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Research Paper

Cell cycle is disturbed in mucopolysaccharidosis type II fibroblasts, and can be improved by genistein

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ABSTRACT

Mucopolysaccharidoses (MPSs) are inherited metabolic diseases caused by mutations resulting in deficiency of one of enzymes involved in degradation of glycosaminoglycans (GAGs). These compounds accumulate in cells causing their dysfunctions. Genistein is a molecule previously found to both modify GAG metabolism and modulate cell cycle. Therefore, we investigated whether the cell cycle is affected in MPS cells and if genistein can influence this process. Fibroblasts derived from patients suffering from MPS types I, II, IIIA and IIIB, as well as normal human fibroblasts (the HDFa cell line) were investigated. MTT assay was used for determination of cell proliferation, and the cell cycle was analyzed by using the MUSE® Cell Analyzer. While effects of genistein on cell proliferation were similar in both normal and MPS fibroblasts, fractions of cells in the G0/G1 phase were higher, and number of cells entering the S and G2/M phases was considerably lower in MPS II fibroblasts relative to control cells. Somewhat similar tendency, though significantly less pronounced, could be noted in MPS I, but only at longer times of incubation. However, this was not observed in MPS IIIA and MPS IIIB fibroblasts. Genistein (5, 7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) was found to be able to partially correct the disturbances in the MPS II cell cycle, and to some extent in MPS I, at higher concentrations of this compound. The tendency to increase the fractions of cells entering the S and G2/M phases was also observed in MPS IIIA and IIIB fibroblasts treated with genistein. In conclusion, this is the first report indicating that the cell cycle can be impaired in MPS cells. The finding that genistein can improve the MPS II (and to some extent also MPS I) cell cycle provides an input to our knowledge on the molecular mechanisms of action of this compound.

1. Introduction

Mucopolysaccharidoses (MPSs) are lysosomal storage diseases (LSDs) characterized by accumulation of glycosaminoglycans (GAGs) in lysosomes and outside the cells (Muenzer, 2011). They are caused by mutations in one of genes coding for enzymes responsible for GAG degradation. Eleven enzymatic deficits are responsible for seven different MPS types: I, II, III (with subtypes A, B, C, and D), IV (with subtypes A and B), VI, VII and IX. Depending on the MPS type, accumulation of following product(s) occurs: dermatan sulfate (DS; stored in MPS I, II, VI, VII), a component of conjunctive tissues, heparan sulfate (HS; stored in MPS I, II, III, VII), a constituent of cellular membranes, keratan sulfate (KS; stored in MPS IV), chondroitin sulfate (CS; stored in MPS IV, VII), present in the cartilages and in the cornea, and hyaluronic acid (HA; stored in MPS IX). Incomplete degradation of these GAGs causes severe complications in patients, resulting in a progressive damage of the affected tissues and organs, including the heart, respiratory system, bones, joints and central nervous system. Although each MPS type differs clinically from others, most patients generally experience a period of normal development followed by a severe decline in physical and/or mental function. The average life span of MPS patients is between one and two decades (Cimaz and La Torre, 2014).

Although there are intensive studies on various therapies for MPS, and enzyme replacement therapy has already been introduced for some types of this disease (MPS I, II, IVA, and VI), there is still a need for development of novel, effective treatment procedures, especially to manage the dysfunction of central nervous system (Cox, 2015). One of possible options is the use of small molecules, able to cross the blood–brain-barrier, which could impair synthesis of GAGs. This strategy is called substrate reduction therapy (Cox, 2015). It was demonstrated that genistein (5, 7-dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) inhibited GAG synthesis in MPS fibroblasts *in vitro* (Piotrowska et al., 2006). This inhibition appears to be due to impairment of the epidermal growth factor receptor activity, and further

Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate; GAG, glycosaminoglycan; KS, keratan sulfate; MPS, mucopolysaccharidosis; TFEB, transcription factor EB.

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down-regulation of the signal transduction, which normally leads to stimulation of expression of genes coding for enzymes involved in GAG synthesis (Jakobkiewicz-Banecka et al., 2009). Despite different laboratories reported various effects of genistein on GAG production and accumulation in different cell lines (Piotrowska et al., 2006; Jakobkiewicz-Banecka et al., 2009; Arfi et al., 2010; Kloska et al., 2011; Otomo et al., 2012; Kingma et al., 2014), very recent studies, employing microarray analyses followed by real-time quantitative RT-PCR identified particular genes coding for enzymes necessary for GAG synthesis which were inhibited by genistein, while most of genes for GAG lysosomal hydrolases were stimulated by this isoflavone (Moskot et al., 2014, 2015a). This stimulation was apparently due to positive regulation of the transcription factor EB (TFEB), a master regulator for lysosomal biogenesis and function (Moskot et al., 2015a).

Interestingly, similar studies with global analyses of gene expression indicated that genistein can modulate expression of genes coding for proteins involved in DNA replication and cell cycle regulation, including MCM2-7, CDKN1A, CDKN1C, CDKN2A, CDKN2B, CDKN2C and GADD45A (Moskot et al., 2015b). In this light, we aimed to investigate whether cell cycle is affected in MPS fibroblasts and if genistein can influence this process in these cells.

2. Materials and methods

2.1. Cell lines, culture media, supplements and genistein solutions

Human Dermal Fibroblasts, adult (HDFa) (Cascade Biologics, Portland, USA) and MPS fibroblasts (types I, II, IIIA and IIIB) (Children's Memorial Health Institute, Warsaw, Poland) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich, Steinheim, Germany), 5% CO_2 at 37 °C. The human material (fibroblasts) was obtained, and the experiments have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and with approval of the local bioethical committee. Genistein was synthetized at the Pharmaceutical Research Institute (Warsaw, Poland), and was dissolved in dimethyl sulfoxide (DMSO).

2.2. Cytotoxicity and proliferation assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed to estimate cell growth and proliferation. Cells were plated in flat-bottomed 96-well plates and treated with 30, 60, and 100 μM of genistein or 0.05% DMSO as a control for 7 days at 37 °C. After incubation period, medium was replaced with RPMI (Sigma-Aldrich) supplemented with MTT (1 mg/ml) for another 4 h. The purple formazan crystals were dissolved in 150 ml DMSO, and absorbance was determined at 570 nm using Wallac 1420 Multilabel Counter (Perkin Elmer).

2.3. Cell cycle assay

The effect of genistein on cell cycle was evaluated by seeding HDFa and MPS fibroblasts into 6-well plates at a density of 1×10^4 per well. The cells were incubated for 24 h, and medium was replaced with a fresh one supplemented with different concentrations of genistein or DMSO in control experiments. The cultivation was continued for another 24, 48 or 72 h, and cell cycle phase was determined by MUSE® Cell Analyzer (Merck Millipore, Germany) using a Muse® Cell Cycle Assay Kit (Merck Millipore, Germany) according to the manufacturer's instructions. An average of at least 10,000 cells was analyzed for each condition. Triplicate independent experiments were conducted.

3. Results

It was demonstrated previously that genistein inhibits proliferation of cancer cells (Pavese et al., 2010). However, the effect of this isoflavone on normal fibroblasts was found to be moderate (Moskot et al., 2015b). We have tested the influence of genistein on proliferation of MPS fibroblasts. The effects of this isoflavone were dose-dependent, but similarly to normal fibroblasts (HDFa cell line), moderate slow down of proliferation was observed for MPS fibroblasts (Table 1).

To test whether cell cycle is affected in MPS cells, we have analyzed this process, using the MUSE® Cell Analyzer, in MPS I, II, IIIA and IIIB fibroblasts relative to normal fibroblasts (HDFa). Fractions of cells in G0/G1, S, and G2/M phases were determined after 24, 48 and 72 h of incubation. We found that the cell cycle was disturbed in MPS II fibroblasts in comparison to HDFa cells (Fig. 1). Namely, fractions of cells in the G0/G1 phase were significantly higher ($p < 0.05$), while number of cells entering the S and G2/M phases was lower in MPS II relative to HDFa (Fig. 1). Similar tendency could be noted also in MPS I fibroblasts at later times of incubation, however, the differences did not reach statistical significance (Fig. 1). No such effects were observed in MPS IIIA and MPS IIIB fibroblasts.

Since genistein was found to modulate expression of genes involved in the regulation of the cell cycle (Moskot et al., 2015b), we asked whether this isoflavone may influence the effects observed in MPS fibroblasts. Three concentrations of genistein were used (30, 60 and 100 μM), and the experiments were repeated under conditions described above. In these experiments, distributions of fractions of MPS I and II cells at particular phases of the cell cycle were changed towards the pattern observed in normal fibroblasts. The G0/G1 fraction decreased while the S and G2/M fractions (counted together) increased, particularly (in MPS II) or exclusively (in MPS I) at longer times of incubation and at higher genistein concentrations (Fig. 1). Although no significant changes in the cell cycle were observed in MPS IIIA and IIIB fibroblasts relative to HDFa cells without genistein, in the presence of this compound, the tendency to increase the S and G2/M fractions was similar to that found in MPS I and II fibroblasts (Fig. 1).

4. Discussion

Different cellular defects in MPS were reported previously (for reviews, see (Muenzer, 2011; Cimaz and La Torre, 2014)), however, this study demonstrates for the first time specific changes in MPS cell cycle. Less effective progression into S and G2/M phases, observed in this work (Fig. 1) may be compatible with the phenotypes of patients which include slower growth and delayed development (Cimaz and La Torre, 2014; Wraith, 2013). Interestingly, the body growth is not inhibited in MPS IIIA and IIIB (Cimaz and La Torre, 2014), and the cell cycle did not differ significantly in corresponding fibroblasts, relative to normal (HDFa) fibroblasts. Since the primary effects of dysfunctions

Table 1
Effect of genistein on growth of HDFa and MPS fibroblasts.

Genistein (μM)	Absorbance at 570 nm[a]	
	HDFa	MPS[b]
0	1.7 ± 0.1	1.4 ± 0.6
30	1.9 ± 0.1	1.3 ± 0.5*
60	0.9 ± 0.0	0.7 ± 0.4
100	0.6 ± 0.0	0.5 ± 0.3

[a] The values were determined for cultures treated for 7 days with the tested compound at indicated concentrations. Data represent mean values ±SD from $n \geq 3$.

[b] MPS represents collected results from MPS I, MPS II, MPS IIIA and MPS IIIB fibroblasts. This form of results' calculation has been chosen to achieve presentation simplicity and clarity, taking into consideration the fact that no significant differences were found between data obtained for each separate MPS type vs. another MPS type ($p > 0.05$ in *t*-Student test).

* $p < 0.05$ in *t*-Student test (HDFa vs. MPS).

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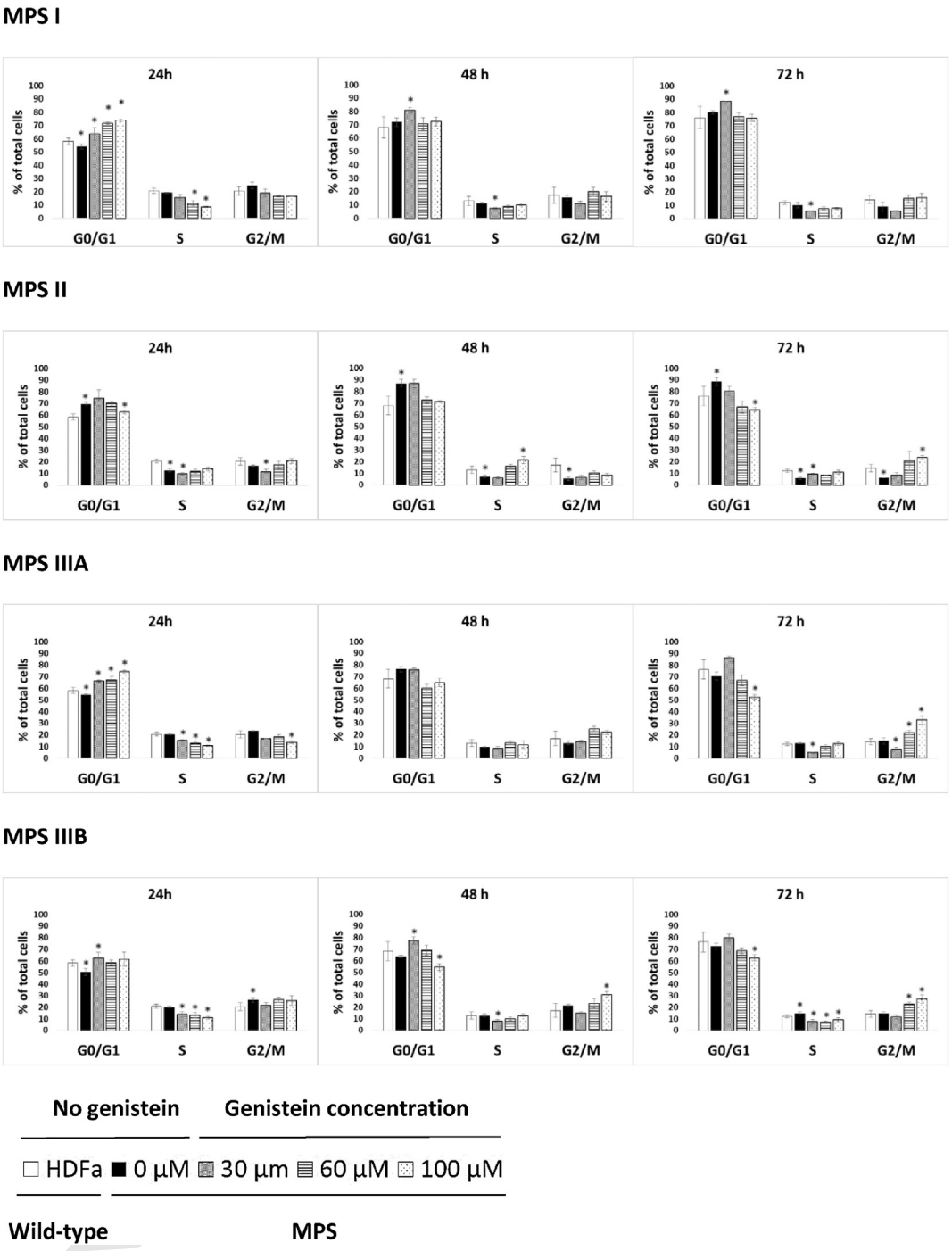

Fig. 1. Cell cycle analysis in HDFa and MPS fibroblasts, and effects of genistein on cell cycle progression in MPS cells. HDFa, MPS I, MPS II, MPS IIIA and MPS IIIB fibroblasts were investigated using the MUSE® Cell Analyzer at 24, 48, and 72 h of incubation with either no genistein (empty columns for HDFa, and closed columns for MPS) or 30 (grey columns), 60 (lined columns) or 100 (dotted columns) µM genistein. The indicated data represent mean values ± SD from $n \geq 3$ independent experiments. Asterisks indicate $p < 0.05$ in *t*-Student test (MPS vs. non-treated HDFa). Results of control experiments (with HDFa cells) are as described previously (Moskot et al., 2015b) (one should note a high reproducibility of results of these experiments).

of α-L-iduronidase (in MPS I) and iduronate-2-sulfatase (in MPS II) are accumulations of GAGs (DS and HS), the disturbance of the cell cycle in MPS I and II cells is likely a secondary or tertiary effect. In fact, GAGs may interfere with various metabolic pathways, and can disturb basic cellular functions (for a review, see (Wegrzyn, 2012)). Moreover, abnormalities in basic metabolism may interfere with the cell cycle, as demonstrated recently for glycolysis and the Krebs cycle (Konieczna et al., 2015). Considering differences observed between MPS I/MPS II and MPS III fibroblasts, one might suggest that the DS and HS storage (characteristic for MPS I and II), but not necessary the sole storage of HS (observed in MPS IIIA and IIIB), could disturb (perhaps indirectly) the cell cycle.

In our experiments, the duration of fibroblasts' treatment was 7 days when cell growth was tested, and up to 3 days when effects of genistein on cell cycle were assessed. These differences were due to experimental requirements, employed in order to obtain interpretable and reliable results. Estimation of effects of a tested compound on cell growth requires a relatively long time (like a weak), particularly if the effects are not strong (as in the case of genistein). On the other hand, to test cell cycle progress, continuation of experiments over 3 days would not

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make sense, as cells could enter further replication cycles which would make the results impossible to interpret.

For the reasons mentioned in the preceding paragraph, a contradiction between slowing down the cell proliferation (Table 1) and improvement of the cell cycle in MPS I and MPS II fibroblast (Fig. 1) is ostensible. The latter parameter was assessed in short-term experiments, contrary to the former one. More importantly, the cell culture growth was not inhibited but slowed down, which does not preclude cell cycle improvement. Moreover, different methods were used in both types of experiments, thus, results of measurement of cell culture growth and assessment of particular stages of the cell cycle should not be compared directly.

Genistein was reported to improve GAG storage in MPS cells due to inhibition of GAG synthesis and stimulation of lysosomal biogenesis and function through positive regulation of the TFEB transcription factor (Piotrowska et al., 2006; Jakobkiewicz-Banecka et al., 2009; Arfi et al., 2010; Kloska et al., 2011; Otomo et al., 2012; Moskot et al., 2014, 2015a). Despite the fact that some other authors could not confirm such effects of genistein in cell culture experiments (Kingma et al., 2014) and in the mouse model of MPS I (Kingma et al., 2015), positive results of studies on other animal models (Malinowska et al., 2009, 2010; Friso et al., 2010) encouraged researches to perform clinical trials with this isoflavone. Pilot clinical investigations indicated some biochemical improvements in blood and urine samples (Piotrowska et al.; Malinova et al., 2012; de Ruijter et al., 2012), as well as some functional benefits for patients (Piotrowska et al.; Marucha et al., 2011). Moreover, it was found that high dose genistein therapy is safe for MPS patients (Kim et al., 2013). Currently, a phase III, double blinded, randomized, placebo controlled clinical trial with high dose genistein aglycone administered orally to patients suffering from Sanfilippo syndrome (MPS III) is ongoing (http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=16209). We assume that results reported in this paper may facilitate understanding of genistein effects on MPS cells.

In conclusion, this study demonstrates that genistein can improve disturbances in the cell cycle which are evident in MPS II fibroblasts, and might also appear in MPS I. At the current stage of our knowledge it is not possible to determine whether these effects of genistein are due to a decreased GAG storage or modulation of expression of genes which products are involved in the cell cycle regulation or both. Nevertheless, the partial correction of the cell cycle indicates another kind of cellular processes (apart from previously reported decreased GAG synthesis and storage (Piotrowska et al., 2006; Jakobkiewicz-Banecka et al., 2009; Arfi et al., 2010; Kloska et al., 2011; Otomo et al., 2012) and enhanced lysosomal biogenesis and function (Moskot et al., 2014)) that can be improved by genistein in MPS cells.

Conflict of interest

The authors declare no conflict of interest.

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