is a hallmark of malignant phenotype. Cancer cells use carbohydrate moieties to escape recognition by the immune cells as they migrate through the body. During metastasis, the carbohydrates are involved in tumor cell-extracellular membrane or tumor cell-endothelial cell reactions (15,16).

Glycosylation inhibitors are used to recognize carbohydrate structure and function. The inhibitors of N-glycosylation: tunicamycin and brefeldin A, reduce protein transport and surface localization of some membrane proteins (3).

Human MUC1 mucin is a transmembrane glycoprotein with extracellular heavily O-glycosylated domain, differing in glycosylation between normal and malignant cells (17,18). MUC1 expression is clearly increased in most breast carcinomas. MUC1 causes a limitation of polarization in tumour cells and it is found in the basolateral plasma membrane or its soluble form in tissue culture supernatants and body fluids (19,20). The overexpression of MUC1 may decrease cell to cell and cell to matrix contacts, causing inhibition of cancer cell killing by cytotoxic T lymphocytes. The appearance of novel oligosaccharide structures and higher level of MUC1 expression over the external cell surface may change adhesive properties of tumour cells (21).

According to our present results, detection of MUC1 using immunohistochemistry and flow cytometry analysis demonstrates that GalNAc-O-bn inhibits mucin appearance on the surface of breast cancer cells more than on endometrial cancer cells. Stronger than GalNAc-O-bn glycoside, tunicamycin inhibits MUC1 glycosylation, greatly reduces MUC1 concentration on the cell surface in both lines of cancer cells, and affects the expression of α2ß1 integrins. The family of integrins play an important role in cell adhesion. Different combinations of α and ß subunits form different receptors for extracellular matrix. For example, α2ß1 is a receptor for collagen and laminin. We studied the expression of α2ß1 integrin since it has been reported that the alterations in expression and function of integrins may play an important

role in each step of cancer development (22). The expression of α2ß1 integrin was reduced after treatment with the high concentration of glycosylation inhibitors (mostly tunicamycin).

Immunohistochemical results related to surface expression of MUC1 were confirmed by an increase in the level of adhesion to collagen in GalNAc-O-bn and tunicamycin-treated cells, in both cancer lines showing that the reduction of MUC1 expression on the cell surface increases cell adhesion. Thus, overexpression of MUC1 can reduce both cell to cell and cell to basement membrane adhesion. Coating of MUC1 with monoclonal antibody (to tandem repeat of MUC1 tetrapeptide) did not change adhesive properties of these cells, suggesting that anti-adhesive properties are derived from steric hindrance rather than from MUC1 chemical structure.

Our earlier study showed that long-time exposure of human endometrial carcinoma cells to benzyl-N-acetyl-α-D-galactosaminide caused a reduction in the quantity of sialic acid residues and T antigen contents in MUC1 mucin included in cellular lysates. Various glycoforms of MUC1 were synthesized in the presence of GalNAc-O-bn and only fully glycosylated glycoforms could be incorporated into the cell membrane (13).

In human carcinoma cells, three short carbohydrate epitopes: Tn (-GalNAc), STn (sialyl-α2-6GalNAc-) and T (Galß1-3GalNAc-) are strongly expressed (23). STn antigens are carried by several high molecular weight membrane-bound O-glycoproteins, including MUC1. It has been suggested that sialyl-Tn expression induces a decrease in cell growth and cell adhesion (24). We confirm the presence of these carbohydrate epitopes in glycoproteins of MCF-7 and Ishikawa cancer cells. Our results with glycosylation inhibitors corroborate indirectly the possible anti-adhesive role of the sialyl-Tn antigens.

The presence of antigen Tn or STn is demonstrated in the glycoforms of MUC1 (near 205 kDa and above 116 kDa) in both lines (endometrial and breast) of cancer cells, but antigen T is found in breast cancer cells only in a higher molecular form. Tunicamycin, at a concentration of 1 mg% and 2 mM of GalNAc-O-bn may be used to regulate glycosylation of glycoproteins and their surface exposure. Endometrial cells are more sensitive to tunicamycin treatment than breast cancer cells.

These experiments provide *in vivo* evidence for the role of O-glycosylation in adhesion of cancer cells, and have implications for understanding the mechanism involved in metastatic progression, and possibility the use of O-glycosylation inhibitors in the treatment of malignant diseases.

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