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Epigenetic modulation of the HeLa cell membrane N-glycome ☆

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ABSTRACT

Background: Epigenetic changes play a role in all major events during tumorigenesis and changes in glycan structures are hallmarks of virtually every cancer. Also, proper N-glycosylation of membrane receptors is important in cell to cell and cell–environment communication. To study how modulation of epigenetic information can affect N-glycan expression we analyzed effects of epigenetic inhibitors on HeLa cell membrane N-glycome.
Methods: HeLa cells were treated with DNA methylation (zebularin and 5-aza-2-deoxycytidine) and histone deacetylation (trichostatin A and Na-butyrate) inhibitors. The effects on HeLa cell membrane N-glycome were analyzed by hydrophilic interaction high performance liquid chromatography (HILIC).
Results: Each of the four epigenetic inhibitors induced changes in the expression of HeLa cell membrane N-glycans that were seen either as an increase or a decrease of individual glycans in the total N-glycome. Compared to DNA methylation inhibitors, histone deacetylation inhibitors showed more moderate changes, probably due to their higher gene target selectivity.
Conclusions: The results clearly show that composition of HeLa cell membrane N-glycome can be specifically altered by epigenetic inhibitors.
General significance: Glycans on the cell membrane are essential elements of tumor cell's metastatic potential and are also an entry point for nearly all pathogenic microorganisms. Since epigenetic inhibitors used in this work are registered drugs, our results provide a new line of research in the application of these drugs as anticancer and antimicrobial agents. This article is part of a Special Issue entitled Glycoproteomics.

1. Introduction

Nearly all membrane and secreted proteins of higher eukaryotes [1], as well as numerous cytoplasmic proteins [2,3] are glycosylated. *N*-glycosylation is essential for multicellular life and its complete absence is embryonically lethal [4]. However, gene mutations resulting in modifications of glycan structures are common and lead to large individual phenotypic variations in humans and other higher organisms. The most prominent example, arising from the existence of three allelic variants of a single glycosyltransferase gene, is the ABO system in blood type classification, which is based on the presence or absence of specific glycoproteins at the cell surface. Recent studies revealed that the glycome composition of specific proteins or the total plasma glycome varies significantly between individuals [5,6]. However, living cells in an organism have a highly organized glycomic compensation system which preserves N-glycan branch complexity even when multiple genes are silenced in parallel [7].

Changes in the attached glycans significantly affect the structure and function of polypeptide parts of many glycoproteins [8]. Proper glycosylation of membrane receptors is particularly important since it modulates adaptive properties of the cell membrane and affects communication between the cell and its environment [9]. Alternative glycosylation of proteins gives the cell an opportunity to quickly react to changes in the environment and adapt the properties of its membrane [10]. A fascinating example of the role of glycans in the interaction between the cell and the environment is the modulation of intestinal glycome composition. Commensal bacteria regulate intestinal physiology, development and function [11], and are essential for the maintenance of immune homeostasis in the gut [12]. An active dialog between commensal microflora and the host mucosal glycans apparently affects immunological tolerance and homeostasis within the gut and can explain some of the differential host responses to commensal and pathogenic bacteria [13]. The mechanisms behind this phenomenon are not known, but apparently some signals from the cell membrane that is exposed to specific non-pathogenic commensal bacteria affect the glycosylation machinery and instruct it to produce glycans which promote successful symbiosis with intestinal commensal bacteria [14].

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The expression of eukaryotic genes is largely regulated by chromatin structure [15]. Methylation of DNA and post-translational covalent modifications of histone tails such as acetylation, methylation and phosphorylation are all involved in the establishment of chromatin conformation that will determine gene transcriptional status (activity *vs*. silencing). One of the proposed mechanisms establishing the cross-talk between DNA methylation and various histone modifications is mediated by methylation-driven DNA-binding proteins, which can in turn recruit enzymatic protein complexes (including histone acetylases and histone deacetylases) responsible for setting up the histone code of a genomic region [16,17]. It is through dynamics of this cross-talk that changes in environmental conditions can induce alteration of chromatin conformation and consequently accessibility of promoter sequences to transcription factor complexes. In normal cells, chromatin around promoters of actively transcribed genes is commonly hypomethylated and hyperacetylated. Aberrant DNA hypermethylation and histone deacetylation, leading to silencing of some tumor suppressor genes [18,19] and other cancer-associated genes [20], are linked to cancer initiation and progression [21]. Glyco-genes are one of the groups of cancer-associated genes since changes in glycan structures are hallmarks of many cancers (for a review see [22,23]). Cancer-specific changes in glycan biosynthetic pathways are resulting from aberrant expressions of glycosyltransferases and glycosidases [24,25].

The epigenetic changes, unlike genetic mutations, are potentially reversible and the search for drugs that would aid to re-establish proper DNA methylation and histone acetylation patterns resulting in resumption of normal gene expression levels is ongoing. So far, the best characterized and widely used inhibitors of the enzymes that establish and maintain DNA methylation patterns (DNA methyltransferases, DNMTs) are cytidine analogs including 5-azacytidine (5-azaC), 5-aza-2′-deoxycytidine (5-azadC) and zebularine [26]. The variety of compounds identified as inhibitors of histone deacetylases (HDACs) include short-chain fatty acids (such as sodium butyrate and 4-phenylbutyrate), cyclic tetrapeptides, benzamides and the hydroxamic acid class of inhibitors (trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA)), which bind the HDAC catalytic site with higher affinity [27]. The synergistic effects of DNMT and HDAC inhibitors can thus potentially restore gene function silenced by aberrant epigenetic changes in cancer cells, which makes them interesting candidates in epigenetic therapy.

The role of epigenetic mechanisms in the aberrant glycosylation process resulting in altered expression of cancer-associated carbohydrate antigens (glycans) has recently been shown [28,29]. Therefore, it appeared crucial to characterize the potential effects of DNMT and HDAC inhibitors on the entire N-glycome of a tumor cell. In the present work, we examine the potential effects of DNMTs inhibitors 5-azadC and zebularine and HDACs inhibitors TSA and Na-butyrate on the composition of membrane N-glycome of HeLa cells. Prominently changed glycan profiles following the treatment of HeLa cells with each of the four drugs indicate that epigenetic phenomena are important in transcriptional regulation of glyco-genes and other N-glycosylation related genes. Further, these tools could potentially allow the modulation of glyco-genes' epigenetic profiles in order to manipulate the glycan fingerprint of a cell, thus avoiding undesirable physiological consequences during disease.

2. Material and methods

2.1. Cell culture techniques and immobilization in polyacrylamide gel blocks

Human HeLa cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogene) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 200 mM glutamine, in a humidified chamber with 5% CO_2 at 37 °C. The cells were plated in 6 cm cell culture dishes at various concentrations and treated with different epigenetic inhibitors until grown to confluence. Cells were then detached with 1 mM EDTA and washed 3–7 times in phosphate buffered saline (PBS). For the immobilization of intact cells, the cells were resuspended in 70 μl of polyacrylamide gel (18.5% acrylamide:bisacrylamide 29.2:0.2, 375 mM Tris–HCl pH 7.5, 3 M urea, 0.1% APS, 1 μl TEMED) and left to polymerize on ice.

2.2. Treatment with epigenetic inhibitors

Zebularine, 5-aza-2-deoxycytidine (5-azadC), and sodium (NA-) butyrate (all purchased from Sigma) were dissolved in phosphate buffered saline (PBS) and Trichostatin A (TSA; Sigma) was dissolved in dimethyl sulfoxide. For treatment with 50 μM or 100 μM Zebularine, Hela cells were plated at a density of 1.8 or 3.6×10^5 cells in 6 cm dishes 24 h prior to the treatment. 48 h after the first treatment, the medium was replaced with fresh medium containing same concentrations of zebularine. Cells were collected and embedded in polyacrylamide gels, 72 h after the beginning of the treatment. As for other three epigenetic inhibitors, cells were plated at a density of 6.4×10^5 cells in 6 cm dishes 24 h prior to the treatment. We used the following concentrations of inhibitors: TSA—5 ng/ml, 40 ng/ml and 100 ng/ml; sodium butyrate—0.5 mM, 2 mM and 6 mM; 5-aza-2′-dC—0.1 μM, 1 μM and 10 μM. 24 h after the treatment with sodium butyrate cells were embedded and processed as described. 24 h after the first treatment with TSA and 5-azadC, the medium was replaced with fresh medium containing same concentrations of the inhibitor and embedded 48 h following the beginning of the treatment. Control cells were left untreated.

2.3. Glycan release and labeling

Glycans were released from the cell membrane and labeled as recently reported (Dezeljin et al., manuscript under review). Gels were transferred into wells of UNIFILTER protein precipitation (PP) fast flow (FF) plate (Whatman, 96 well plate, 2 ml, glass polypropylene). Acetonitrile (1 ml) was added to the wells and after 10 min of shaking, the liquid was vacuumed to waste. The washing procedure has been continued with 20 mM $NaHCO_3$, ACN, 20 Mm $NaHCO_3$, and finished with ACN (1 ml of solvent for each step). Unifilter PP FF plate was then placed on a clean collection 96 well plate and gels were soaked with 1 μl of PNGase F (ProZyme N-glycanase ; peptide-N-glycosidase F 2.5 U/ml) diluted in 99 μl of 20 mM $NaHCO_3$. Gels were covered with another 50 μl of 20 mM $NaHCO_3$, sealed with adhesive sealing film and left to incubate for 18 h at 37 °C.

Released N-glycans were eluted from gels by washing with 200 μl water, shaking for 10 min, and collecting the liquid to the collection plate. The procedure was repeated two more times, and continued with 200 μl of ACN, 200 μl of water, and finished with 200 μl of ACN. Released N-glycans were then dried in vacuum centrifuge and fluorescently labeled with 2-aminobenzamide as described by Royle et al. [30]. Labeled glycans were dried in vacuum centrifuge and redissolved in known volume of water for further analysis.

2.4. Hydrophilic interaction high performance liquid chromatography (HILIC)

Released glycans were subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a 250×4.6 mm i.d. 5 μm particle packed TSKgel Amide 80 column (Tosoh Bioscience, Stuttgart, Germany) at 30 °C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. 60 min runs were performed with a fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers from which the