The International Journal of Biochemistry & Cell Biology 43 (2011) 586–593

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

5-Aza-2′-deoxycytidine increases sialyl Lewis X on MUC1 by stimulating β-galactoside:α2,3-sialyltransferase 6 gene

Vishwanath B. Chachadi[a], Helen Cheng[a], David Klinkebiel[a], Judith K. Christman[a,b], Pi-Wan Cheng[a,b,*]

[a] Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5870 United States
[b] Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5870 United States

ARTICLE INFO

Article history:
Received 9 September 2010
Received in revised form 9 December 2010
Accepted 10 December 2010
Available online 17 December 2010

Keywords:
Mucin biosynthesis
sLe^x
Cancer metastasis
5-Aza-2′-deoxycytidine
Epigenetic regulation of glycosyltransferase gene expression

ABSTRACT

Sialyl Lewis X is a tumor-associated antigen frequently found in the advanced cancers. However, the mechanism for the production of this cancer antigen is not entirely clear. The objective of this study is to examine whether epigenetics is involved in the regulation of the formation of this antigen. We observed an increase of sialyl Lewis X in HCT15 cells, a colon cancer cell line, treated with 5-Aza-2′-deoxycytidine. This treatment enhanced the expression of β-galactoside:α2,3-sialyltransferase 6 gene and sialyl Lewis X on MUC1, and the adherence of these cells to E-selectin under dynamic flow conditions. In addition, 5-Aza-2′-deoxycytidine treatment inhibited methylation of β-galactoside:α2,3-sialyltransferase 6 gene and siRNA knockdown of this gene drastically reduced sialyl Lewis X without affecting MUC1 expression. We conclude that 5-Aza-2′-deoxycytidine treatment increases sialyl Lewis X on MUC1 by stimulating the β-galactoside:α2,3-sialyltransferase 6 gene via inhibition of DNA methylation. Increased sialyl Lewis X by 5-Aza-2′-deoxycytidine raises a concern about the safety of this chemotherapeutic drug. In addition, β-galactoside:α2,3-sialyltransferase 6 gene may be a potential therapeutic target for suppressing tumorigenicity of colon cancer.

1. Introduction

Alterations of mucin glycans have been frequently observed in cancer. Some of these glycans have been identified as tumor-associated antigens and used as markers for diagnosis and prognosis of cancer (Dube and Bertozzi, 2005) or targets for therapy (Taylor-Papadimitriou and Epenetos, 1994). Among these tumor-associated antigens, sialyl Lewis X (sLe^x) receives the most attention because it is associated with poor prognosis. For example, colon carcinoma cells with a high potential for liver metastasis express more abundant amounts of sLe^x than counterparts with low metastatic potential (Amado et al., 1998; Magnani et al., 1982; Matsushita et al., 1990; Nakamori et al., 1993). Increased expression of sLe^x correlated with strong adherence of cancer cells to E-selectin on vascular endothelial cells (Bresalier et al., 1996; Izumi et al., 1995; Saitoh et al., 1992; Sawada et al., 1994). SLe^x is also a key component of the carbohydrate ligands for P and L-selectins (Kim et al., 1999). These results indicate that sLe^x plays an important role in hematogenous cancer metastasis.

SLe^x is a tetrasaccharide located at the non-reducing terminus of a glycan chain. It contains a Galβ1-4GlcNAc backbone with the Gal decorated with α2-3NeuAc and the GlcNAc with α1-3Fuc. The synthesis of sLe^x is initiated by adding α2-3NeuAc to the Gal of *N*-acetyllactosamine as catalyzed by βgal:α2,3-sialyltransferases (ST3Gal) (Carvalho et al., 2010). Then, α1-3Fuc is added to the GlcNAc as catalyzed by α1,3/4-fucosyltransferases (de Vries et al., 1995; Holmes et al., 1986). There are at least four *ST3Gals*, including *ST3Gal3*, *ST3Gal4*, *ST3Gal5*, and *ST3Gal6*, that can produce α2→3 sialyl *N*-acetyllactosamine on Galβ1-4GlcNAc *in vitro* (Carvalho et al., 2010; Kitagawa and Paulson, 1993; Okajima et al., 1999; Sasaki et al., 1993). However, the true target of each of these ST3Gals *in situ* is not known. Similarly, there are at least five α1,3/4-fucosyltransferases which are capable of synthesizing sLe^x (de Vries et al., 1995). The intracellular glycoprotein targets for these enzymes also remain elusive.

In an effort to elucidate the potential epigenetic regulation of glycosyltransferase genes, we treated colon carcinoma HCT15 cells with 5-Aza-2′-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor (Palii et al., 2008), and then monitored subsequent changes in the expression of glycogenes involved in mucin glyco-

Abbreviations: 5-Aza-dC, 5-Aza-2′-deoxycytidine; CHO, Chinese hamster ovary; ST3Gal, β-galactoside:α2,3-sialyltransferase; FUT, fucosyltransferase; Gal, galactose; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; PNGase F, peptide-*N*4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase F; sLe^x, sialyl Lewis X; ST, sialyltransferase; r.t., room temperature; MAA, Maackia amurensis lectin.

* Corresponding author at: Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, United States. Tel.: +1 402 559 5776; fax: +1 402 559 6650.
E-mail address: pcheng@unmc.edu (P.-W. Cheng).

1357-2725/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2010.12.015

sylation, particularly the synthesis of sLe^x. We found that 5-Aza-dC treatment induced the production of sLe^x on MUC1 and enhanced the binding of HCT15 cells to E-selectin under dynamic flow conditions through induction of *ST3Gal6* gene by inhibiting methylation of this gene. The results establish that ST3Gal6 is responsible for the synthesis of sLe^x on MUC1 and this contributes to the adhesive property of HCT15 cells. Because expression of sLe^x correlates with cancer metastatic potential, these results raise a concern about the safety of 5-Aza-dC for cancer treatment.

2. Methods

2.1. Cell line and cell culture

Colon carcinoma HCT15 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). These cells were maintained at 37 °C under a 5% CO_2 and water saturated environment in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml Penicillin and 100 μg/ml Streptomycin.

2.2. 5-Aza-2′-deoxycytidine treatment

A stock solution (1 mg/ml) of 5-Aza-dC (Sigma Chemical Co., St. Louis, MO) was prepared fresh in sterilized PBS within 1 h of the treatment. Final 5-Aza-dC concentrations (0.1–4 μM) were prepared by adding an appropriate amount of the stock solution directly to the culture medium. Drug and PBS (control) treatments of the cells were initiated at 20% confluence and then continued daily for 5 days (d).

2.3. Western blotting

Aliquots of the cell lysates from vehicle and 5-Aza-dC treated cells were boiled (5 min) and then run on 6% SDS-PAGE (7.5 cm × 8.5 cm) under reducing conditions. After electrotransfer, the PVDF membrane (Immobilon-P, 0.2 μ., Millipore, Bedford, MA) was blocked with TBS containing 0.05% Tween 20 and 5% (w/v) nonfat dried milk for 60 min and then exposed to anti-sLe^x (CSLEX1; BD Biosciences), anti-MUC1 (VU-4H5; Life Span Biosciences), anti-MUC4 (8G-7, Santa Cruz Biotechnology, Inc. and 6A134; Abcam, Cambridge, MA), anti-MUC6 (CLH5; Vector Laboratories) or anti-MUC16 (X75; Abcam, Cambridge, MA) antibodies overnight at 4 °C in same buffer at 1:500 for anti-sLe^x, and 1:1000 dilutions for other antibodies. After five washings in same buffer, the membrane was treated for 1 h at room temperature (r.t.) with HRP-conjugated donkey anti-mouse IgM (Jackson Laboratories, West Grove, PA) for anti-sLe^x and donkey anti-mouse IgG (Jackson Laboratories, West Grove, PA) for other antibodies. The membrane was then washed five times (5 min each) with TBS containing 0.05% Tween 20 and once with milli-Q water. Then, the blot was developed with ECL-sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden). β-Actin probed with mAb (Santa Cruz, Inc.) was used for normalizing the protein loadings.

2.4. Immunoprecipitation

To isolate glycoproteins for characterization of conjugated glycans, cells were lysed in lysis buffer and precleared either in protein L-agarose or in protein G-agarose (Pierce). The precleared cell lysate was incubated with anti-sLe^x (or anti-MUC1) mAb (5 μg antibody per 100 μg protein) for 12 h at 4 °C. The immunocomplexes isolated with protein L-agarose (sLe^x), and protein G-agarose (MUC1) for 1 h at 4 °C, were analyzed for sLe^x, MUC1, or MAA ligand by western blotting. Immunoprecipitates with nonspecific mouse IgM and IgG antibodies were served as negative control. Protein concentration was measured by Coomassie blue dye (Pierce).

2.5. Peptide-N-glycosidase (PNGase) F digestion

The anti-sLe^x immunoprecipitate was boiled (100 °C) for 10 min in 0.5% SDS and 1% 2-mercaptoethanol and then treated with PNGase F (5 U/mg protein in 50 mM phosphate, pH 7.5 containing 1% NP-40) (37 °C, 16 h). The sample was analyzed by anti-sLe^x western blotting.

2.6. Lectin blotting

For lectin blotting, the PVDF membrane prepared above was blocked with TBS containing 3% BSA (60 min), followed by incubation with 2 μg/ml of biotin-conjugated MAA (in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.5) for 60 min. The membrane was washed (5×) with TBS containing 0.05% Tween 20 and then incubated with streptavidin-HRP (Pierce) (1:20,000) for 30 min. Finally, the blot was washed (5×) with TBS containing 0.05% Tween 20 and then developed using ECL method as described above.

2.7. Quantitative real-time PCR analysis of glycogene expression

Cells cultured in a T-25 flask were lysed with 1 ml TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) followed by the addition of bromo-3-chloropropane (0.1 ml) after transferred to a 1.5 ml Eppendorf tube. After shaken vigorously for 15 s, the sample was incubated at r.t. for 15 min, and then centrifuged (12,000 rpm) for 15 min at 4 °C. The upper, colorless phase was transferred to a clean Eppendorf tube, which was followed by mixing with 0.5 ml isopropanol, incubated at r.t. for 10 min, and then centrifuged (12,000 rpm) at 4 °C for 8 min. The pelleted RNA was rinsed with 1 ml of cold 75% ethanol in DEPC-treated water and then dissolved in 30–50 μl of DEPC-treated water at r.t. for about 10 min. One half of the total RNA was submitted to the Consortium for Functional Glycomics for GLYCOv4 DNA microarray analysis (see Supplementary materials for detailed protocol) and the other half was utilized for real-time PCR analysis. To prepare cDNA, 2 μg RNA was used in a 20 μl reaction mixture using a Verso reverse transcriptase kit (Thermo scientific) as follows: 5 min at r.t., 60 min at 42 °C, and 2 min at 95 °C. Quantitative real-time PCR was performed in 10 μl reaction volume in a 96-well plate using 2 μl of diluted cDNA with SYBR® *Premix ExTaq*™ (TAKARA BIO INC.) on a Mastercycler Epgradient realplex (Eppendorf AG, Hamburg, Germany). The PCR conditions included 1 cycle at 95 °C for 2 min followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The data were analyzed using Eppendorf realplex software, version 1.5 (Eppendorf). The amounts of various glycogene transcripts were normalized to the amount of *GAPDH* transcript in same cDNA sample. Relative fold differences in transcript expression were determined using the following comparative CT method: $2^{-[\Delta C_t(5\text{-Aza})-\Delta C_t(\text{control})]} = 2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t(Target) - C_t(GAPDH)$ as described previously (Tassone et al., 2000). The results were expressed as the amount (%) relative to that (100%) of *GAPDH* and plotted as mean fold changes ± SEM. Primer sequences used for expression analysis of all genes including *GAPDH* are summarized in Table S1 in Supplementary materials.

2.8. Transient transfection of HCT15 cells with ST3Gal6 gene-specific siRNAs

Four different siRNAs targeting *ST3Gal6* RNA along with scrambled siRNA were purchased from Dharmacon (IL, USA). After