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Inhibition of human HT-29 colon carcinoma cell adhesion by a 4-fluoro-glucosamine analogue

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Cell surface glycoconjugates play an important role in cellular recognition and adhesion. Modification of these structures in tumour cells could affect tumour cell growth and behaviour, including metastasis. 2-Acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-α-D-glycopyranose (4-F-GlcNAc) was synthesized as a potential inhibitor and/or modifier of tumour cell glycoconjugates. The effect of this sugar analogue on the adhesive properties of human colon carcinoma HT-29 cells was evaluated. Treatment of HT-29 cells with 4-F-GlcNAc led to reduced cell surface expression of terminal lactosamine, sialyl-Le^x and sialyl-Le^a, as determined by Western blotting and flow cytometry. The aberrant expression of these oligosaccharide structures on the HT-29 cell surface resulted in: (1) decreased E-selectin mediated adhesion of human colon cells to human umbilical cord endothelial cells (HUVEC); (2) impaired adhesion of HT-29 cells to β-galactoside binding lectin, galectin-1; and (3) reduced ability to form homotypic aggregates. After exposure to 4-F-GlcNAc, lysosomal associated membrane proteins (lamp) 1 and 2, and carcinoembryonic antigen (CEA) detected in HT-29 cells were of lower molecular weight, probably due to impaired glycosylation. These results strongly suggest that modification of tumour cell surface molecules can alter tumour cell adhesion and that tumour cell surface oligosaccharides may be suitable targets for therapeutic exploitation.

Keywords: inhibition of tumour cell adhesion, glucosamine analogue, carcinoembryonic antigen

Abbreviations: 4-F-GlcNAc: 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-α-glucopyranose; GlcNAc: N-acetylglucosamine; s-Le^x: sialyl-Lewis^x; s-Le^a: sialyl-Lewis^a; lamp-1 and lamp-2: Lysosomal Associated Membrane Protein 1 and 2; CEA: carcinoembryonic antigen, DMEM: Dulbecco's Modified Eagle Medium; PBS: Phosphate Buffered Saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.5 mM Na_2HPO_4, pH 7.3); BSA: Bovine Serum Albumin; PMSF: Phenylmethylsulfonylfluoride; TBS: Tris Buffered Saline (10 mM Tris, 20 mM NaCl, pH 7.3); TCA: Trichloroacetic Acid; DSA: *Datura stramonium* agglutinin.

Introduction

Tumour metastasis results from specific interactions between tumour and host cells, mediated in many instances by cell surface glycoconjugates [1–4]. These cell surface molecules influence specific adhesion processes, invasiveness and immunogenicity that collectively determine the metastatic potential of tumor cells.

Tumour cell adhesion to endothelial cells plays a pivotal role in the metastatic process and may be responsible for organ specific metastases. Adhesion of human colon tumor cells to endothelium is mediated by endothelial leukocyte adhesion molecule-1 (ELAM or E-selectin) [5]. This adhesion molecule, along with intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), are expressed on endothelial cell surfaces after stimulation by inflammatory cytokines, such as interleukin-1β (IL-1β). The presence of these cell surface adhesion molecules was shown to induce tumour cell adhesion [6–8]. However, to date, only ELAM-1 (E-selectin) has been shown to be

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specifically involved in the adhesion of human colorectal cells to endothelial cells [5]. It has been suggested that sialyl-Le^x and sialyl-Le^a present on human colon cell surfaces are the ligands for E-selectin [5, 9, 10].

In sublines of human colon carcinoma, highly metastatic cells express more lysosomal membrane glycoproteins, lamp-1 and lamp-2 on their cell surface than their non-metastatic counterparts [11]. Although the majority of lamp molecules are localized in lysosomes, a small fraction is found on the tumour cell surface [12–15]. Lamp-1 and lamp-2 are the major carriers of polylactosaminoglycans, which are high molecular weight oligosaccharides composed of Galβ1 → 4GlcNAcβ1 → 3 repeats [16, 17]. The structure of polylactosaminoglycans is often characteristic for a particular cell type and their stage of differentiation [17–19]. The lysosomal membrane glycoproteins isolated from metastatic carcinomas contain more poly-N-acetyllactosaminyl side chains with branched galactose residues than low metastatic cells [11]. These polylactosaminoglycans carry various antigenic determinants including the Lewis antigens: Le^x [Galβ1 → 4(Fucα1 → 3)GlcNAcβ → R], sialyl Le^x [NeuAcα2 → 3Galβ1 → 4(Fucα1 → 3)GlcNAcβ → R], and sialyl Le^a [NeuAcα2 → 3Galβ1 → 3(Fucα1 → 4)GlcNAc → R]; and these structures have been found in tumour cells [20–23]. Additionally, increased levels of Le^x and sialyl Le^x, or sialyl-dimeric Le^x, have been found to correlate with the degree of malignancy in human colorectal carcinoma and a poorer prognosis [24–28].

Homotypic, Ca^{2+}-independent, aggregation of human colon carcinoma cells is mediated by carcinoembryonic antigen (CEA) [29, 30]. CEA functions as an adhesion molecule involved in cellular interactions relevant to metastasis [31]. It is a 180–200 kDa glycoprotein consisting of approximately 60% carbohydrate, with the majority of its tetra-antennary oligosaccharide structures terminating with Galβ1 → 4GlcNAc and/or NeuAcα2 → 6Galβ1 → 4GlcNAc-R units [32, 33]. Some of these lactosamine units are also fucosylated (Fucα1 → 3) to form structures corresponding to the Le^x antigen [34]. The expression of colon tumour cell surface CEA *in vitro* is directly correlated with the degree of cellular differentiation, while serum levels of CEA and the levels of expression of lactose-binding lectin *in vivo* have been associated with the stage of malignant progression of colorectal carcinoma [3, 31, 35].

In this report we describe the effect of the fluoroglucosamine analogue, 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-α-D-glucopyranose (4-F-GlcNAc), on the adhesion of HT-29 human colon carcinoma cells. Treatment of HT-29 cells with this sugar analogue significantly inhibited the adhesion of tumour cells to stimulated human umbilical vein endothelial cells (HUVEC). This was probably a result of decreased expression of sialyl-Le^x and sialyl-Le^a structures on the HT-29 cell surface, along with a reduction of lactosamine units. HT-29 cell aggregation mediated by CEA was also reduced after exposure to 4-F-GlcNAc, again suggesting oligosaccharide involvement in this adhesion process. Related to these findings was the observation that both CEA and lamp molecules detected in drug treated cells demonstrated lower molecular weights than species derived from control cells. These results suggest that the tumor cell surface may be a suitable target for therapeutic exploitation.

Materials and methods

Materials

Normal human umbilical vein endothelial cells (HUVEC), endothelial cell growth medium and trypsin neutralizing solution were purchased from Clonetics. RPMI 1640, DMEM (lacking glucose), heat inactivated foetal bovine serum, glutamine and 0.05% trypsin/0.53 mM EDTA were purchased from Life Technologies, Inc. Nu-serum, mouse monoclonal anti-ELAM-1 IgG2a and anti-s-Le^x IgM$_k$ were obtained from Collaborative Research. [Methyl-^{3}H]thymidine (spec act 20 Ci mmol^{-1}) was obtained from NEN Products. Purified human recombinant interleukin-1 (hr-IL-1β) was obtained from Cistron Technology. Rainbow protein standards and ECL Western blotting detection kit were purchased from Amersham. *Datura stramonium* biotin-conjugates were from Boehringer Mannheim. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG were purchased from Accurate Chemical and Scientific Corp. Rabbit antiserum to carcinoembryonic antigen (anti-CEA), mouse monoclonal anti-Le^a IgG$_1$ and anti-sLe^a IgG$_1$ were purchased from Signet Labs. X-Omat AR X-ray film was purchased from Kodak, and Immulon 1 microenzyme-linked immunosorbent assay plates were obtained from Dynatech Laboratories. Avidin-peroxidase, avidin-FITC rabbit IgG, mouse anti-rabbit-peroxidase IgG, goat IgG, fluorescein isothiocyanate goat anti-mouse IgG (Fab′), FITC-goat anti-mouse IgM conjugate, DNase and all others chemicals were from Sigma. Previously characterized rabbit lamp-1 and lamp-2 polyclonal antibodies (IgG fraction) [17] were generously provided by Dr Minoru Fukuda (La Jolla Cancer Research Foundation). 4-F-GlcNAc was synthesized as described earlier [36].

Cell culture

The human colorectal tumour cell line, HT-29, was maintained in RPMI 1640 medium supplemented with 5% heat-inactivated foetal bovine serum (HIFBS), 5% Nu-serum, 20 mM HEPES and 2 mM glutamine at 37 °C in a humidified atmosphere. The estimated doubling time for this cell line was 30 h, and cells were passaged every six days after detachment with 0.05% trypsin, 0.53 mM

EDTA. For attachment experiments, cells were routinely collected using 0.53 mM EDTA in PBS.

Normal human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and maintained in complete HUVEC growth medium supplemented with 10 ng ml^{-1} epidermal growth factor, 1 μg ml^{-1} hydroxycortisone, 2% HIFBS, 50 μg ml^{-1} Amphothericin B, and bovine brain extracts according to manufacturer's recommended procedure. HUVEC were subcultured after reaching 60–80% confluency.

Flow cytometry

Flow cytometry was performed by the RPCI Flow Cytometry Facility. Control and 4-F-GlcNAc treated (0.5 mM, 48 h) HT-29 cells (5×10^5 cells in 50 μl PBS) were blocked with 10 μg goat IgG for 10 min at 4 °C. One microgram of anti-sLe^a IgG$_1$, anti-Le^a IgG$_1$ or anti-sLe^x IgM$_k$ were added, and the incubation was carried out for the next 15 min on ice. The cells were washed three times with PBS, resuspended in PBS, and the secondary antibodies added at the following dilutions: FITC-goat anti-mouse F(ab') IgG 1:3; FITC-goat anti-mouse IgM 1:128, and allowed to bind for 15 min on ice. After three washes with ice-cold PBS, the cells were fixed by addition of 0.5 ml 1% formaldehyde and analysed by FACS (Fluorescence Activated Cell Sorter). The results represent the percentage of positively stained cells after correction for autofluorescence and non-specific binding.

HT-29 cell adhesion to endothelial cells

Normal HUVEC were seeded onto 96-well plates at 1×10^4 cells per well in complete growth medium. To stimulate E-selectin expression, recombinant human interleukin-1β (10 U ml^{-1}) was added to confluent cultures of HUVEC for 4 h [5]. The cells were washed twice with fresh growth medium before addition of 0.2 ml of HT-29 cells at 5×10^4 cells ml^{-1} in DMEM, 1% BSA. HT-29 cells were prepared as described for adhesion to polymerized galaptin (galectin-1). The number of adherent cells was determined as described earlier [15]. To determine the effect of anti-sLe^x, anti-Le^a, anti-sLe^a and anti-ELAM antibodies on HT-29 cell attachment, cells were incubated with 50 μg ml^{-1} corresponding antibodies for 15 min prior to attachment assay.

Indirect immunofluorescence

Cells were grown in complete growth medium on glass coverslips in the absence and presence of drug for 48 h. Monolayers of viable cells were treated with PBS containing 0.4% BSA for 15 min at 4 °C to block non-specific protein binding. Cells were exposed to lamp-1 or lamp-2 rabbit polyclonal antibodies diluted 1:100 in PBS containing 0.4% BSA or to biotinylated polymerized galectin-1 (10 μg ml^{-1}) for 45 min at 4 °C. Control cells were incubated with normal rabbit serum. After extensive washing, bound anti-lamp antibody was detected by incubating cells with FITC-donkey-anti-rabbit IgG diluted 1:100 in PBS containing 0.4% BSA for 30 min at 4 °C, while polymerized galectin-1 was detected with avidin-FITC diluted 1:200 in 0.4% BSA in PBS. Cells were washed in PBS, fixed for 30 min in 2% paraformaldehyde; the coverslips then were mounted in Aqua-Poly Mount onto microscopic slides. Fluorescence micrographs were prepared using a Nikon microscope equipped with Nikon FX-35WA camera and appropriate objectives and filter modules [15].

HT-29 cell adhesion to polymerized galectin-1 (galaptin)

Lectin was isolated from human spleen and polymerized as described previously [15]. Polymerized galaptin (35 μg ml^{-1} in 10 mM Tris, 20 mM NaCl, pH 7.3) was added to wells (50 μl per well) of Immulon 1 micro-enzyme-linked immunosorbent assay plates and allowed to adsorb for 24 h at 4 °C. Non-adsorbed lectin was aspirated, wells were washed twice with PBS and used for cell adhesion experiments.

Tumour cell adhesion to polymerized galectin experiments were performed as described previously [37]. Briefly, 7.5×10^5 cells were seeded in tissue culture flasks in 10 ml of growth medium and allowed to attach overnight. Cell medium was exchanged with 5 ml fresh medium containing sugar analogue at the indicated concentrations, and tumour cell cultures were incubated for 48 h. To radiolabel the cells, [^{3}H]thymidine was added (1 μCi ml^{-1}) for the last 24 h of incubation. Cells were detached with 0.53 mM EDTA in PBS, washed with PBS and resuspended at 10^5 cells ml^{-1} in DMEM containing 15 mM HEPES and 0.1% BSA. Cell suspensions (100 μl) were added to polymerized galaptin coated wells and the percentage adherent cells was estimated as described earlier [15].

Aggregation assay

The cell aggregation assay was performed according to Benchimol *et al.* [29]. Briefly, monolayers of HT-29 cells were collected by treatment with 0.53 mM EDTA. Cells were washed with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution and resuspended at 1×10^6 cells ml^{-1} in Ca^{2+}- and Mg^{2+}-free RPMI 1640, 0.8% HIFBS, 10 μg ml^{-1} DNase. Anti-CEA rabbit polyclonal antibodies (1 mg ml^{-1} of protein, 1.7 μg ml^{-1} of IgG) or non-immune rabbit serum (1 mg ml^{-1} of protein) was added and the cells were incubated for 10 min at 4 °C. The cell suspension was passed three times through a 30-gauge needle and incubated at 37 °C with stirring at 100 rpm for 2 h. The results are expressed as the per cent of single cells out of the total number of cells counted. To evaluate the effect of sugar analogue on homotypic cell–cell aggregation, HT-29 cells were exposed for 48 h to the

agent at a given concentration prior to performing an aggregation assay as described above.

Preparation of HT-29 cell extracts, SDS-PAGE and Western blotting

Cultures of control and sugar analogue-treated HT-29 cells were washed with PBS and detached with 0.53 mM EDTA. Cell pellets (10^6 cells) were lysed by incubation for 30 min on ice in 100 μl TBS (10 mM Tris/8.3 mM NaCl pH 7.3), containing 2 mM EDTA, 5 mM $MgCl_2$, 1.5% Triton X-100, 50 $\mu g\,ml^{-1}$ DNase I, 50 $\mu g\,ml^{-1}$ RNase, 1 mM PMSF and 10 $\mu g\,ml^{-1}$ each of: pepstatin A, aprotinin and leupeptin. The cell lysate was sonicated and centrifuged at 100 000 × **g** for 1.5 h at 4 °C. The resulting cell protein extracts were separated on 7% minigels by SDS-PAGE [38], and electroblotted onto nitrocellulose membranes.

Nitrocellulose blots were probed with rabbit lamp-1 and lamp-2 polyclonal antibodies (1:50 in TBS, 2% BSA), rabbit polyclonal anti-CEA (1:50 in TBS, 2% BSA), *D. stramonium* agglutinin biotin conjugates (10 $\mu g\,ml^{-1}$ in TBS, 2% BSA) or biotin-conjugated polymerized galaptin (10 $\mu g\,ml^{-1}$ in TBS, 2% BSA) as described earlier [15, 37]. When probed with *D. stramonium* agglutinin and polymerized galectin biotin conjugates, an additional 1 h incubation with avidin-horseradish peroxidase conjugate (1:20 000 in TBS/2% BSA) was performed. Bound antibody or lectin was detected using enhanced chemiluminescence reaction (ECL Western blotting detection, Amersham) according to the manufacturer's protocol. Blots were exposed to X-Omat AR X-ray films, and the obtained autoradiograms were scanned using a Computing Densitometer (Molecular Dynamics).

Protein assay

Protein was determined by a modified Lowry procedure, according to Schacterle and Pollack [39].

Results

The HT-29 human colon adenocarcinoma cell line was used as a model system to evaluate the biological activity of 4-F-GlcNAc. Based on a lack of growth inhibition, 0.5 mM 4-F-GlcNAc and a 48 h continuous exposure time was chosen for further study. Under these conditions, there was no significant effect on cell viability, however inhibition of [^{3}H]glucosamine and [^{3}H]fucose incorporation (40–50%) followed by [^{3}H]galactose incorporation into TCA precipitates was observed (data not shown). No inhibitory effect on [^{3}H]leucine incorporation was noted, indicating that under these selected conditions, protein synthesis was not affected by 4-F-GlcNAc in HT-29 cells.

4-F-GlcNAc was synthesized as a potential oligosaccharide chain terminator and/or modifier. We have anticipated that the incorporation of this sugar analogue into oligosaccharide would result in a block in the elongation of polylactosamine chains (Galβ1 → 4GlcNAc) and in fucosylation of type I chains (Fucα1 → 4). These effects would lead to changes in oligosaccharide structures, including Lewis antigens.

The effect of 4-F-GlcNAc on the expression of Le^a, s-Le^a, and s-Le^x on the HT-29 cell surface was evaluated by flow cytometry. These studies revealed that 72%, 30% and 25% of HT-29 cells were positive for s-Le^x, s-Le^a, and Le^a, respectively (Table 1). Treatment with 0.5 mM 4-F-GlcNAc for 48 h resulted in reduced cell surface expression of s-Le^x and s-Le^a by 42% and 37%, respectively. There was no significant effect on the binding of specific antibody to Le^a.

The onco-developmental carbohydrate antigens, sialyl Lewis X (sLe^x, NeuAcα2 → 3Galβ1 → 4(Fucα1 → 3)GlcNAc → R) and sialyl Lewis A (sLe^a, NeuAcα2 → 3Galβ1 → 3(Fucα1 → 4)GlcNAc → R), are postulated to be tumour cell ligands for endothelial leukocyte adhesion molecule (ECAM-1 or E-selectin) [5–10]. Thus, a decrease in cell surface expression of these structures should lead to altered adhesion of HT-29 cells to endothelial cells mediated by E-selectin.

The effect of 4-F-GlcNAc on E-selectin mediated adhesion was determined by measuring the number of adherent, control and 4-F-GlcNAc treated, HT-29 cells to confluent monolayers of HUVEC stimulated with IL-1β. The adhesion of control HT-29 cells to IL-1β stimulated endothelial cells reached a level of 50% after 15 min of incubation and increased up to 75% after 60 min (Fig. 1). Attachment of HT-29 control cells to non-stimulated HUVEC did not exceed 40%. The treatment of HT-29 cells with 0.25 mM and 0.5 mM 4-F-GlcNAc for 48 h resulted in a 60% decrease in attachment (Fig. 1). The percentage of 4-F-GlcNAc-treated cells that attached to stimulated endothelial cells was relatively low, constant over the time, and was comparable to the level of attachment found for the control HT-29 cells to the non-stimulated endothelial cells. From our results, there appeared to be no inhibitory effect of 4-F-GlcNAc on the adhesion of HT-29 cells to *non*-stimulated HUVEC

Table 1. The effect of 4-F-GlcNAc on HT-29 human colon carcinoma cell surface expression of Lewis antigens.

Addition	*Control (%)*	*+4-F-GlcNAc (%)*
Anti-s-Le^x	74.2 ± 2.1	48.4 ± 6.5
Anti-s-Le^a	30.1 ± 0.2	20.0 ± 0.9
Anti-Le^a	25.5 ± 0.5	23.6 ± 0.4

Control and 4-F-GlcNAc-treated (0.5 mM, 48 h) HT-29 cells were incubated with anti-s-Le^x, anti-s-Le^a, and anti-Le^a as described in Materials and methods. After incubation with FITC-conjugated secondary antibodies, cells were fixed with 1% formaldehyde and analysed by FACS. The results represent the percentage of positively stained cells after correction for autofluorescence and nonspecific binding.

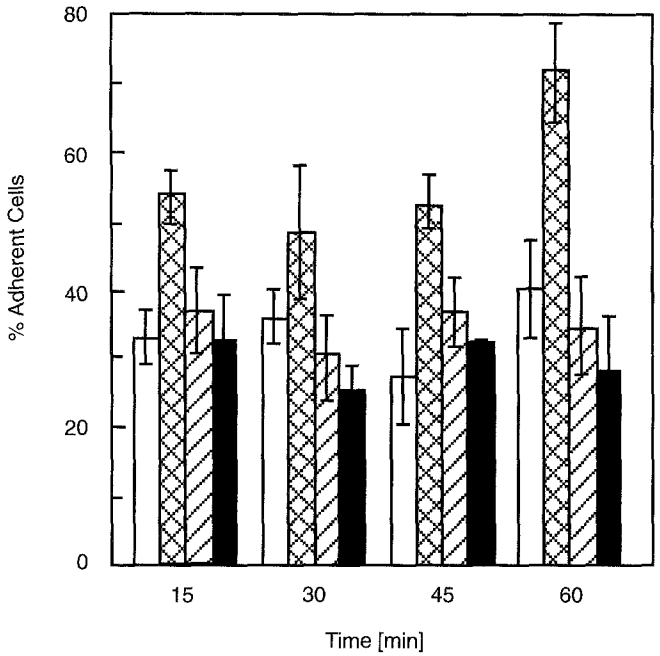

Figure 1. Adhesion of HT-29 cells to IL-1β stimulated human umbilical vein endothelial cells (HUVEC). HT-29 cells (10^4 cells 0.2 ml^{-1}) treated with 0.25 mM and 0.5 mM 4-F-GlcNAc and radiolabelled with [^{3}H]-thymidine were added to the wells containing monolayers of confluent IL-1β stimulated or non-stimulated HUVEC cells. The number of attached HT-29 cells was determined as described in Materials and methods. Open bars: control cell adhesion to non-stimulated HUVEC; crossed bars: control cell adhesion to IL-1β stimulated HUVEC; diagonally hatched bars: adhesion of 0.25 mM 4-F-GlcNAc-treated HT-29 cells; closed bars: adhesion of 0.5 mM 4-F-GlcNAc-treated HT-29 cells.

(data not shown). Thus, the effect of 4-F-GlcNAc on HT-29 adhesion to endothelial cells that depended on IL-1β induced expression of E-selectin, in all probability, was related to the reduced availability of E-selectin ligands (e.g. s-Le^x and s-Le^a) on the HT-29 tumour cells.

In experimental controls, we tested the effect of anti-s-Le^x, anti-s-Le^a, anti-Le^a and anti-ELAM antibodies on HT-29 cell adhesion to stimulated HUVEC. In our hands, a maximum inhibitory effect of 33% was observed with the anti-s-Le^x antibody. Anti-s-Le^a caused a 23% inhibition, while anti-Le^a inhibited adhesion only by 19%. However, when anti-ELAM antibody was used to block HT-29 cell adhesion, a 57% decrease in the number of adherent cells was determined, a level comparable to that caused by 4-F-GlcNAc (data not shown).

A previous study from our laboratory has shown that modifications of tumour cell surface glycoconjugates, by 4-F-GlcNAc and 3-F-GlcNAc resulted in the inhibition of adhesion of human ovarian carcinoma cells to polymerized galaptin (galectin-1) [37]. This process has been shown to be mediated via an interaction between terminal galactose residues on cell surface lamps and β-galactoside binding lectin, galectin-1 [40].

Lamp-1 and lamp-2 were detected on the cell surface of several human colon cell lines [11]. To assess the effect of 4-F-GlcNAc on cell surface expression of lamp molecules, the control and drug treated cells were subjected to indirect immunofluorescence. Both lamp-1 and lamp-2 were detected on cell surfaces in control (Fig. 2A and C) and sugar analogue-treated cells (Fig. 2B and D). However, the intensity of staining after 4-F-GlcNAc treatment was significantly decreased for lamp-1 (Fig. 2B) and almost absent for lamp-2 (Fig. 2D) suggesting that the effect of 4-F-GlcNAc was on the structure of the epitope(s) recognized by anti-lamp-1 and 2 antibodies. While the precise structure of epitope(s) for the employed polyclonal antibodies is unknown, it has been suggested that the carbohydrate structure might carry these antigenic properties [17].

The incubation of HT-29 cells with 4-F-GlcNAc at 0.25 mM for 48 h resulted in a 70% decrease in adhesion of these cells to polymerized galectin (Fig. 3). While up to 60% of control cells attached within a 45 min period of time, only 20% of the 4-F-GlcNAc treated cells attached after 15 min, and the number of attached cells did not increase by prolonging the incubation time (Fig. 3). This decreased attachment of 4-F-GlcNAc-treated cells to polymerized galaptin correlates well with reduced levels of cell surface lamp (Fig. 2), and the significant inhibition of galactose incorporation after the same treatment. Moreover, the availability of galectin ligands on HT-29 cell surface after exposure to sugar analogue was also decreased as assessed by indirect immunofluorescence. As shown in Fig. 4, a 48 h incubation with 4-F-GlcNAc resulted in a reduction of bound polymerized galaptin to the cell surface of viable HT-29 cells, indicating a decrease of terminal galactose residues on polylactosaminoglycans, which are necessary for the galaptin-mediated adhesion to occur [15, 41, 42].

Lysosomal associated membrane proteins, which are often found on tumour cell surfaces, contain large amounts of polylactosamine residues [11]. Their expression on human colon carcinoma cell surfaces has been correlated with their ability to attach to endothelial cells and their increased metastatic potential [11, 43]. Homotypic cellular aggregation, on the other hand, is thought to facilitate the implantation and/or arrest of cancer cells during the metastatic cascade. The formation of cellular aggregates may enhance the survival of circulating cancer cells by protecting the inner cells from the host immune system. In human colon cells, homotypic aggregation was suggested to be mediated by carcinoembryonic antigen, CEA, a heavily glycosylated cell surface glycoprotein [29, 33].

To evaluate the effect of 4-F-GlcNAc on CEA mediated intercellular adhesion, HT-29 cells were treated

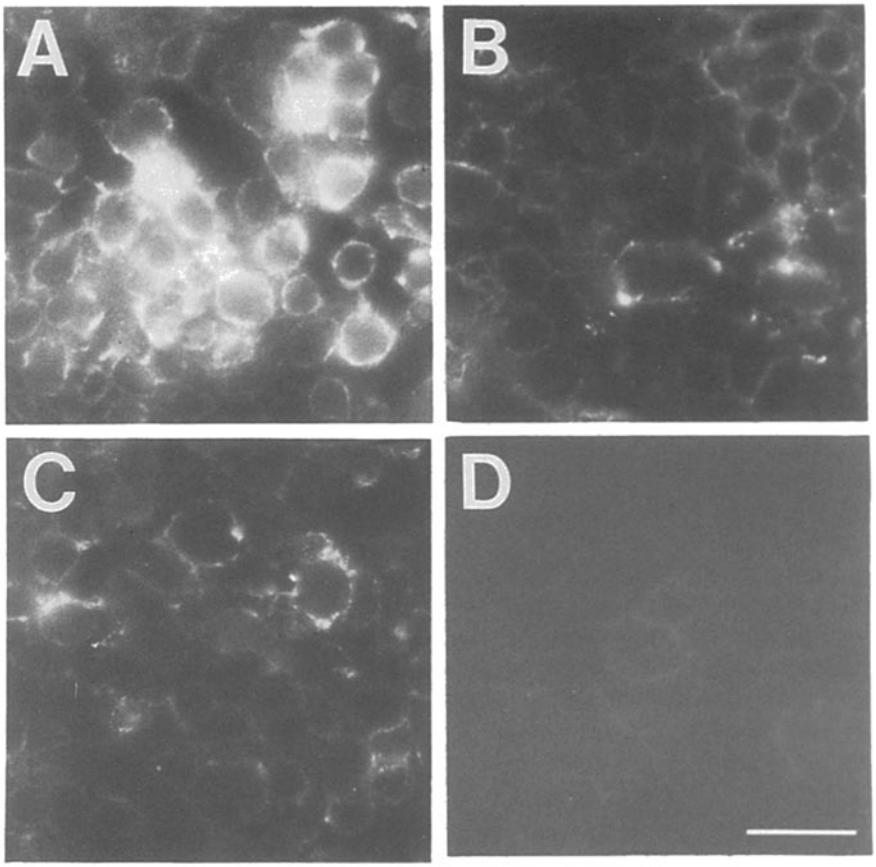

Figure 2. HT-29 human colon carcinoma control (A and C) and 0.5 mM 4-F-GlcNAc treated (B and D) cells grown on coverslips were incubated with a 1:100 dilution of lamp-1 (A and B) and lamp-2 (C and D) polyclonal antibodies. Cells were washed and incubated with a 1:100 dilution of FITC-donkey-anti-rabbit IgG and fixed in 2% paraformaldehyde as described in Materials and methods. Magnification, 40×, (scale bar = 20 μm).

with 0.25 mM and 0.5 mM 4-F-GlcNAc for 48 h, detached with EDTA, resuspended in Ca^{2+} and Mg^{2+} free medium. The number of remaining single cells was determined after 2 h incubation with gentle rotation. The percentage of single cells in the control samples was at the level of 30%, while ~60% of 4-F-GlcNAc-treated cells remained in a form of single cell suspension (data not shown). The similar (~50% single cells) low level of aggregation was observed in the presence of anti-CEA antibodies. These data strongly imply an active role of CEA oligosaccharides in homotypic aggregation and a possibility of affecting this process through alteration of cell surface glycoproteins using specific sugar analogues.

CEA and lamp oligosaccharides contain polylactosaminoglycans with galactose residues available for binding to galectin-1. To distinguish the role of HT-29 cell surface lamps and CEA in lectin mediated heterotypic adhesion, we measured the attachment of HT-29 cells to polymerized galectin in the presence of antibodies directed against lamps and CEA. A previous study showed that polymerized galectin binds to the F_c fragment of rabbit IgG heavy chain [15]. Therefore, Protein A was used to mask the F_c region, preventing binding between the oligosaccharides on the F_c fragments to polymerized galectin. As shown in Table 2, preincubation of HT-29 cells with anti-lamp-2 antibodies for 15 min at 4 °C followed by incubation with Protein A resulted in ~30% inhibition of cell attachment to polymerized galaptin-coated wells. In contrast, there was no inhibitory effect observed when either anti-lamp-1 or anti-CEA antibodies were used. Preincubation of HT-29 cells with Protein A, non-immune rabbit serum alone or the combination of both did not have any effect on HT-29 cell adhesion. The observed selective inhibition of HT-29 cell adhesion to polymerized galaptin by anti-lamp-2 antibodies along with the strong effect of 4-F-GlcNAc on cell surface expression of lamp-2, suggest the importance of lamp-2 in lectin mediated HT-29 cell adhesion.

Treatment of HT-29 cells with 4-F-GlcNAc resulted in impaired adhesive properties of these cells. Since evaluated adhesion processes are mediated by different mechanisms and different cell surface glycoproteins, we

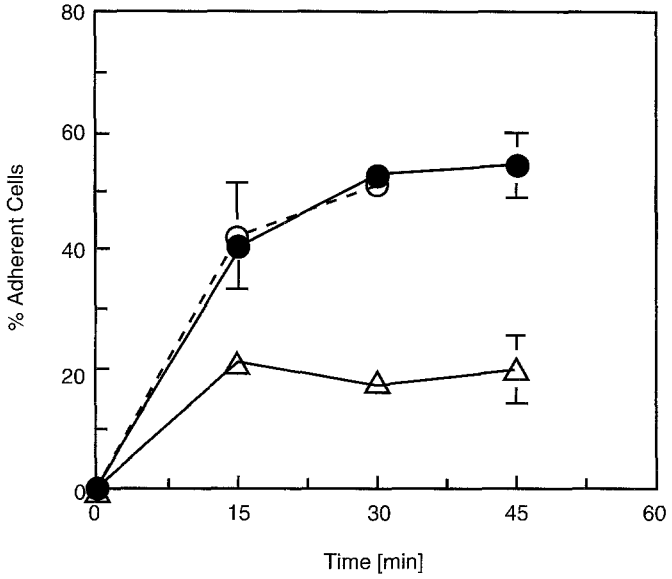

Figure 3. Adhesion to polymerized galaptin. HT-29 cells were incubated for 48 h without (–●–) or with 0.05 mM (–○–) and 0.25 mM (–△–) 4-F-GlcNAc. [^{3}H]Thymidine (1 μCi ml^{-1}) was added for the last 24 h of incubation and 10^4 cells per 100 μl in DMEM/15 mM HEPES/0.1% BSA were added to the wells coated with polymerized galaptin (1.6 μg per well). The number of adherent cells was measured at the indicated time points as described in Materials and methods.

examined whether lamp and CEA underwent any similar structural changes after treatment with sugar analogue.

CEA and lamps contain a large amount of poly-N-acetyllactosaminoglycans with repeating Galβ1 → 4GlcNAc linkages that are specifically recognized by *D. stramonium* agglutinin [44]. Protein extracts from control and 0.5 mM 4-F-GlcNAc treated cells were separated by electrophoresis under reducing conditions, transferred to nitrocellulose membrane and probed with this lectin. Significant differences in *D. stramonium* lectin binding to protein extracts from control (Fig. 5, lanes 2 and 4) and 4-F-GlcNAc treated (Fig. 5, lanes 1 and 3) HT-29 cells were noted. The effect of 4-F-GlcNAc on lectin binding was observed on glycoproteins of molecular weight in the

Table 2. The effect of anti-CEA and anti-lamp-1 and 2 antibodies on HT-29 human colon carcinoma cell adhesion to polymerized galectin-1 coated plates.

HT-29 pretreatment	*Adherent cells (%)*
None	74.9 ± 6.2
Protein A	80.6 ± 4.7
Non-immune rabbit serum (NRS)	72.7 ± 7.6
NRS + protein A	70.8 ± 14.3[a]
Anti-CEA + protein A	75.8 ± 10.8
Anti-lamp-1 + protein A	63.9 ± 1.7
Anti-lamp-2 + protein A	51.0 ± 5.8[a]

[a] $p < 0.01$; paired t-test

[^{3}H]Thymidine-labelled HT-29 cells (3.3×10^4) were suspended in 300 μl of RPMI 1640 containing 15 mM HEPES, 1% BSA. Samples were treated for 15 min with 30 μl of polyclonal anti-lamp-1, anti-lamp-2, anti-CEA or non-immune rabbit serum (NRS). Protein A (15 μg) was added for additional 15 min incubation. Aliquots containing 10^4 cells (100 μl) were added to polymerized galaptin-coated wells and the percentage of adherent cells was determined as described in Materials and methods. The results represent the mean value ± SD from three independent experiments performed in triplicate.

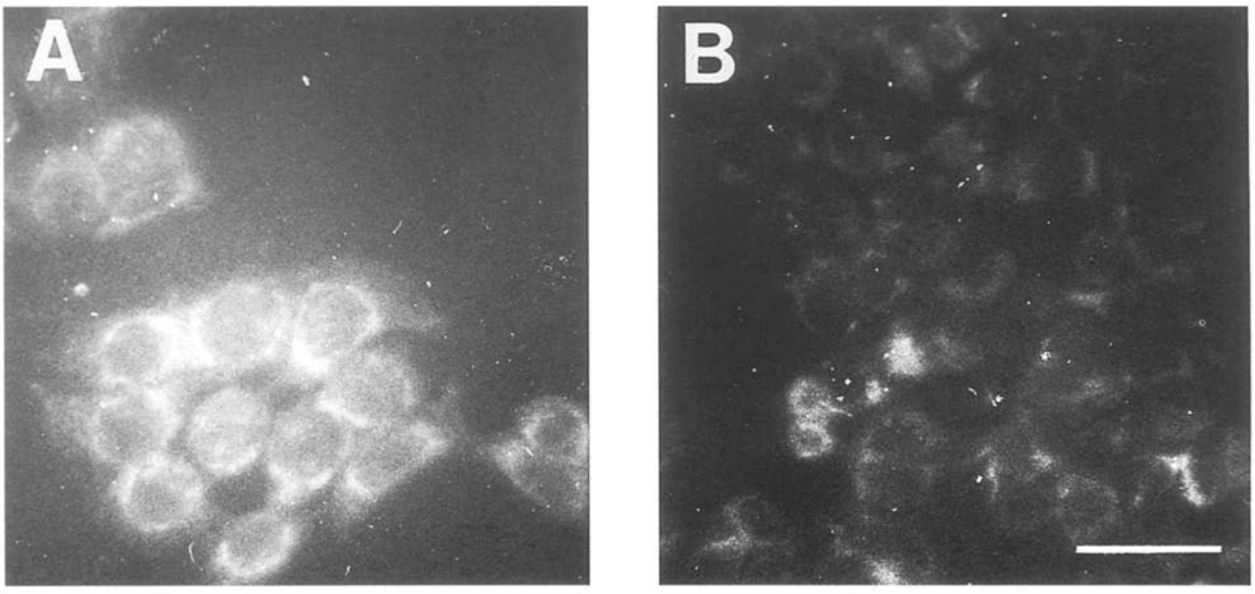

Figure 4. Binding of polymerized galaptin to HT-29 cells. HT-29 human colon carcinoma control (A) and 0.5 mM 4-F-GlcNAc treated (B) cells grown on coverslips were incubated with biotin conjugates of polymerized galaptin (10 μg ml^{-1} in 0.4% BSA in PBS). Cell were washed and bound lectin was detected with a 1:200 dilution of avidin-FITC conjugates in 0.4% BSA in PBS, and fixed in 2% paraformaldehyde as described in Materials and methods. Magnification, 40×, (scale bar = 20 μm).

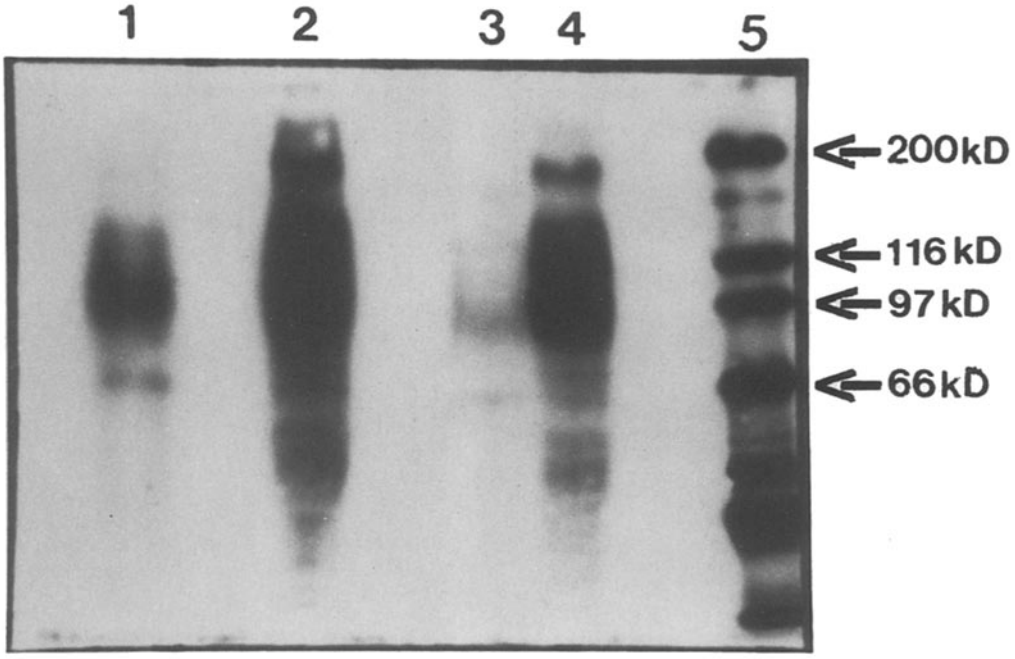

Figure 5. Protein extracts from HT-29 control (lanes 2, 4) and 0.5 mM 4-F-GlcNAc treated (lanes 1, 3) HT-29 cells were applied at 10 (lanes 1, 2) and 5 μg (lanes 3, 4) cellular protein/lane and separated by SDS-PAGE on 7% minigels, transferred to nitrocellulose membrane and probed with 10 $\mu g\,ml^{-1}$ *D. stramonium* agglutinin as described in Materials and methods. Lane 5: biotinylated high MW markers.

range of 66–180 kDa (Fig. 5). Also, there was a complete disappearance of binding at a molecular weight range below 66 kDa. Thus, although the 4-fluoro-analogue had a significant inhibitory effect on formation of Galβ1 → 4GlcNAc linkages, this effect was not limited to lamp and CEA molecules.

Cell extracts from control and 4-F-GlcNAc treated HT-29 cells were probed with rabbit antiserum against lamp-1 and 2, and CEA. The treatment of HT-29 cells with 4-F-GlcNAc resulted in decreased antibody binding to lamp-1 and lamp-2 and a shift of the detected bands towards a lower molecular weight. As shown in Fig. 6A, protein extracts from 4-F-GlcNAc treated cells showed a ~46% decrease in anti-lamp-1 antibody reactivity and a shift towards a lower molecular weight. In the control samples (Fig. 6A, lane 1) the protein band recognized by anti-lamp-1 antibody corresponded to an intact, mature lamp-1 molecule with a molecular weight of ~110 kDa. In the protein extracts from 0.5 mM 4-F-GlcNAc treated cells (Fig. 6A, lane 2), the same antibody recognized a lamp-1 species with molecular weight of ~90 kDa. Probing the same protein extracts with anti-lamp-2 antibody showed a 50% decrease in the intensity of anti-lamp-2 reactivity and shift of the reactive band towards a lower molecular weight in the cell extracts obtained from drug treated HT-29 cells (Fig. 6A, lane 3). When probed with biotin conjugates of polymerized galaptin, a ~110 kDa protein in extract from control cells reacted with galaptin (Fig. 6B, lane 3). Very weak binding, at the area of lower molecular weights, was observed in extracts from 4-GlcNAc treated cells (Fig. 6B, lane 2).

A similar pattern in antibody reactivity and molecular weight were observed for carcinoembryonic antigen (Fig. 7). The CEA species of ~180 kDa was found in protein extracts from control cells (Fig. 7 lanes 1 and 3), while a less intense anti-CEA reactive band was detected at ~160 kDa (Fig. 7 lanes 2 and 4) from protein extracts obtained from sugar analogue treated HT-29 cells.

Discussion

In this report, we evaluated the ability of 4-F-GlcNAc to modulate HT-29 human colon carcinoma cell glycoconjugate mediated adhesion reactions. Substitution of fluorine at position C4 was designed to result in an agent capable of blocking the elongation of type 2 backbone lactosamine units [Galβ**1** → **4**(Fucα1 → 3)GlcNAc → R] and decreasing fucosylation of type 1 structures [Galβ1 → 3(Fucα**1** → **4**)GlcNAc → R]. These oligosaccharide structures, often found on polylactosamine chains of lysosomal associated membrane proteins, correspond to Lewis antigens which have been reported to play an important role in leukocyte and tumour cell adhesion to endothelial cells [45–51].

We have shown previously that adhesion of human

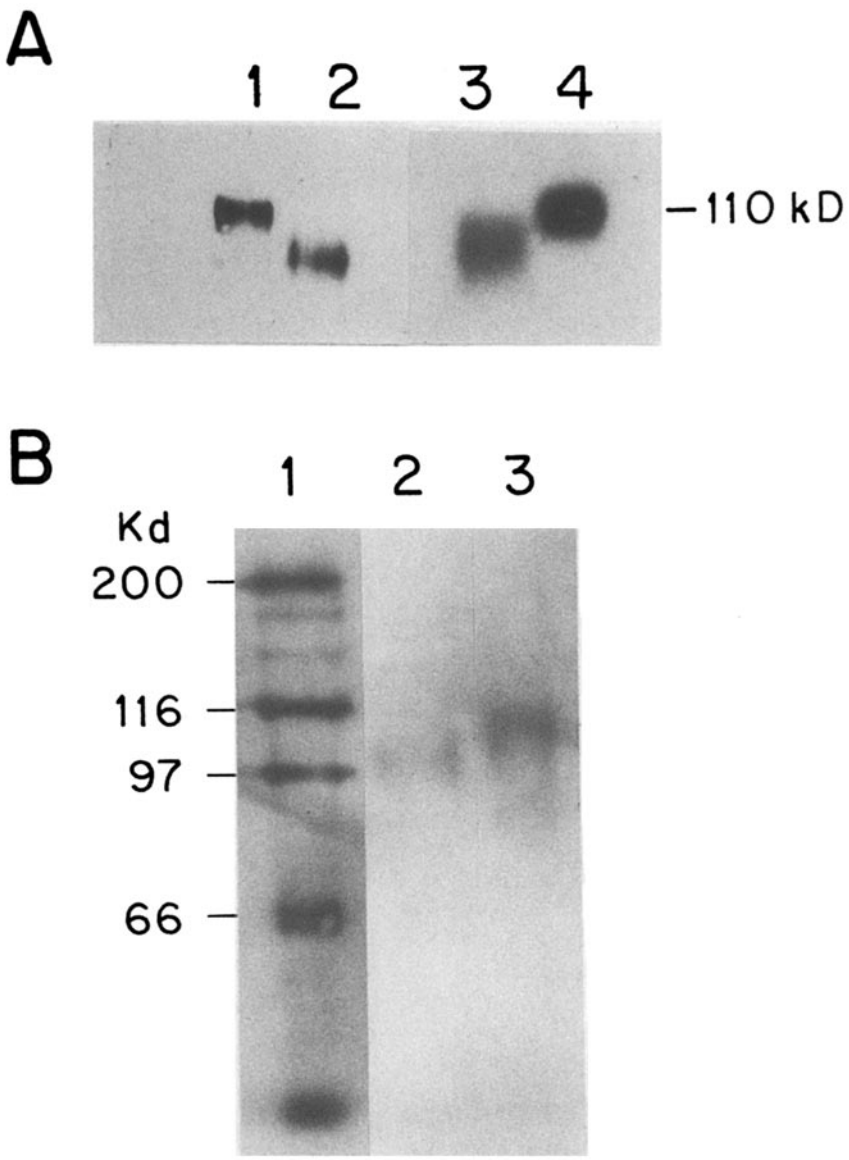

Figure 6. Protein extracts from HT-29 control and 0.5 mM 4-F-GlcNAc treated HT-29 cells were separated by SDS-PAGE on 7% minigels, transferred to nitrocellulose membrane and probed with (A) anti-lamp-1 and anti-lamp-2 polyclonal antibody (1:50) and (B) biotin conjugated polymerized galaptin ($10\,\mu g\,ml^{-1}$) as described in Materials and methods. (A) lamp-1: protein extract from control cells (lane 1); protein extracts from drug treated cells (lane 2) at $5\,\mu g$ per lane; lamp-2: protein extract from control cells (lane 4); protein extracts from drug treated cells (lane 3) at $5\,\mu g$ per lane. (B) polymerized galaptin: extract from 0.5 mM 4-F-GlcNAc treated (lane 2) and control (lane 3) cells, molecular weight markers (lane 1).

ovarian carcinoma cells is mediated partially by an interaction between cell surface lamp molecules and galectin-1 (galaptin), a β-galactoside specific lectin present in extracellular matrix [15, 52]. The galaptin mediated adhesion of ovarian cells *in vitro* was inhibited by 4-F-GlcNAc and 3-F-GlcNAc, as a result of the modification of cell surface lamps [37]. Similarly, the adhesion of HT-29 colon carcinoma cells to polymerized galectin was inhibited by the 4-F-GlcNAc, suggesting an involvement of HT-29 cell surface lamps in attachment, mainly, lamp-2. We have observed almost undetectable cell surface expression of lamp-2 in 4-F-GlcNAc treated cells that could result from (1) structural changes in lamp-2 epitope recognized by antibodies, (2) impaired transport of lamp-2 to cell surface due to affected glycosylation, (3) inhibition of lamp biosynthesis, or combination of all of the above effects. However, a lack of protein synthesis inhibition as measured by leucine

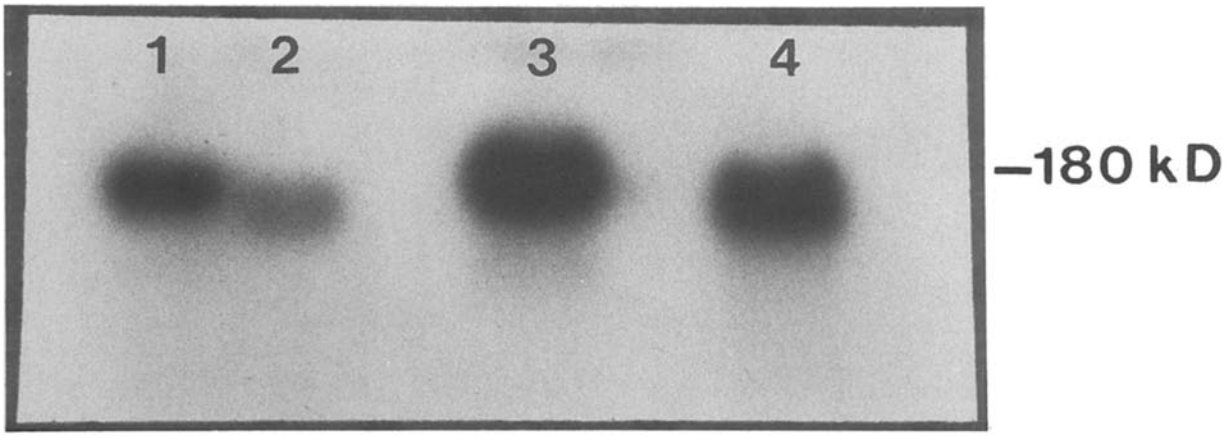

Figure 7. Protein extracts from HT-29 control (lanes 1, 3) and 0.5 mM 4-F-GlcNAc-treated cells (lanes 2, 4) applied at 5 μg protein per lane (lanes 1, 2) and 10 μg protein per lane (lanes 3, 4) were separated by SDS-PAGE on 7% minigels, transferred to nitrocellulose membrane and probed with anti-CEA rabbit polyclonal antibody (1:50) as described in Materials and methods.

incorporation, and appearance of lower molecular species of lamp in 4-F-GlcNAc treated cells point to incomplete glycosylation.

It is likely that in HT-29 cells, lamp molecules are involved in the adhesion to polymerized galectin to a different degree. While the anti-lamp-2 antibody reduced the adhesion of HT-29 cells to polymerized galectin, anti-lamp-1 and anti-CEA antibodies did not show any significant effect. These results suggest that lamp-2 molecules on HT-29 cell surface may contain more specific and accessible ligands for polymerized galectin. Also, these findings point to tumour-specific, subtle, structural and functional differences in interaction between lamp molecules and β-galactoside binding lectin(s).

Recently, the concomitant increases in expression of galectin-1, lamp and CEA in human colon carcinoma cells have been reported by Lotan's group [53]. The authors implicated lamps and CEA as ligands for galectin and suggested complementary interactions between CEA and galectin during intercellular adhesion. In our hands, however, anti-CEA antibody did not affect binding of HT-29 cells to polymerized galectin-coated surface. On the other hand, CEA-mediated homotypic aggregation of HT-29 cells was significantly inhibited by 4-F-GlcNAc and anti-CEA polyclonal antibody. In view of homotypic aggregation of colonic cells as a process facilitating metastasis formation, these data convey an important role for CEA oligosaccharides in intercellular adhesion and possibility of affecting this process by using glycoconjugate modifiers [30].

Saitoh *et al.* reported that cell surface expression of lamp-1 and lamp-2 in human colon cancer sublines correlates with their metastatic potential [11]. Increased levels of cell surface lamp-1 was associated with enhanced expression of cell surface sialyl-Lewis^x antigen and more efficient E-selectin mediated adhesion of colon carcinoma cells [43]. The E-selectin-dependent adhesion of human colon carcinoma cells to IL-1β stimulated endothelial cells was inhibited by soluble lamp-1 and leukosialin that contained sialyl-Le^x [54]. Thus, reduced adhesion was achieved by an oligosaccharide dependent competitive type of inhibition.

In our studies, treatment of human colon HT-29 cells with 4-F-GlcNAc led to decreased levels of expression and/or changes in structure of ELAM ligands (e.g. s-Le^x, s-Le^a), as shown by flow cytometry. As a result, a significant inhibition of E-selectin mediated adhesion of HT-29 to endothelial cells was observed. The treatment of HT-29 cells with 4-F-GlcNAc reduced attachment of these cells by 60%, the highest level of inhibition, equal to that caused by ELAM directed antibody. In contrast, anti-s-Le^x or anti-s-Le^a antibodies, when given separately, caused only a 20–30% inhibition of colon carcinoma cell attachment to endothelial cells. Thus, it is likely that 4-F-GlcNAc treatment of HT-29 cells eliminated more than one of the available ligands (containing type-2 and/or type-1 chains) for E-selectin present on endothelial cells. This inhibitory effect on HT-29 cell adhesion to endothelial cells did not require the continued presence of the drug, indicating a non-competitive type of inhibition. The lack of complete inhibition of adhesion was probably due to other, non-lectin mediated attachment mechanisms involved in human colon and endothelial cell interactions [55].

Huang *et al.* reported a decrease in colon cancer cell binding to E-selectin following treatment of these cells with benzyl-α-GalNAc. However, in this case, benzyl-α-GalNAc affected adhesion of HM7 colon carcinoma cells by inhibiting mucin biosynthesis and reducing the level

of peripheral mucin associated carbohydrate antigens such as sialyl-Le^a and sialyl-Le^x, while increasing T and T_n antigenic levels [56].

4-F-GlcNAc inhibited the incorporation of labelled sugars into TCA precipitates in HT-29 cells without inhibition of leucine incorporation. It is likely that in 4-F-GlcNAc treated cells, incomplete glycosylation was responsible for detection of lamp and CEA species with lower molecular weight. We have observed a decrease in the amount of Galβ1 → GlcNAc units as detected by *D. stramonium* agglutinin. Reduction in binding of polymerized galaptin to the HT-29 cell surface suggests limitation in the availability of terminal galactose. Moreover, 4-F-GlcNAc treated HT-29 cells had less sialyl-Le^a. This may suggest that the sugar analogue affects the structure of type 1 chain-containing epitopes. However, further investigation is necessary to provide direct evidence for the 4-F-GlcNAc mechanism of action and its effect on the cellular glycosylation pathway.

The results described herein show that inhibition of tumour cell adhesion to endothelial cells and tumour cell aggregation can be achieved through the modulation of glycoconjugates by the glucosamine analogue, 4-F-GlcNAc. Inhibition of these processes could result in decreased metastatic potential for colon carcinoma cells. Preliminary findings from our laboratory have demonstrated a reduction in the number of liver metastases without affecting primary tumour formation following intrasplenic injection in nude athymic mice of HT-29 cells pretreated with 4-F-GlcNAc. However, further systematic studies are needed to confirm 4-F-GlcNAc activity in oligosaccharide mediated intercellular adhesion and aggregation during metastasis formation *in vivo*.

In summary, we have demonstrated that treatment with a specific, 4-fluoro-glycosamine analogue decreased expression of Lewis antigens on HT-29 human colon carcinoma cells, reduced E-selectin mediated adhesion of these cells to endothelium, and decreased their homotypic cellular aggregation. Interference with these carbohydrate-mediated interactions may lead to reduced tumour cell adhesiveness *in vivo* and decreased metastatic potential.

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