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Interaction of colon cancer cells with glycoconjugates triggers complex changes in gene expression, glucose transporters and cell invasion

ROMANA KŘIVOHLAVÁ, VALIKA GROBÁROVÁ, EVA NEUHÖFEROVÁ, ANNA FIŠEROVÁ and VERONIKA BENSON

Laboratory of Molecular Biology and Immunology, Department of Immunology, Institute of Microbiology, Czech Academy of Sciences, 14220 Prague 4, Czech Republic

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Abstract. Glycan metabolism balance is critical for cell prosperity, and macromolecule glycosylation is essential for cell communication, signaling and survival. Thus, glycotherapy may be a potential cancer treatment. The aim of the present study was to determine whether combined synthetic glycoconjugates (GCs) induce changes in gene expression that alter the survival of colon cancer cells. The current study evaluated the effect of the GCs *N*-acetyl-D-glucosamine modified polyamidoamine dendrimer and calix[4]arene scaffold on cancer cell proliferation, apoptosis, invasion and sensitivity to immune cell-mediated killing. Using reverse transcription-quantitative polymerase chain reaction, the expression of genes involved in the aforementioned processes was measured. It was determined that GCs reduce the expression of the glucosaminyltransferases *Mgat3* and *Mgat5* responsible for surface glycosylation and employed components of the Wnt signaling pathway *Wnt2B* and *Wnt9B*. In addition, the calix[4]arene-based GC reduced cell colony formation; this was accompanied by the downregulation of the metalloproteinase *Mmp3*. By contrast, the dendrimer-based GC affected the expression of the glucose transporter components *Sglt1* and *Egfr1*. Therefore, to the best of our knowledge, the present study is the first to reveal that *N*-acetyl-D-glucosamine-dendrimer/calix[4]arene GCs alter mRNA expression in a comprehensive way, resulting in the reduced malignant phenotype of the colon cancer cell line HT-29.

Correspondence to: Dr Veronika Benson, Laboratory of Molecular Biology and Immunology, Department of Immunology, Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 14220 Prague 4, Czech Republic
E-mail: benson@biomed.cas.cz

Abbreviations: GC, glycoconjugate; GlcNAc, *N*-acetylglucosamine; GN4C, *N*-acetyl-D-glucosamine-coated calix[4]arene; GN8P, *N*-acetyl-D-glucosamine-coated polyamidoamine dendrimer; PAMAM, polyamidoamine; PBMC, peripheral blood mononuclear cells; RTCA, Real-Time Cell Analyzer

Key words: calix[4]arene, polyamidoamine dendrimer, glycosylation, N-acetyl-glucosaminyl-transferase, WNT, colon cancer cell

Introduction

Cell surface glycans are molecules that regulate interactions among neighboring cells or contact between cells and the extracellular matrix during cell adhesion, recognition and communication (1). An aberrant glycosylation pattern promotes the development and progression of certain pathologies, including cancer (1). Glycan alterations may result in tumor development and progression, as well as tumor-cell dissociation and invasion, which subsequently promote metastasis and tumor-associated neoangiogenesis (2). Importantly, aberrant glycosylation of the tumor cell surface results in the impairment of its recognition by cells of the immune system, including natural killer (NK) cells (2,3).

Alterations in glycosylation primarily arise from changes in the expression of *N*-acetyl-D-glucosamine (GlcNAc) transferases in the Golgi system (4). The family of β-1,4-mannosyl-glycoprotein 4-β-*N*-acetyl glucosaminyltransferases consists of several members, including Mgat*3* and *Mgat5*, which are involved in linking terminal residues to glycans on the cell surface. The competition between *Mgat3* and *Mgat5* results in the branching or bisecting of surface glycans and this final pattern influences intercellular recognition (5). *Mgat5* is responsible for adding β1-6 GlcNAc residues and forming branched structures, which are particularly abundant in cancer tissues with high metastatic potential (4). *Mgat3* facilitates the addition of β1-4 GlcNAc residues and constructs a bisecting structure that inhibits further addition of GlcNAc by other glucosaminyltransferases, including *Mgat5* (6). *Mgat3* and *Mgat5* enzymes tend to be overexpressed in tumor cells (2,7). Song *et al* (8) reported that bisecting GlcNAc on N-glycans inhibits the signaling of growth factors and attenuates the progression of mammary tumors. Therefore, the resulting effect of *Mgat3*/*Mgat5* activity is likely to be tumor specific. The expression of *Mgat5* is regulated via the NM23 regulator, which is encoded by *Nme1* gene (9) and the expression of glucosaminyltransferases, including *Mgat3* and *Mgat5*, is associated with their surface glycosylation activity (10).

In vitro, cell glycosylation may be studied using synthetic glycoconjugates (GCs) that modulate cell surface glycosylation. GCs are able to interfere with cancer cell processes and the cancer microenvironment; therefore they may be able to modulate the malignant phenotype of cancer cells (11).

The present study focused on GC-triggered alterations in the mRNA expression of surface glycosylation regulators, as well as key components of signaling pathways responsible for cell-extracellular matrix adhesion, neovascularization and invasion, each of which contribute to the metastatic potential of cancer cells. The GCs assessed included GlcNAc moieties linked with a calix[4]arene or with a polyamidoamine dendrimer (PAMAM) core. Calix[4]arenes possessing distinctive geometry act as carriers of anticancer drugs (12) or as direct anticancer agents via enzyme inhibition (13), angiogenesis inhibition (14) or innate immunity modulation (15). PAMAM dendrimers have previously been used to deliver genes and drugs (16).

Cells internalize exogenous glucosamine via the glucose transporter and process it, as well as cellular glucosamine, via lysosomal degradation into uridine diphosphate glucose (UDP)-GlcNAc (17,18). This UDP-GlcNAc is further used in the post-transcriptional modification of glycosylate substrates, such as proteins. Glycosylation of nuclear proteins, including histone H2B, contributes to transcriptional regulation (19). The genes encoding the sodium glucose cotransporter 1 (*Sglt1*) and epidermal growth factor receptor (*Egfr1*) are components of a glucose co-transporter. *Sglt1* is expressed primarily in the human intestine and kidney (20). An example of a *Egfr1* ligand is *N*-acetylglucosamine (21). Glycosylation of the epidermal growth factor receptor improves its ability to bind to epidermal growth factor (22). The coupling of *Egfr1* to *Sglt1* further stabilizes the whole complex and enables cancer cells to take up high levels of glucose (23).

The authors of the present study previously reported that a GC consisting of four GlcNAc residues on a *N*-acetyl-D-glucosamine-coated calix[4]arene core (GN4C) alters the glycosylation of human NK cells and promotes the cell-mediated cytotoxicity of human NK cells against K562 and HT-29 target cells via the phosphoinositide 3-kinase pathway (2). In addition, it has been demonstrated that a GC consisting of eight GlcNAc residues on a PAMAM core *N*-acetyl-D-glucosamine-coated polyamidoamine dendrimer (GN8P) mediates alterations in cytokine profile specific to mouse NK T-cells and macrophages (24). Therefore, the present study aimed to determine whether these GCs directly affect cancer cells by modulating the regulation of gene expression and causing changes in the phenotypes of malignant cells. The effect of synthetic GCs on the mRNA expression of the cell glucosaminyltransferases *Mgat3* and *Mgat5*, members of the Wnt signaling family (*Wnt2B* and *Wnt9B*), regulators of glucose metabolism (*Sglt1* and *Egfr1*) and regulators of cell adhesion and invasion matrix metalloproteinase 3 (*Mmp3*) or transforming growth factor-β1 (*Tgfb1*) were measured.

Materials and methods

Preparation and treatment of cells with GCs. The HT-29 adenocarcinoma cell line (ATCC number: HTB-38) was obtained from American Type Culture Collection (Manassas, VA, USA) and was authenticated following the guidelines of the International Cell Line Authentication Committee (https://www.lgcstandards-atcc.org). Cells were maintained in 37˚C and in an RPMI-1640 medium with 10% fetal calf serum (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood of healthy donors (4 males, mean age 46.3±9.4; samples collected August 2012) using a Ficoll-Paque (GE Healthcare Life Sciences, Little Chalfont, UK) at density 1,077 g/ml and centrifugation for 40 min at room temperature and 400 x g. Blood samples were taken from material remaining following a routine donor check-up at the transfusion unit of the Thomayer Hospital (Prague, Czech Republic). All donors signed informed consent for the use of their blood for experimental purposes. The current study was approved by the Ethical Committee of Thomayer Hospital. The chemically defined GC calix[4]arene containing of four terminal N-acetyl-D-glucosamine moieties (GN4C) was synthesized and kindly provided by Vladimir Křen and Karel Křenek (Czech Academy of Sciences, Prague, Czech Republic). A GC consisting of PAMAM with eight terminal N-acetyl-D-glucosamine moieties (GN8P) was synthesized and kindly provided by Thisbe Lindhorst (Christiana Albertina University, Kiel, Germany). The synthesis, purity, nuclear magnetic resonance data and dose-dependent effect of the GCs on human PBMC cells have been previously described (2,24-26). The optimal concentration of 10 nM GN4C or GN8P were used for the experiments. Fludara® (1 mM, FLU; Genzyme, Cambridge, MA, USA), which is a conventional anticancer drug, was used to compare the anticancer effect of the tested GCs with a known anticancer agent.

Glyco-gene profiling array. The HT-29 cell line was incubated with GN8P for 24 h and total RNA was isolated using an RNAeasy Mini kit involving DNAse I treatment following the manufacturer's protocol (DNAse I was a component of RNAeasy Mini kit; Qiagen GmbH, Hilden, Germany). A Glyco-gene Chip array (GLYCOv3 Gene Chip; Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing probe sets of 2,000 human transcripts was provided by the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/resources/resourcecoree.shtml). Microarray experiments were conducted by The Microarray Gene Core of Consortium for Functional Glycomics, National Institutes of Health (NIH)/National Institute of General Medical Sciences (NIGMS), both Bethesda, MD, USA (http://www.functionalglycomics.org) and were performed in triplicate. BRB ArrayTools version 4.3.0 beta 3 [Biometric Research Branch, NIH/National Cancer Institute (NCI)] were used to filter and analyze experimental data sets. Class comparison used a two-sample t-test with a random variance model. A P-value of log-ratio <0.05 was considered significant.

Gene Ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software version 6.7 available from NCI (http://david.abcc.ncifcrf.gov). The Gene Card database was used for basic gene identification and characterization (http://www.genecards.org; Weizmann Institute of Science, Rehovot, Israel).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The HT-29 cell line was incubated with GCs and FLU for 24 h and total RNA was isolated using an RNAeasy Mini kit involving DNAse I treatment, as described by the manufacturer (Qiagen). A total of 5 μg RNA was transcribed into cDNA using a cDNA Archive kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using PowerSybr Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an iCycler5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR product specificity was analyzed by melt curve analysis. The cycling conditions recommended by the Master mix manufacturer were followed: initiation 95˚C 10 min and 40 cycles of 95˚C 15 sec and 60˚C 1 min. The primers used for PCR were designed using Primer3 Input software version 0.4.0 (National Center for Biotechnology Information, Bethesda, MD, USA). The sequences of primers were as follows: *Mgat5,* forward, 5'-CTT CTTTCTTCCAGCACCTCAAC-3' and reverse, 5'-AAACAC ACAGTGCTTATTCTTAGGG-3'; *Nme1,* forward, 5'-ACC TTCATTGCGATCAAACC-3' and reverse, 5'-GGCCCTGAG TGCATGTATTT-3'; *Siglec5,* forward, 5'-CAAGTGCAGAAG TCGGTGAC-3' and reverse, 5'-GGGTCTCTGGCTTCACTC TT-3'; *Siglec8,* forward, 5'-TGCAACCCTCAGCTTCCATA-3' and reverse, 5'-ACTTCTTTGCTGGAGGGGTT-3'; *Wnt9B,* forward, 5'-TGGGCAGACTGTCATCACAT-3' and reverse, 5'-AACAAGGTTGGGGATGCTTG-3'. Sequences of the primers used to amplify *B2M*, *Egfr1* and *Sglt1*, *Mki67*, *Mmp3*, *Tgfb1* and *Wnt2B* were described previously (27-31): *B2M,* forward, 5'-GAGTATGCCTGCCGTGTG-3' and reverse, 5'-AATCCAAATGCGGCATCT-3'; *Egfr1,* forward, 5'-TTT CGATACCCAGGACCAAGCCACAGCAGG-3' and reverse, 5'-AATATTCTTGCTGGATGCGTTTCTGTA-3'; *Sglt1,* forward, 5'-TGGCAGGCCGAAGTATGGTGT-3' and reverse, 5'-ATGAATATGGCCCCCGAGAAGA-3'; *Mki67,* forward, 5'-GGAGGCAATATTACATAATTTCA-3' and reverse, 5'-CAGGGTCAGAAGAGAAGCTA-3'; *Mmp3,* forward, 5'-ATGCCCACTTTGATGATGATGAAC-3' and reverse, 5'-CCACGCCTGAAGGAAGAGATG-3'; *Tgfb1,* forward, 5'-TGACAGCAGGGATAACACACT-3' and reverse, 5'-GTA GGGGCAGGGCCCGAGGCA-3'; *Wnt2B,* forward, 5'-CAC CTGCTGGCGTGCACTCTCAGA-3' and reverse, 5'-GGG CTTTGCAAGTATGGACGTCCACAGTA-3'. Primers for *Mgat3* were obtained from SA Biosciences; Qiagen (product ID: PPH01058A). The expression of the genes of interest were normalized to that of the control gene *B2M* and quantified using the $2^{-\Delta\Delta Cq}$ method (32). Differences in the expression of genes between the untreated and GC-treated cells were evaluated using Bio-Rad iQ5 version 2.0 (Bio-Rad Laboratories, Inc.).

Cell proliferation and colony formation assays. To determine the effect of GCs on cell growth, $5x10^3$ cells/well were seeded in triplicate on a 96-well plate and incubated with GCs for 126 h. FLU (1 mM) was used as a positive control of tumor cell growth inhibition. Negative controls represent cells with no additional treatment. Cell growth was monitored continuously using a Real-Time Cell Analyzer (RTCA; xCELLigence System; Acea Biosciences, San Diego, CA, USA; https://www.aceabio.com/products/rtca-dp/) and evaluated using RTCA software version 1.2 (Acea Biosciences).

To study colony formation, a standard colony forming assay and an impedance-based assay using the RTCA instrument (Acea Biosciences) were performed. For colony formation assays, $1x10^3$ were seeded in a Petri dish 100 mm in diameter. After 2 weeks, colonies were fixed for 30 min with 70% ethanol (Sigma-Aldrich; Merck KGaA) at room temperature and subsequently stained for 10 min at room temperature with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA). RTCA was also used to evaluate colony growth in order to obtain quantitative data. Here, 100 cells/well were seeded in triplicate on a 96-well plate and colony growth was monitored using RTCA for 2 weeks.

Apoptosis assay and cell-mediated cytotoxicity test. GC-mediated toxicity in the HT-29 cancer cell line was assessed using propidium iodide (PI) staining and subsequent flow cytometry with a BD LSRII (BD Biosciences, Franklin Lakes, NJ, USA). Apoptosis was measured using an FITC Annexin V Apoptosis Detection kit I (Annexin V-fluorescein isothiocyanate, BD Biosciences) following the manufacturer's protocol. The results were analyzed using FlowJo version 7.2.2 software (FlowJo LLC, Ashland, OR, USA).

Cell-mediated cytotoxicity was performed using a standard chromium release assay, as previously described (2). Briefly, HT-29 cells ($2x10^4$ cells/well) pretreated with or without GCs for 30 min were incubated with ^{51}Cr for 2 h and served as target cells for PBMCs (total volume 0.1 ml/well). All samples were tested in triplicate. The effector:target ratio of 16:1 was optimal for the experimental conditions. Following 4 h co-incubation of target and PBMC cells, ^{51}Cr release was measured in cell-free supernatants (obtained as supernatants following centrifugation of cells at 2,000 x g for 5 min at room temperature) using a Wallac Microbeta Trilux scintillation counter (PerkinElmer, Inc., Waltham, MA, USA). The percentage specific lysis was calculated using the following formula: [Experimental counts per minute (cpm) - spontaneous cpm]/(maximum cpm - spontaneous cpm) x 100, where maximum cpm was determined by addition of 10% Triton X-100.

Statistical analysis. Statistically significant differences in the parameters tested in HT-29 cells cultured in the presence or absence of GCs were assessed using a one-way analysis of variance followed by Dunnett's post hoc test and a confidence interval of 95%. Statistical analysis was conducted using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

GC stimulation modulates the mRNA expression of glucosaminyltransferases, components of glucose metabolism and adhesion molecules. Previous research has noted alterations in the phenotype of cancer cells following treatment with GCs. Therefore, the present study investigated a wide range of candidate molecules and the results revealed that the interaction of cancer cells with GCs induced complex alterations in gene expression.

Candidate molecules were selected based on the novel results of a glyco-gene array investigating GN8P, as well

Table I. List of GN8P-responsive genes in the HT-29 cell line.

Gene	Entrez gene ID	Biological function	Disease association	(Refs.)
Upregulated genes				
Mpl	4352	GFR, ST, CP	CAMT, TR, PV, MD	(33-35)
Flt3	2322	Ig, TK, ST, CP	C-L (AML, ALL)	(36,37)
Bmp7	655	GF, TGF	RD, OS, PA	(38-40)
Ptprt	11122	ST, PP	CoCa, glioma	(41,42)
Ppbp	5473	GF, GTTA, CP, I	IN, ET, TP	(43-45)
Cxcl2	2920	I, CP	C, N, S	(46,47)
Gpc3	2719	CP	HepCa, WT	(48,49)
Downregulated genes				
Siglec12	89858	CB, adheze	-	(50)
Nrg1	3084	GF, TF	C, SC	(51)
Ccl7	6354	I, ST	IN, AS	(52)
Lgals13	29124	CB, GAL, LPL	Pre-eclampsia	(53)
Csf2ra	1438	ST, CP	C, N	(54)
Wnt9B	7484	ST	C	(55)
Clec4C	170482	CB, I	-	(56)
Wnt2B	7482	ST	C	(57)
Mrc1	4360	CB, L, RI	TBC, ALL	(58,59)
Cxccl9	4283	ST, I	N, IN	(60)
Sgsh	6448	hydrolase, GB	MPS	(61)
Clec3B	7123	CB, L, PAP	C	(62)
Gdf11	10220	TGF, GF	ALL	(63)
Emr2	30817	ST	CoCa	(64)
Hs3st5	222537	GB	CoCa	(65)

CAMT, congenital amegakaryocytic thrombocytopenia; TR, thrombocytosis; PV, polycythemia vera; MPS, mucopolysaccharidosis; C-L, leukemia; MD, myeloproliferative disorders; OS, osteosarcoma; PA, pseudarthrosis; RD, renal disease; CoCa, colon cancer; HepCa, hepatocellular carcinoma; WT, Wilm's tumor; KS, Kaposhi sarcoma; SC, schizophrenia; IN, inflammation; ET, essential hypertension; TP, thrombocytopenia; C, cancer; N, necrosis; S, sepsis; DA, dermatitis atopic; AS, asthma; GFR, growth factor receptor; TK, tyrosin kinase; CP, cell proliferation; ST, signal transduction; GTTA, glucose transmembrane transporter activity; I, immune processes; CB, carbohydrate binding; T, transferase; GB, glycan biosynthesis; SFT, sulfotransferase; Ig, immunoglobulin protein domain; L, lectin pr dom; RI, ricin B lectin pro dom; GAL, galectin pr dom; PAP, pancreatis associated protein; TGF, transforming growth factor β receptor binding; PP, protein tyrosine phosphatase activity; UDP, glucuronosyl/UDP glucosyl transferase; LPL, lysophospholipase activity.

as previously published results of glyco-gene profiling of GN4C in NK-92 cancer cells (2). Regarding the glyco-gene array performed on cell samples incubated for 24 h in the presence or absence of GN8P, the present study excluded all genes in which the percentage of absent data (all data where two out of three parallels were missing) >50% and where P>0.05. Subsequently, class comparison identified 22 genes that exhibited significantly different expression between control and GN8P-treated HT-29 cells (P<0.05). The responsive genes were functionally categorized according to Gene Ontology classification and the Gene Card database. A total of 64% of the genes listed were linked to cancer and 14% of the genes were linked to inflammation. Differentially expressed genes were primarily involved in signal transduction (28%), carbohydrate binding (23%), proliferation (14%) and immune processes (14%). A complete list of the differentially expressed genes, including their function and disease association, is presented in Table I. Along with other genes, GN8P mediated the downregulation of *Wnt* signaling molecules that serve an important role in cancer progression; thus inhibition of their expression is of particular interest. Upregulated genes included protein tyrosine phosphatase *Ptprt* and tyrosine kinase *Flt3* (Table I). Candidate genes were validated by RT-qPCR (data not shown).

For a detailed examination of the effect of GCs on the glycosylation mechanism in HT-29 cells, the expression of glucosaminyltransferases involved in terminal glycan elongation and genes involved in glucose uptake and cell adhesion, were measured. When compared with the NTC group, treatment with the GN4C and GN8P significantly downregulated expression levels of *Mgat3* (P=0.0002 and P=0.0001, respectively) and *Mgat5* (each, P=0.0001) glucosaminyltransferases. FLU also inhibited the expression of *Mgat5* (P=0.0001) compared with the NTC group. The *Nme1* gene encodes a transcription regulator controlling expression of the *Mgat5* gene (9). Compared with the NTC group, the mRNA expression of this transcription factor was significantly downregulated by GN8P and FLU (P=0.0009 and P=0.002, respectively; Fig. 1A).

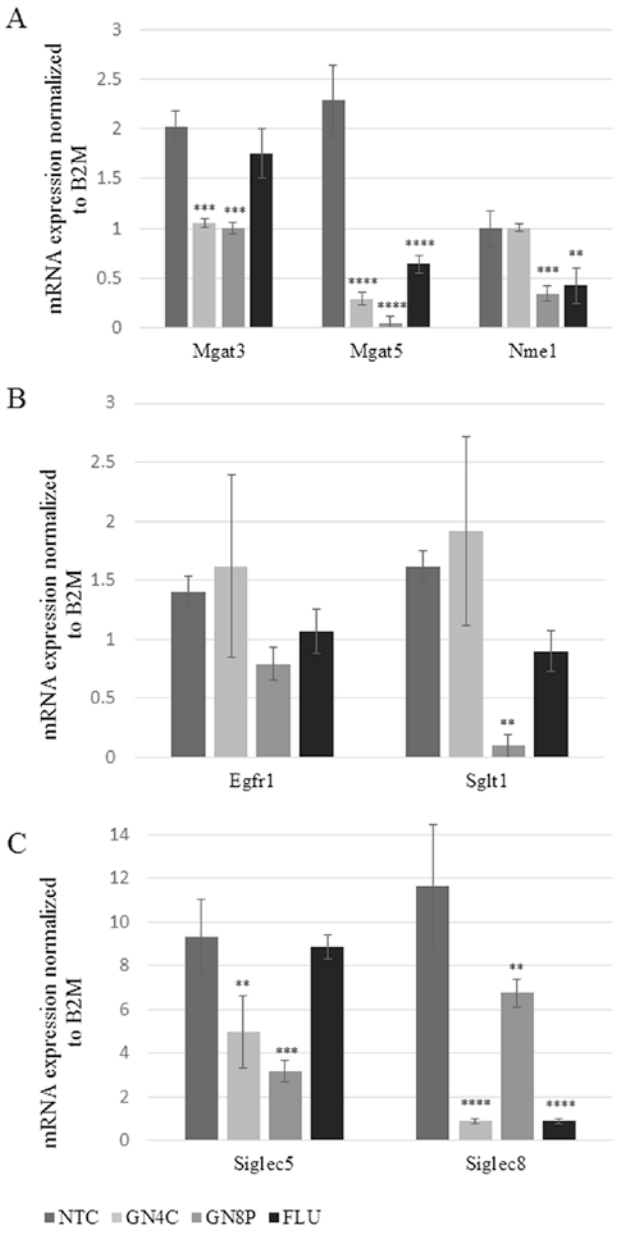

Figure 1. Synthetic glycoconjugates modulate the mRNA expression of glycosylation regulators in colon cancer cells. Graphs present the mRNA expression (fold change) of (A) the glucosaminyltransferases *Mgat3*, *Mgat5* and the transcription factor *Nme1*, (B) the glucose cotransporter components *Egfr1* and *Sglt1* and (C) expression of the control *B2M*. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. **P<0.01, ***P<0.001 and ****P<0.0001 vs. NTC group. NTC, non-treated control; GN4C and GN8P, cells treated with GN4C or GN8P, respectively; FLU, Fludara positive control treated cells; GN4C, *N*-acetyl-D-glucosamine-coated calix[4]arene; GN8P, *N*-acetyl-D-glucosamine-coated polyamidoamine dendrimer.

Sglt1 and *Egfr1* are components of a glucose cotransporter in human cells. The mRNA expression of these genes was significantly reduced by GN8P (P=0.0054 for SGLT1). However, the reduction of EGFR1 expression following exposure to GN8P was of borderline significance (P=0.2) (Fig. 1B). Furthermore, the mRNA expression of the glycan binding adhesion molecules *Siglec5* and *Siglec8* were downregulated by GN4C (P=0.0078 and P=0.0001, respectively) as well as by GN8P (P=0.0009 and P=0.0081, respectively; all Fig. 1C). However, FLU only inhibited the mRNA expression of *Siglec8* (P=0.0001; Fig. 1C).

GCs inhibit HT-29 cell proliferation, induce apoptosis and promote PBMC-mediated cytotoxicity. The present study questioned whether GCs alter the proliferation of cancer cells. To test this hypothesis, real-time monitoring of cell proliferation was performed for 5 days. A proliferation protocol was optimized using an RTCA system allowing the continuous monitoring of treated and untreated cells for a set period of time. The results indicated that the proliferation of HT-29 cells treated with GN4C, GN8P or FLU was significantly inhibited from ~5 h following induction. The greatest inhibition of cell growth was detected 60 h following treatment in the cells treated with FLU, GN8P and GN4C (P=0.0001 for all treatments vs. NTC group; Fig. 2A). A significant reduction in the expression of the proliferation antigen *Mki67* 48 h following treatment with GN4C (P=0.0017), GN8P (P=0.0005) and FLU (P=0.0003; Fig. 2B), was observed, compared with the NTC group.

To determine the toxicity of GCs in the HT-29 cell line, Annexin V positivity (specific marker of apoptosis) and the incorporation of PI into cells were measured. The percentage of early apoptotic, Annexin V-positive and PI-negative cells was significantly increased following treatment with GN4C, GN8P and FLU (P=0.0001, P=0.002 and P=0.0001, respectively), compared with the NTC group. Treatment with FLU induced a significant decrease in the population of cells in the late apoptotic and necrotic phases compared with the NTC group (all P=0.0001; Fig. 2C) However, treatment with GCs had no effect on the proportion of cells in the late apoptotic and necrotic stages of apoptosis (Fig. 2C). The proportion of untreated HT-29 cells that underwent spontaneous necrosis (PI positive, Annexin V-negative population) was 5.8%; comparable with the percentage of necrotic cells found in GN4C and GN8P treated cells (Fig. 2C). These results indicate that GCs preferably induce apoptosis over necrosis.

Surface glycosylation is a tool facilitating the recognition of cancer cells by immune cells. The current study questioned whether alterations in cancer cell glycosylation sensitizes them to PBMC-mediated toxicity. HT-29 cells, acting as target cells, which were naturally resistant to human PBMC-mediated cytotoxicity, were pretreated with GCs and subsequently exposed to PBMCs from healthy donors, acting as effector cells. Significantly higher cytolytic activity was observed in HT-29 cells pretreated with GN4C and GN8P (P=0.0002 and P=0.0001, respectively; Fig. 2D). The rate of spontaneous toxicity in untreated HT-29 cells against human PBMCs was ~1.2% (Fig. 2D).

Synthetic GCs reduce cancer cell colony formation. As cell invasion is associated with extracellular matrix remodeling and neoangiogenesis, levels of regulatory components, including *Mmp3*, *Tgfb1*, *Wnt2B* and *Wnt9B*, were measured. There was a significant decrease in the expression of *Mmp3* mRNA following treatment with GN4C and FLU (P=0.0005 and P=0.0006, respectively; Fig. 3A). GN4C inhibited the expression of *Tgfb1* (P=0.0016; Fig. 3A). GN4C and GN8P decreased the expression of *Wnt2B* (P=0.0002 and P=0.0066, respectively) and *Wnt9B* (P=0.0004 and P=0.0101,

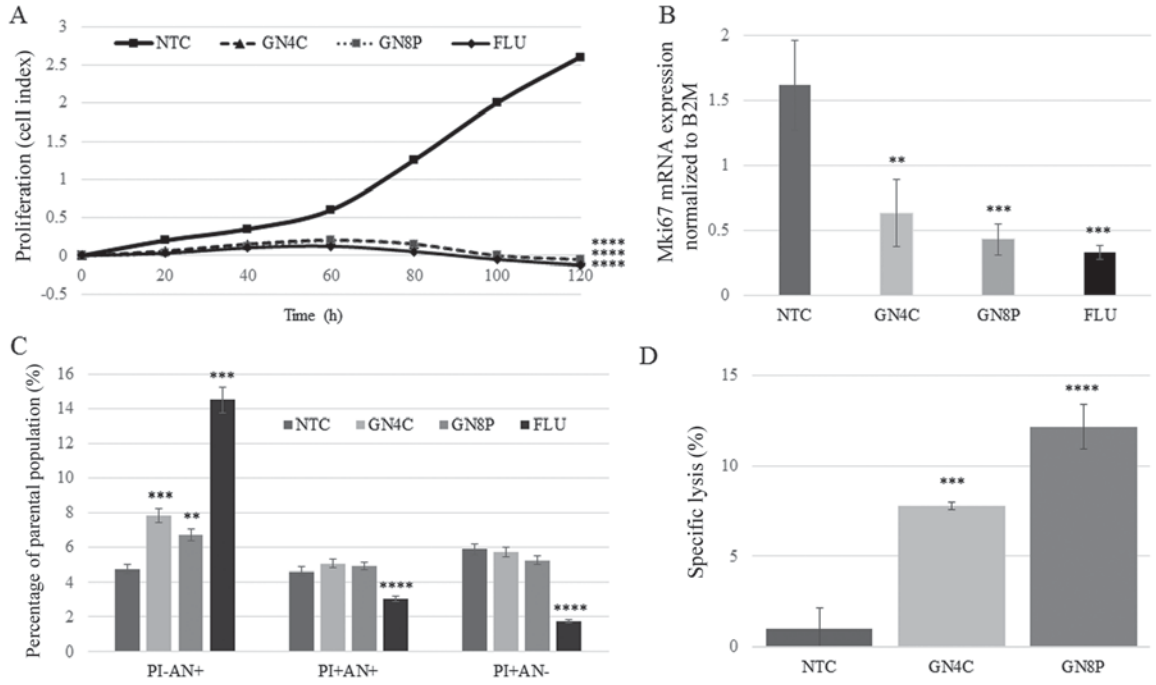

Figure 2. Effect of synthetic glycoconjugates on cell proliferation, apoptosis and sensitivity to cell-mediated killing. (A) Proliferation curve of cancer cells measured using a Real-Time Cell analyzer. The cell index reflects the number of live, attached cells. Average values of triplicates are plotted. (B) mRNA expression (fold change) of the proliferative antigen *Mki67*. (C) Detection of apoptotic and necrotic cells by flow cytometric analysis. The early apoptotic population was represented by cells that were PI- and AN+; the late apoptotic population was represented by cells that were PI+ and AN+; the necrotic population was represented by cells that were PI+ and AN-. (D) The cell mediated cytotoxicity assay employed PBMCs. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. **P<0.01, ***P<0.001 and ****P<0.0001 vs. NTC control group. PBMCs, peripheral blood mononuclear cells; NTC, glycoconjugate-non-treated control with basic toxicity of PBMC to HT-29 cancer cells; GN4C and GN8P, cells treated with GN4C or GN8P, respectively; FLU, cells treated with Fludara that represented the positive control; GN4C, *N*-acetyl-D-glucosamine-coated calix[4]arene; GN8P, *N*-acetyl-D-glucosamine-coated polyamidoamine dendrimer; PI, propidium iodide; AN, Annexin V; +, positive; -, negative.

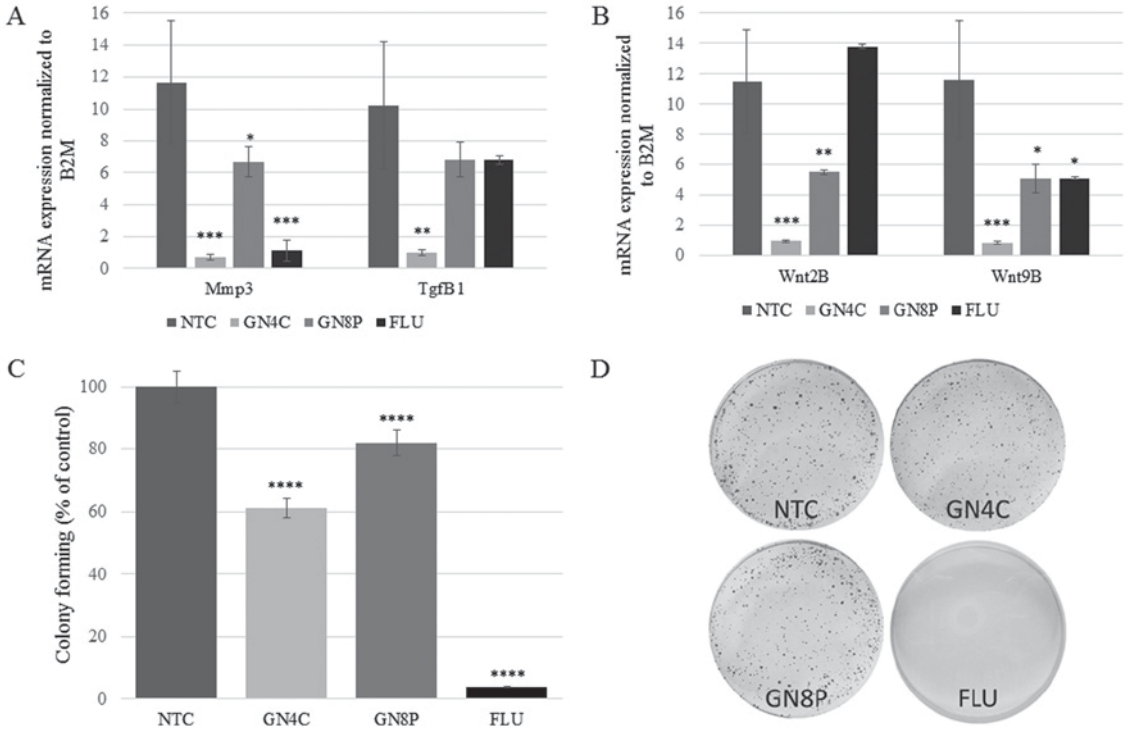

Figure 3. Colony forming inhibition triggered by glycoconjugates associated with altered expression of cell invasion regulators. Fold change in mRNA expression of the cell invasion and migration regulators (A) *Mmp3* and *Tgfb1* and (B) *Wnt2B* and *Wnt9B*. (C) Inhibition of colony formation measured in real-time. The proportion of inhibition compared with the non-treated control cells is presented. (D) Conventional colony formation assay. Representative images are presented. Data are presented as the mean ± standard deviation. Experiments were performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. NTC group. NTC, non-treated control; GN4C and GN8P, cells treated with GN4C or GN8P, respectively; FLU, positive control cells treated with Fludara; GN4C, *N*-acetyl-D-glucosamine-coated calix[4]arene; GN8P, *N*-acetyl-D-glucosamine-coated polyamidoamine.

respectively; Fig. 3B). FLU significantly decreased the expression of *Wnt9B* (P=0.01; Fig. 3B) but not *Wnt2B*. Treatment of HT-29 cells with GN4C and GN8P led to decreases in cell colony formation by 39 and 18%, respectively (P=0.0001 vs. NTC). FLU treated (positive control) cells exhibited a decrease in cell colony formation of 96% compared with the NTC group (P=0.0001; Fig. 3C). These changes in colony formation were confirmed following standard crystal-violet staining. There was a reduction in colonies in samples incubated with GCs and FLU (Fig. 3D).

Discussion

Previous studies have focused on the identification of genes involved in the response of NK cells isolated from PBMC and the permanent NK-92 cell line to the synthetic GC GN4C (2), or on the role of GN8P in the activation of immune cells (24).

Phenotypic alterations in cancer cells following exposure to GCs has been observed. Calix[6]arenes exhibit an anticancer effect by modulating AXL and Mer tyrosine kinase receptor gene expression (66); therefore, the present study questioned whether the phenotypic changes in cancer cells were modulated by changes in gene expression. The current study specifically focused on the expression of cancer development-related genes and how gene expression is affected by two GlcNAc-modified GCs that contain different cores: First generation PAMAM or calix[4]arene.

Previous studies have reported that exogenous glucosamine, the terminal moiety of the tested GCs, may be internalized via a glucose transporter (17,18). In HT-29 cells, clathrin-mediated endocytosis has been described as a mechanism of uptake of third generation PAMAM (67). Cancer cells, such as HT-29, exhibit a high glucose intake due to high-energy requirements (22). The sodium-glucose cotransporter (SGLT1) transports glucose into cells independent of its concentration (68). SGLT1 is stabilized by interaction with EGFR1, facilitating cancer cell survival (27). In colon cancer, the high expression of *Sglt1* and *Egfr1* is associated with poor patient prognosis (69). The results of the present study demonstrated that the PAMAM-based GC significantly decreased the expression of the two genes *Sglt1* and *Egfr1* that code for the glucose cotransporter complex. This, in turn, inhibited cell growth and reduced levels of the proliferation antigen *Mki67*.

It has been reported that higher generation dendrimers (G2, G4 and G6) promote cell growth at lower concentrations but induce cell death at higher concentrations. The critical concentration at which dendrimers induced cell death was 500 nM. Also, toxicity was enhanced by higher generation dendrimers (70). The current study observed that a first generation PAMAM dendrimer with eight GlcNAc moieties induced alterations in gene expression and altered the properties of cancer cells even at low concentrations (10 nM). Comparing the responses of cells to GCs and FLU identified the different underlying mechanisms of action of each compound. FLU is a potent inducer of apoptosis and affected cells undergo rapid disintegration, thus explaining the decrease in the number of cells in the late apoptotic and necrotic phases following treatment with FLU.

The growth of cancer cells was markedly affected by the GC GN8P and changes in cell adhesion and invasion were associated with the altered expression of specific mRNAs. The Siglec family contains proteins that serve an important role in cancer cell adhesion and invasion. Human cell-surface-receptors, including sialic acid-binding Ig-like lectin (SIGLEC) 5 and SIGLEC8, are members of the cluster of differentiation 33-related Siglec subfamily, expressed predominantly by immune cells (71) and they are overexpressed in acute myeloid leukemia, chronic eosinophilic and myelogenous leukemias (72,73). In solid tumors, Siglecs are overexpressed in tumor-associated immune cells, such as macrophages; however there is little evidence regarding their overexpression in actual cancer cells (74). The alteration in Siglec mRNA expression observed in the present study following the incubation of tumor cells with GC may be due to alterations in *Mgat5* expression.

In colon cancer, glycosylation performed by MGAT5 may regulate colon cancer stem cells and tumor progression via Wnt signaling (75). As GCs downregulate the expression of *Mgat5* and exhibit reduced colony formation, the present study measured the expression of the Wnt family members *Wnt2B* and *Wnt9B*, the metalloproteinase *Mmp3,* which is responsible for extracellular matrix remodeling (76) and *Tgfb1,* which is involved in tumor neoangiogenesis (77). These genes were selected based on the preliminary results of cDNA profiling in GN8P-treated cancer cells (unpublished results). GCs reduced the expression of *Wnt2B* and *Wnt9B*; however, only GN4C inhibited the expression of *Mmp3* and *Tgfb1*. This may be due to the fact GN4C is more effective at inhibiting cell invasion; indeed, it has been demonstrated that calix[4]arene inhibits cancer angiogenesis (78).

The present study identified a specific pattern in gene expression, common to the two GCs, which included downregulation of i) the glucosaminyltransferases *Mgat3* and *Mgat5*, ii) the adhesion molecules *Siglec5* and *Siglec8*, iii) *Wnt2B* and *Wnt9B* and iv) the proliferation marker *Mki67*. The two GCs increased the proportion of cells in the early apoptotic phase and the sensitivity of cancer cells to PBMCs. The current study focused the effect of GCs in cancer cells and revealed associations that may allow for the investigation of individual components in a different perspective or focus on specific signaling pathways induced by the GCs. It has been demonstrated that glycosylation in NK cells involves the phosphoinositide 3-kinase signaling pathway (2). Based on information from a recently published report (79), alterations regarding the sensitivity to PBMCs may be due to the GlcNAc section of the GCs. In the case of the downregulation of the *Sglt1/Egfr1*, the PAMAM core may induce this change since GN8P significantly lowered expression of the Sglt1 component but GN4C demonstrated no such effect. In myotubes, Wnt signaling affects glucose transport via a glucose transporter (80). In the present study, the same members of the Wnt pathway (*Wnt2B* and *Wnt9B*) were downregulated by GN4C. No differences in *Sglt1/Egfr1* expression following treatment with GN4C were detected. This suggests that these factors are not involved in colon cancer cell signaling, however, further analysis may reveal if there is a similar association between the SGLT1/EGFR1 cotransporter and other members of Wnt pathway.

It is likely that other members of the Wnt signaling pathway are involved and responsible for this distinctive

response to particular cores. The present study demonstrated that Wnt signaling may be involved in the response of cancer cells to synthetic GCs, possibly via modulation of glucosaminyltransferases. Subsequent detailed studies focusing on the Wnt pathway may identify response-specific members of the Wnt family.

The evolutionarily conserved Wnt signaling mechanism is an important pathway and Wnt proteins undergo post-translational glycosylation. Therefore, anticancer therapies that target Wnt-signaling members, based on glycosylation modulation, may be developed as a novel therapeutic strategy.

In conclusion, the present study demonstrated that the interaction of colon cancer cells with specifically designed GCs results in a complex commitment of different cellular pathways and induces alterations in the phenotypes of cells. The results of the current study revealed that alterations in the expression of particular genes following treatment with GCs are associated with specific outcomes in cancer cells, including their higher sensitivity to immune cell-mediated killing. The GCs used in the current study exhibited multiple effects following their application to cancer cells. These results, together with those of previous studies determining the immunostimulatory effects of GCs, support the importance of glycosylation-targeted anticancer therapy and provides a basis for further studies.

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