Original Paper

Genistein Enhances or Reduces Glycosaminoglycan Quantity in a Cell Type-Specific Manner

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Key Words

Glycosaminoglycan • Isoflavones • Genistein • Monosaccharide • Disaccharide • LC-MS

Abstract

Background/Aims: Genistein is a natural isoflavone enriched in soybeans. It has beneficial effects for patients with mucopolysaccharidose type III through inhibiting glycosaminoglycan biosynthesis. However, other studies indicate that genistein does not always inhibit glycosaminoglycan biosynthesis. **Methods:** To understand the underlying molecular mechanisms, CHOK1, CHO3.1, CHO3.3, and HCT116 cells were treated with genistein and the monosaccharide compositions and quantity of all glycans from the cell lysate were measured after thorough acid hydrolysis followed by HPLC analysis. In addition, the glycosaminoglycan disaccharide compositions were obtained by stable isotope labeling coupled with LC/MS analysis. **Results:** Genistein treatment reduced the amount of glycans but increased the amount of glycosaminoglycans in HCT116 cells. In contrast, genistein treatment reduced both glycan and glycosaminoglycan quantities in CHOK1, CHO3.1, and CHO3.3 cells in addition to differential changes in glycosaminoglycan disaccharide compositions. **Conclusion:** Genistein treatment reduced overall glycan quantity but glycosaminoglycan quantities were either increased or decreased in a cell type-dependent manner.

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Introduction

Genistein (4', 5, 7-trihydroxyisoflavone or 5, 7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (Fig. 1) is the main bioactive ingredient of soy isoflavones [1]. Isoflavones extracted from plants have the similar structure with estrogen and are therefore called phytoestrogens. Soybean isoflavones have numerous biological activities and have been

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used not only as cancer-preventing agents [2] but also in prevention of hypertension, cardiovascular diseases, diabetes, and osteoporosis [3-7]

Animal cells are covered by a surface layer of glycans that is referred to as the 'glycocalyx'. The major components of the glycocalyx are glycoproteins, glycolipid, and glycosaminoglycans (GAGs). The thickness of the glycocalyx is 50 to 100 times more than that of the cell membrane lipid bilayer [8]. The

glycans are defined as all the sugar chains synthesized by an organism [9, 10] Glycosylation, the addition of glycans to proteins and lipids, can result in a complex array of glycoforms, which are estimated to be 10^4 times larger than the proteome in different species [11]. Over 70% proteins expressed in animal cells are covalently modified by O-linked and N-linked glycans where ~50 core proteins can be modified with O-linked GAGs or mucopolysaccharides [12]. GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit. Chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA) are major types of GAGs. The repeating disaccharide unit consists of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) along with a uronic sugar (glucuronic acid or iduronic acid) for HS, CS, DS and HA or galactose for KS. In vertebrate, 95% glycans are O- and N-linked glycans and 5% glycans are GAGs or polysaccharides.

The mucopolysaccharidoses (MPSs) are part of the lysosomal storage disease family, a group of more than 40 genetic disorders that result when the lysosome organelle in animal cells malfunctions [13, 14]. MPS are caused by the absence or malfunction of lysosomal enzymes needed to break down HS, CS, DS, KS, or HA GAGs [15] that leads to GAG accumulation in lysosomes in virtually all cells of the affected organism. It causes a progressive damage of tissues leading to severe dysfunctions of various organs, including the heart, bones, respiratory system, joints, and central nervous system (CNS), with an average expected lifespan of one or two decades [13, 14, 16-19]. Depending on the lacking or deficient enzyme associated with the accumulated GAGs, 11 types and subtypes of MPS have been recognized [13, 16, 18-23]. Apart from MPS II, which is an X chromosome-linked disease, all other MPS types are inherited in an autosomal recessive manner [13, 16, 21-24]. No effective and approved treatment was available for all types of MPS until 2003 when several approaches for the cure of MPS diseases have been proposed. The methods rely on direct (enzyme replacement therapy - ERT) or indirect (hematopoietic stem cells' transplantation - HSCT, gene therapy - GT) delivery of the appropriate lysosomal enzyme into the patients. However, each of them has limitations.

During the past decade, both clinical and preclinical studies showed that genistein has beneficial effects for patients and mice with MPS type III [13, 16, 18, 24, 25] through inhibiting HS biosynthesis, the action of endoglycosidases that degrade GAGs, e.g., heparanase in combination with functional GAG-specific hydrolases [21], and by enhancing lysosomal biogenesis [14, 19]. Piotrowska et al [21]. reported that genistein inhibits synthesis of GAGs considerably in cultures of fibroblasts of MPS types I, II, IIIA and IIIB patients. Chatzinikolaou et al [26]. reported that genistein could alter the biosynthesis of GAGs in two human colon cancer cell lines, HT-29 and SW-1116. On one hand, genistein reduces biosynthesis of GAGs in fibroblasts from MPS type I, II, IIIA and IIIB patients [21]. In contrast, genistein not only increases GAG levels in MPS type I cell models [27] but also causes adverse effects in this mouse model [28]. The discrepancy raises the question how to explain the different observations at molecular level.

HS or CS GAGs are assembled in the Golgi on a common tetrasaccharide that is present in different sites of proteoglycan core proteins [12]. Both HS and CS are polymerized and modified by enzymes that are encoded by more than 40 genes. Because of the expression

Fig. 1. The chemical structure of genistein.

repertoire of the GAG assembly enzymes, each HS and CS chain has a sulfation pattern, chain length, and fine structure that is potentially unique to each cell. Cell surface GAGs turn over within 1/8 to 1/3 of a cell cycle [29]. This means their structures are able to rapidly change in response to a variety of environmental factors. Therefore, different from template-guided the biosynthesis of RNAs and proteins, GAGs are synthesized on a dynamic assembly line contributed by environment factor-regulated biosynthesis and modification enzymes produced by specific cells. Moreover, certain critical enzymes responsible for both HS and CS assembly are 2-3-fold overexpressed in certain cell lines [30]. Even genistein inhibits the expression of one or several enzymes responsible for HS and/or CS biosynthesis, it is hard to predict if genistein would enhance or reduce the amount of GAGs in a specific cell line.

By using two novel analytical methods developed in our laboratory, the quantitative changes in glycans and GAGs induced by genistein in four different cell lines were analyzed. We discovered in current study that genistein treatment reduced overall glycan quantity but GAG quantities were either increased or decreased in a cell type-dependent manner. Our results were in line with the reported beneficial effects of genistein in treating MPS type III by inhibiting HS biosynthesis and enhancing lysosomal biogenesis. It also demonstrated that genistein could stimulate GAG biosynthesis in certain cells. Mostly important, the many biological functions reported for genistein might be due to its ability in affecting the quantity of both glycans and GAGs in animal cells.

Materials and Methods

Reagent, culture conditions and treatment

Genistein was purchased from Aladdin Reagent of China. Colon cancer cell line HCT116 was obtained from Shanghai Cell Bank of Chinese Academy of Science, and CHOK1, CHO3.1, CHO3.3 cells were generous gifts of Dr. Jeffrey D. Esko (University of California, San Diego). Cells were cultured in McCoy's 5A or F12 medium supplemented with 10% FBS (Gibco, USA), 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin with 5% CO₂ and 95% air at 37°C in the incubator. All cells used were between 3 and 30 passages. Genistein was dissolve in DMSO and the final concentration of DMSO in all cell culture experiments was 0.1%.

Cell growth inhibition assay

For cell growth inhibition assay was performed as described previously [31]. In brief, HCT116, CHOK1, CHO3.1, CHO3.3 cells were seeded in 96-well plates with 2000 cells in each well. After 24 h, cells were treated with serial concentrations of the genistein (0, 6.25, 12.5, 25, 50, 100 µM for CHOK1, CHO3.1, CHO3.3 cells and 0, 15, 30, 45, 60, 75, 90 µM for HCT116) for 24 h, 48 h, 72 h. Then, each well was added 20 µL 2 mg/mL resazurin (Sigma, USA) solution. After 16 h of incubation at 37°C, the fluorescent signal was monitored by using Spectramax M5 plate reader (Molecular Devices, USA) at 544 nm excitation wavelength and 595 nm emission wavelength. The relative fluorescence unit (RFU) generated from the assay was proportional to the number of living cells in each well.

Extraction and purification of GAGs from control and genistein treated cells

Cells (10⁶) were seeded in each 15 cm diameter cell culture dish. Five dishes of control cells (0.1% DMSO treated) and 8 dishes of genistein treated cells were used for GAG preparations, 20 mL per dish in McCoy's 5A or F12 medium containing 10% FBS for 24 h. Different amount of genistein (according to the IC₅₀ values of the respective cells, 38.27 µM for CHOK1, 33.13 µM for CHO3.1, 36.13 µM for CHO3.3, 41.75 µM for HCT116) were then added to the medium of the cultured cells and incubated for 48 h at 37°C with 5% CO₂ and 95% air in the incubator. GAGs were extracted from control and genistein treated cells following a minor modified protocol [32]. NaOH (100 mM) was added to the petri dish, and the cell lysates were incubated at 4°C overnight to cleave GAG chains from core proteins, then taking 300 µL cell lysis from each group was used for monosaccharide analysis and protein quantification to calculate the GAG/protein ratio. The remaining cell lysate were adjusted the pH to 6.0 before adding protease (0.1g/sample) (Tianshi corporation, China) to digest GAG-binding proteins by incubating at 37°C overnight. Anion exchange resin (0.5 mL) was added into each tube and shook for 3 h. The beads were washed three times with 3 mL 0.25

M NaCl, 0.01% Triton X-100, 20 mM NaAc pH 6.0 to remove some impurities. The GAGs were eluted four times with 500 μ L 1.0 M NaCl in 0.01% Triton X-100, 20 mM NaAc, pH 6.0 followed by pooling the four times supernatants, and precipitated with 8 mL 100% ethanol to ensure the final ethanol concentration of 80% overnight at 4°C. The precipitated GAGs were collected by centrifugation for 15 min at 1970 $\times g$ followed by washing with 1 mL of 75% ethanol, the supernatant was aspirated. Finally, suspending GAGs in 200 μ L water was frozen in a siliconized tube for further analysis.

PMP labeling for monosaccharide analysis

One% cell lysis was used for monosaccharide analysis. The polysaccharides in cell lysis were degraded into monosaccharide under the condition of 1 mL of 2 M trifluoroacetic acid at 105°C for 6 h. Afterwards, the reaction was terminated by evaporating the residual trifluoroacetic acid under vacuum. The precipitate was dissolved in 40 μ L ddH₂O and 40 μ L 0.3 M NaOH followed by reacting with 60 μ L 0.5 M PMP (Sigma, Missouri, USA) solution at 70 °C for 60 min. Then, 4 μ L 3 M HCl was added while vortexing. Trichloromethane (500 μ L) was used to extract the extra PMP used in the labeling reaction for three times. An Agilent 1260 liquid chromatography system with an Agilent poroshell EC-C18 chromatogram column (4.6 $\times$ 100 mm) was used for HPLC analysis. Solvent A was 100% acetonitrile and solvent B was 0.1 M ammonium acetate adjusted pH to 4.5 by using acetic acid. The gradient used was 15–22% A for 10 min. The column was then washed with 24% A for 5 min and equilibrated with 15% solvent B for 5 min. The flow rate was 1 mL/min. The UV signal of the eluted PMP-labeled samples was measured at 254 nm by using an online DAD detector.

GAG depolymerization and LC/MS analysis

For HS digestions, GAGs were incubated with 0.33 mU each of heparin lyase I, II, III (Biokangtai Pharmaceuticals, China) in HS buffer (a final volume of 100 μ L 100 mM NH₄Ac pH 7.0 and 0.125 mM CaCl₂) at 30°C overnight. The next day, adding another round of the enzymes is to ensure the completion of the digestion. For CS digestions, GAGs were incubated with 1 mU chondroitinase ABC (Biokangtai Pharmaceuticals, China) in CS buffer (100 μ L of digestion solution containing 100 mM ammonium acetate pH 7.0) at 30°C overnight. Digestion was monitored by increased absorbance at 232 nm by using a Spectra MAX M2 plate-reading spectrophotometer (Molecular Devices, USA) with the temperature of the plate chamber set at 30°C.

Evaporating the enzyme-digested samples, the samples were dissolved in 30 μ L ddH₂O followed by 5 μ L NH₃·H₂O and 50 μ L 0.5 M PMP (Sigma, USA) or 0.5 M D5PMP (synthesized by our laboratory) at 70 °C for 60 min. The extra PMP or D5PMP in the labeling reaction was extracted by adding 500 μ L chloroform three times. For genistein treated and untreated cells, respectively the PMP- and D5PMP- labeled GAG disaccharides from the same amount of cellular proteins were mixed. All mixed samples were then ready for LC/MS analysis. The PMP-labeled commercial HS or CS disaccharide standards or the mixture of PMP- and D5PMP-labeled samples were separated by using Agilent 1200 infinity capillary liquid chromatography system with a ZORBAX Stable Bond chromatogram column (5 μ m 0.5 $\times$ 250 mm). Solvent A was 10 mM ammonium acetate (pH 4.5), Solvent B was 100% acetonitrile. The gradient used was 15% B in 10 min, 25% B for another 15 min, 29% B for another 10 min and 50% B for further 5 min, and finally equilibrated with 15% B for 15 min. The flow rate was 15 μ L/min. The UV detector monitored the samples at 245 nm. The total ion current (TIC) was collected using a Thermo LTQ-XL mass spectrometer operating in negative ion mode by scanning the m/z ranged from 300–2000. The mass spectra were processed by using Thermo Xcalibur software.

Statistical analysis of data

All statistical calculations were done using IBM SPSS Statistics 21. All data are represented as the mean $\pm$ S.D. Comparison between groups was made by T-test to analyze the difference. *P < 0.05 and **P < 0.01 were considered to indicate statistical significance.

Results

Cell growth inhibition by genistein in four different cell lines

The effects of genistein on CHOK1, CHO3.1, CHO3.3 and HCT116 cells were tested. CHOK1 is the wild-type control, CHO3.1 and CHO3.3 are HS 3-*osulfotransferase-1* and

3-*osulfotransferase-3* over-expressing CHO cell lines, respectively. Genistein could inhibit the cell growth in all four cell lines tested (Fig. 2). The growth of CHOK1, CHO3.1, CHO3.3 and HCT116 cells was suppressed in the present of genistein in a concentration- and time-dependent manner. The genistein had IC_{50} values of 160.8 μ M, 98.75 μ M, 90.71 μ M, 95.18 μ M at 24 h; 38.27 μ M, 33.13 μ M, 36.13 μ M, 41.75 μ M at 48 h; and 25.24 μ M, 17.6 μ M, 39.93 μ M, 31.92 μ M at 72 h, in CHOK1, CHOK3.1, CHO3.3, and HCT116 cells, respectively.

Monosaccharide analysis for four cell lines

The HPLC profile of eight monosaccharides including mannose (Man), glucosamine (GlcN), galactosamine (GalN), glucuronic acid (GlcA), glucose (Glc), galactose (Gal), xylose (Xyl), fucose (Fuc) from monosaccharide standards or monosaccharide released from glycans of cell lysate of genistein-treated or untreated CHOK1 cells were shown in Fig. 3. The ratio of each monosaccharide released from glycans of cell lysate in genistein-treated to untreated (control) of CHOK1, CHO3.1, CHO3.3 and HCT116 cells were shown in Fig. 4. The data showed that genistein reduced the amount of all eight monosaccharides released from glycans in HCT116 cells and reduced the amount of all monosaccharides except for glucose from CHOK1, CHO3.1, and CHO3.3 cells. Interestingly, fucose was not detected in HCT116 cells.

CS disaccharide analysis for four cell lines

Due to the complicated GAG assembly scheme, the best way to analyze if genistein might disturb the GAG biosynthesis is to perform the GAG disaccharide composition analysis. To this end, our lab has developed a special method to detect disaccharide structural changes of both HS and CS based on LC-MS analysis [29] (See details in Materials and methods). The molecular masses over charge (m/z) for all PMP- or D5PMP-labeled HS and CS disaccharides observed in genistein-treated or control CHO and HCT116 cells detected by MS were

Fig. 2. Growth inhibitory effect of genistein on CHOK1, CHO3.1, CHO3.3 and HCT116 cells. Three Chinese hamster ovary cell lines (CHOK1, CHO3.1, CHO3.3) and one human colon cancer cell line (HCT116) were used to measure the percentage of viable cells. Each type of cells was treated with serial concentrations of genistein (0, 6.25, 12.5, 25, 50, 100 μ M for CHOK1, CHO3.1, CHO3.3 cells and 15, 30, 45, 60, 75, 90 μ M for HCT116) for 24 h, 48 h, or 72 h, respectively. The experiment was repeated three times, and the results were presented as mean $\pm$ S.D. *P < 0.05 and **P < 0.01 vs. control group.

Fig. 3. HPLC profile of eight monosaccharides from the monosaccharide standards (A), monosaccharide-released from glycans of cell lysate from the control (without genistein treatment) CHOK1 cells (B), or genistein-treated CHOK1 cells (C). Eight monosaccharide standards (Man, GlcN, GalN, GlcA, Glc, Gal, Xyl and Fuc) were mixed and diluted in a series of concentrations (0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, and 0.0005 mg/mL) to obtain the standard curve for quantification purpose. The HPLC profile of the monosaccharide mixture at 0.5 mg/mL was shown in Fig. 3A. The monosaccharide peaks from 1 to 8 were Man, GlcN, GalN, GlcA, Glc, Gal, Xyl and Fuc, respectively.

summarized in Table 1. The representing extracted ion current of ten pairs of HS and seven pairs of CS disaccharides from genistein-treated (PMP-labeled, blue) and control (D5PMP-labeled, red) HCT116 cells were shown in Fig. 5. The different types of cells were treated with the concentrations of genistein according to IC_{50} value of the respective cells, 38.27 μ M for CHOK1, 33.13 μ M for CHOK3.1, 36.13 μ M for CHO3.3, or 41.75 μ M for HCT116 for 48 h, respectively. The GAGs were isolated from both cells and cultural medium.

As shown in Fig. 6, CS disaccharides from genistein treated cells exhibited a common trend, that is, monosulfated disaccharides were reduced in the three kinds of CHO cell lines, and the sulfated disaccharides (monosulfated or disulfated) were significantly increased in HCT116 cells. Nonsulfated, monosulfated and disulfated structures of disaccharides from medium generally raised in the four cell lines.

Table 1. Molecular masses for PMP- or D5PMP-labeled HS and CS disaccharides detected in genistein treated or untreated CHO cells and HCT116 cells. The disaccharide structure code (DSC), the abbreviated chemical structure ($\Delta U = 4$, 5-unsaturated uronic acid)

Disaccharide structure Code	Structures	Molecular masses observed	
		m/z: z=1(z=2)	
		PMP-labeled	D5PMP-labeled
CS disaccharides			
D0a0	Δ UA-GalNAc	708(N/A)	718(N/A)
D2a0	Δ UA2S-GalNAc	788(393.5)	798(398.5)
D0a6	Δ UA-GalNAc6S	788(393.5)	798(398.5)
D0a4	Δ UA-GalNAc4S	788(393.5)	798(398.5)
D2a4	Δ UA2S-GalNAc4S	868(433.5)	878(438.5)
D2a6	Δ UA2S-GalNAc6S	868(433.5)	878(438.5)
D0a10	Δ UA-GalNAc4S6S	868(433.5)	878(438.5)
HS disaccharides			
D0A0	Δ UA-GlcNAc	708(N/A)	718(N/A)
D2A0	Δ UA2S- GlcNAc	788(393.5)	798(398.5)
D0A6	Δ UA- GlcNAc6S	788(393.5)	798(398.5)
D2A6	Δ UA2S- GlcNAc6S	868(433.5)	878(438.5)
D0H6	Δ UA-GlcNH ₂ 6S	746(372.5)	756(377.5)
D0S0	Δ UA-GlcNS	746(372.5)	756(377.5)
D2H0	Δ UA2S-GlcNH ₂	746(372.5)	756(377.5)
D0S6	Δ UA- GlcNS6S	826(412.5)	836(417.5)
D2S0	Δ UA2S-GlcNS	826(412.5)	836(417.5)
D2S6	Δ UA2S-GlcNS6S	906(452.5)	916(457.5)

Fig. 4. Genistein reduced the amount of monosaccharides in glycans released from cell lysates of CHOK1, CHO3.1, CHO3.3 and HCT116 cells. Monosaccharides were released from the cell lysates and labeled with PMP followed by HPLC analysis. The different cell lines were treated with different concentrations of genistein based on IC_{50} values of each cell line, i.e., 38.27 μM for CHOK1, 33.13 μM for CHO3.1, 36.13 μM for CHO3.3, 41.75 μM for HCT116 (See details in Materials and Methods). The ordinate was the ratio of genistein-treated vs. control. The red line represents when the ratio is 1, i.e., genistein treatment had no effects on the amount of monosaccharides released from the glycans of specific cell lysates. The experiment was repeated three times, and the results were presented as mean $\pm$ S.D. * $P < 0.05$ and ** $P < 0.01$ vs. control group.

Specific sulfated CS disaccharides were also analyzed as shown in Fig. 6. For CS disaccharides from the cells, monosulfated CS disaccharides (D0a6 or $\Delta U A-GalNAc6S$,

Fig. 5. MS data of ten kinds of HS (A) and seven kinds of CS (B) disaccharides from the PMP labeled (blue, genistein-treated) and D5PMP-labeled (red, the control) samples from HCT116 cells. HS and CS disaccharides from enzymatically digested GAGs extracted from genistein-treated or control HCT116 cells were labeled with PMP or D5PMP, respectively. The two independently labeled samples were proportionally mixed to ensure that amount of cell-proteins of each sample was the same. The mixture was then injected to LC for separation and MS for detection. Because each disaccharide can be tagged with two molecules of PMP or D5PMP, the labeled disaccharide has 10 mass unit difference, however, if the molecule carries two charges, the disaccharide has 5 mass unit difference, which were observed in the MS data shown above. All disaccharides were further identified by being compared with commercially available disaccharide standards.

D0a4 or Δ UA-GalNAc4S, D2a0 or Δ UA2S-GalNAc) in three CHO cell lines were reduced, but they were increased in HCT116 cells, and for disaccharides from the medium, they were basically higher than control in all four cell lines except for D2a0 in CHO3.1 and CHO3.3 cells. For disulfated CS disaccharides from the cells, the differences between CHO cell lines and HCT116 cells were obvious (Fig. 6), D2a4 (Δ UA2S-GalNAc4S) and D2a6 (Δ UA2S-GalNAc6S) did not changed much in CHOK1, CHO3.1 and CHO3.3 cells, but D2a4 and D2a6 had a 3-4-fold increase in HCT116 cells. More specifically, D0a10 (Δ UA-GalNAc4S6S) was not detected in CHOK1, CHO3.1 and CHO3.3 cells, while it increased 3-fold in HCT116 cells. Meanwhile, disulfated disaccharides (D2a4, D2a6, D0a10) from the medium were increasing for all cells except for CHO3.3 cells.

Disaccharide HS analysis for four cell lines

The HS disaccharide analysis results were shown in Fig. 7. All HS disaccharides including nonsulfated (708), monosulfated (788, 746), disulfated (826) and trisulfated (906) disaccharides were decreased or had no significant change from genistein-treated CHOK1, CHO3.1, CHO3.3 cells or cultured media. In contrast, HS disaccharides including nonsulfated (708), monosulfated (788, 746), disulfated (868, 826) and trisulfated (906) disaccharides were increased 2-4-fold in HCT116 cells or the cultured media.

The quantitative changes of specific HS disaccharides were shown in Fig. 7. The monosulfated (D2H0/ Δ UA2S-GlcNH₂, D0S0/ Δ UA-GlcNS, D0H6/ Δ UA-GlcNH₂6S, D2A0/ Δ UA2S-GlcNAc, D0A6/ Δ UA-GlcNAc6S), disulfated (D0S6/ Δ UA-GlcNS6S, D2S0/ Δ UA2S-

Fig. 6. Disaccharide compositions of CS from genistein-treated and untreated CHOK1, CHO3.1, CHO3.3 and HCT116 cells. GAGs were isolated from either cells (A, C, E) or culture media (B, D, F). Disaccharides from enzymatically digested CS isolated from genistein-treated and untreated cells were tagged with PMP or D5PMP, respectively. The PMP- or D5PMP-labeled samples were proportionally admixed to ensure equal cell-protein (See details in Materials and methods). Data are expressed as ratio of genistein-treated group and untreated group of total disaccharide, the experiment was repeated three times, and the results were presented as mean $\pm$ S.D. * P <0.05 and ** P <0.01 vs. control group. The red line represents the ratio value is 1.

Fig. 7. Disaccharide compositions of HS in CHOK1, CHO3.1, CHO3.3 and HCT116 cells with or without genistein treatment. GAGs were isolated from the cells (A, C, E, G) or the cultural media (B, D, F, H). Disaccharides from enzymatically digested HS isolated from genistein-treated and untreated CHO and HCT116 cells were tagged with PMP or D5PMP, respectively. The PMP- or D5PMP-labeled samples were proportionally admixed to ensure equal cell-protein (See details in Materials and methods). Data are expressed as ratio of genistein-treated group and untreated group of total disaccharide, the experiment was repeated three times, and the results were presented as mean $\pm$ S.D. * P <0.05 and ** P <0.01 vs. control group. The red line expresses the ratio value is 1.

GlcNS) HS disaccharides were reduced in all CHO cell lines. In contrast, they were all increased in HCT116 cells (about 2-fold increase in both cells and cultural media).

Discussion

It has been reported that genistein affects proliferations of oviductal epithelial cells [33], melanoma cells [34], telomerase-negative cells [35], and other cancer cells [36]. In consistent, current studies showed that genistein inhibited the proliferation of CHOK1, CHO3.1, CHO3.3 and HCT116 cells in a concentration- and time-dependent manner (Fig. 2). However, we first reported that genistein reduced the amount of monosaccharides (Man, GlcN, GalN, GlcA, Glc, Gal, Xyl) released from glycans of the cell lysates of HCT116 cells and the amount of monosaccharides (Man, GlcN, GalN, GlcA, Gal, Xyl, Fuc) released from glycans of the cell lysates of CHOK1, CHO3.1, CHO3.3 cells (Fig. 4) with glucose (Glc) as an exception. Both N- and O-linked glycans on glycoproteins usually do not contain any Glc and Glc could store in the form of glycogen in cells. Thus, the data shown in Fig. 2 clearly demonstrated that genistein treatment reduced the amount of glycans in the four cell lines tested. In vertebrate, 95% glycans are O- and N-linked glycans and 5% glycans are GAGs, which make GAGs minor species of the glycans. Our data revealed for the first time that genistein treatment reduced overall glycan quantity in all four cell lines tested but glycosaminoglycan quantities were either increased or decreased in a cell type-dependent manner.

The majority of proteins are modified by glycans that have numerous important structural, functional and regulatory roles in various physiological processes [37, 38]. Glycan structures are encoded by 1% human genome within a network of several hundred glycosyltransferases, glycosidases, transporters, transcription factors and other proteins [39], which enables the influence of environmental factors on the quantity and structure of glycans, either by direct effects on individual enzymatic processes or by regulating the amount of glucose available for glycan biosynthesis. We did not know how genistein treatment reduced overall glycan quantity in all four cell lines tested due to the complexity of non-template driven glycan biosynthesis, but the glycan quantification assay used in current study would allow us to screen for more compounds that have similar glycan quantity-reducing properties. We are currently screening for such compounds with fluorescent property that would allow us to observe the cellular distribution of the compound once it enters the cells as a starting point of molecular mechanism study. We have published such techniques previously [40, 41].

GAGs have diverse structures and interact with hundreds of proteins by catalyzing protease inhibition by serpins and regulate multiple signaling pathways including, but not limited to, fibroblast growth factor (FGF)/FGFR, hepatocyte growth factor (HGF)/c-Met, glial cell line-derived neurotrophic factor (GDNF)/c-Ret/GFR α 1, vascular endothelial growth factor (VEGF)/VEGFR, platelet derived growth factor (PDGF)/PDGFR, BAFF/TACI, Indian hedgehog, Wnt, and BMP signaling pathways [10]. Since specific HS structures are responsible for specific biological functions, we used not only wild-type CHO K1 cells but also two CHO cell lines that overexpress different 3-O-sulfotransferases in addition to human colon cancer cell line HCT116 in current study. CHO3.1 overexpress 3-OST-1, which adds 3-O-sulfate to GlcNS in a glucuronic acid (GlcA)-GlcNS 6 S sequence context in HS and facilitate high affinity antithrombin III binding [42]. CHO3.3 cells overexpress 3-OST-3, which adds 3-O-sulfate to GlcNS in a GlcA2S/iduronic acid (IdoA) 2S-GlcNS 6 S sequence context in HS and promote herpes simplex virus (HSV) glycoprotein D binding and entry [43]. 3-O-sulfated HS is also indicated in regulating FGFs/FGFRs signaling pathways [44, 45]. Therefore, we not only used CHOK1 but also CHO 3.1 and CHO 3.3 cell lines in current study to test if genistein had the same or different effects on GAG biosynthesis with different endogenous HS structures in the three cell lines. Our results showed that genistein decreased the biosynthesis of GAGs in CHO K1, CHO 3.1, and CHO 3.3 cells but enhanced GAG biosynthesis in HCT116 cells even though genistein had similar IC₅₀s in all four cell lines tested.

EGFR is expressed in most of epithelial cells and over-expressed in 25% to 82% of colorectal cancers [46]. We have previously showed that HCT116 cells express EGFR [47-49]. In contrast, CHO K1 cells do not express EGFR and such property has been used in various EGFR-related studies [50-52]. Paradoxically, genistein treatment enhanced the GAG quantity in the EGFR-expressing HCT116 cells but reduced the GAG quantity in CHO cells that do not express EGFR. Thus, the data obtained in current study contradicted to the proposed mechanism that genistein-mediated inhibition of GAG synthesis operates through EGF-dependent pathway [53]. Thus, we concluded that EGF/EGFR signaling pathway might not be responsible for the increased or decreased GAG biosynthesis observed in the four cell lines tested.

Apart from impairing efficiency of GAG synthesis through EGF pathway, genistein is also reported to reduce GAG levels by activation of lysosomal biogenesis, particularly through increasing levels of TFEB and stimulation of its translocation to the nucleus. Determination of TFEB levels or its localization or both in all tested cell lines treated and un-treated with genistein would indicate if there are differences between them which could be responsible for various effects of genistein. Our data showed that genistein enhanced GAG biosynthesis in HCT116 cells but decreased GAG biosynthesis in three CHO cell lines (Fig. 7) but exhibited the similar cytotoxicity (Fig. 2). If genistein decreases GAG levels by activation of lysosomal biogenesis, particularly through increasing levels of TFEB and stimulation of its translocation to the nucleus, such molecular mechanism could not explain the similar IC₅₀ values in the four cell lines (Fig. 2) and increased GAG biosynthesis in HCT116 cells. Since TFEB is a master regulator for transcription of genes involved not only in lysosome biogenesis but also in autophagy and mitochondrial biogenesis [54], we felt that determination of TFEB levels or its localization or both in all tested cell lines treated and un-treated with genistein might be the experiment we should do after we obtain the evidence that genistein indeed activates lysosomal biogenesis in the cell lines tested using a fluorescent compound in future.

In addition, we analyzed the structure of the CS and HS disaccharides by LC-MS. It is well known that quantification of GAG disaccharides by mass spectrometry is difficult. However, we used two non-radioactive compounds to observe the drug-induced changes in GAG structures in current studies. Moreover, the PMP- and D5PMP-labeling method made it possible to compare the quantity and disaccharide compositions of both HS and CS from the genistein-treated and control cells and cultural media simultaneously by LC/MS (Figs 5-7).

Previous study showed that genistein could either reduce or increase GAG biosynthesis in different cells [21, 27]. In our study, the amount of HS was reduced in CHOK1, CHO3.1 and

Fig. 8. Summary of the major discoveries: Genistein reduced the amount of all eight monosaccharides released from glycans in HCT116 cells and reduced the amount of all monosaccharides except for glucose in CHOK1, CHO3.1 and CHO3.3 cells. Genistein enhanced the amount of both CS and HS disaccharides in HCT116 cells but reduced the amount of disaccharides in CHOK1, CHO3.1 and CHO3.3 cells. Thus, genistein could enhance or reduce the amount of GAGs in a cell type-dependent manner.

CHO3.3 cells but was increased in HCT116 cells. Such changes were consistent no matter the GAGs were isolated from the cells or the culture media (Fig. 7). In contrast, the amount of CS disaccharides was only slightly reduced in the three CHO cell lines tested (Fig. 6). Thus, these studies confirmed that genistein affected the GAG biosynthesis in a cell type- and HS-/CS-dependent manner. Our results were in line with the reported beneficial effects of genistein in treating MPS type III by inhibiting HS biosynthesis and enhancing lysosomal biogenesis [13, 16, 18, 23-25].

We concluded that genistein was a novel type of small molecule that reduced the amount of glycans in the four cell lines tested in current study. In contrast, genistein enhanced the amount of both HS and CS in HCT116 cells but reduced the amount of HS and CS in CHOK1, CHO3.1 and CHO3.3 cells (Fig. 8). Thus, our study supported the beneficial effects of genistein for patients with MPS type III by reducing HS accumulations and cautioned that genistein was not a general GAG inhibitor due to the complicated GAG biosynthetic pathways.

Abbreviations

GAG (glycosaminoglycan); HS (heparan sulfate); CS (chondroitin sulfate); Man (mannose); GlcN (glucosamine); GalN (galactosamine); GlcA (glucuronic acid); Glc (glucose); Gal (galactose); Xyl (xylose); Fuc (fucose); PMP (1-phenyl-3-methyl-5-pyrazolone); deuterated PMP- (D5PMP-).

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Disclosure Statement

The authors declare that they have no conflict of interests with the contents of this article.

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