

Differential effect of flavonoids on glycosaminoglycan content and distribution in skin fibroblasts of patients with type I osteogenesis imperfecta

ANNA GALICKA¹, JOLANTA NAZARUK² and MARTA BRUCZKO³

Departments of ¹Medical Chemistry, ²Pharmacognosy, and ³Pediatric Orthopaedics and Traumatology, Medical University of Białystok, 15-230 Białystok 8, Poland

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Abstract. We recently reported that, in osteogenesis imperfecta (OI) type I with diminished type I collagen biosynthesis, flavonoids such as apigenin 7-*O*-glucuronide, apigenin 7-*O*-methylglucuronide and pectolinarin normalized the level of collagen type I without affecting total protein synthesis. In addition to collagen, glycosaminoglycans (GAGs) play an important role in the formation of a functional supramolecular complex in the extracellular matrix, and any changes in their content and/or composition may be involved in the OI phenotype. We previously detected a marked increase in sulphated GAG content in the OI fibroblasts of more severely affected patients (OI types II and III). These alterations were more pronounced in medium than in cells. Although, in OI type I cells, the increase observed in medium was much smaller (approximately 1.5-fold), it resulted in an increase of approximately 3-fold of the GAG to collagen type I ratio. Therefore, in the potential pharmacotherapy of OI type I with flavonoids, their effect on GAG level may be of importance. In the OI cells, some of the tested flavonoids applied at a concentration of 30 μ M affected GAG content in quite the opposite way than type I collagen. Aglicones inhibiting collagen synthesis caused a marked increase in GAG concentration in medium, in contrast to the flavonoid glycosides, which exerted a stimulatory effect on type I collagen synthesis, but had a different effect on GAG content and distribution. Among these, apigenin 7-*O*-methylglucuronide did not affect GAG level or secretion, and thus may potentially be used in OI type I pharmacotherapy in patients with normal GAG content. However, in patients with increased concentrations of GAG, pectolinarin, which decreases GAG content by approximately 40%, may be more beneficial.

Introduction

Osteogenesis imperfecta (OI) is a genetically inherited disorder of the connective tissues characterized by bone fragility and skeletal deformities (1-5). OI presents with a broad range of clinical severity, ranging from multiple fracturing and perinatal death to a mild form that may elude clinical detection. OI has been categorized into four major clinical types based on clinical features as well as on radiological and genetic criteria (3). Biochemical and molecular genetic studies have shown that the vast majority of affected individuals have mutations in either the *COL1A1* or *COL1A2* genes, which encode the chains of type I procollagen, the major structural protein of bone (1,2,4). Recently, four new groups of patients have been defined in terms of clinical severity with the exclusion of type I collagen gene mutations (5). The lack of a relationship between collagen mutation and phenotype suggests the involvement of other modifying factors. Studies of this disorder in various tissues reflect an altered structure and/or metabolism of one or more of the extracellular matrix components, including collagen, glycosaminoglycans (GAGs) and/or proteoglycans (PGs) (6-8). GAGs are known to be involved in interactions with collagen type I and in the process of fibrillogenesis (9), thus any alterations in GAG synthesis, modification or secretion could affect these processes.

Previously, we demonstrated that disturbances of collagen type I biosynthesis in the skin fibroblasts of patients with OI type I were normalized by treatment of the cells with certain flavonoid glycosides (10). Flavonoids, which are plant polyphenolic compounds, are known to have a number of biological activities, including anti-oxidant, anti-radical, anti-inflammatory, anti-microbial, anti-allergy and anti-apoptic effects (11-13). This study was undertaken to investigate the influence of bioactive compounds isolated from the thistles *Cirsium rivulare* (Jacq.) Mill., *Cirsium palustre* (L.) Scop. and blue fleabane *Erigeron acris* L. (14-16) on GAG in OI fibroblasts.

Materials and methods

Fibroblast cultures. Skin fibroblast cultures from OI patients and healthy age-matched control subjects were grown to

Correspondence to: Dr Anna Galicka, Department of Medical Chemistry, Medical University of Białystok, 15-230 Białystok 8, Poland

E-mail: angajko@umwb.edu.pl

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Table I. Structure of the investigated flavonoids.

Compound	No.	Substitution pattern					
		R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Apigenin	1	H	H	OH	H	OH	H
Tricin	2	H	H	OH	OCH ₃	OH	OCH ₃
Isokaempferide	3	OCH ₃	H	OH	H	OH	H
Quercetin	4	OH	H	OH	OH	OH	H
Apigenin 7- <i>O</i> -glucuronide	5	H	H	<i>O</i> -glucuronyl	H	OH	H
Apigenin 7- <i>O</i> -methylglucuronide	6	H	H	<i>O</i> -methylglucuronyl	H	OH	H
Apigenin 7- <i>O</i> -glucoside	7	H	H	<i>O</i> -glucosyl	H	OH	H
Skutellarein 7- <i>O</i> -glucuronide	8	H	OH	<i>O</i> -glucuronyl	H	OH	H
Pectolinarin	9	H	OCH ₃	<i>O</i> -rutinosyl	H	OCH ₃	H
Isokaempferide 7- <i>O</i> -glucoside	10	OCH ₃	H	<i>O</i> -glucosyl	H	OH	H
Quercetin 3- <i>O</i> -glucoside	11	<i>O</i> -glucosyl	H	OH	OH	OH	H

confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mmol/l L-glutamine at 37°C in a 5% CO₂ incubator. The biopsies were obtained with the approval of the Ethics Committee of the Medical University of Białystok and with written parental consent. Cells were counted in a hemocytometer and cultured at a density of 1x10⁶ cells/well in 2 ml growth medium in 6-well plates (Costar). For the experiments, confluent cells were used to eliminate growth-related events.

Effect of flavonoids on GAG content in skin fibroblasts. The confluent cells were pre-incubated in fresh serum-free medium for 2 h. The flavonoids were dissolved in dimethyl sulfoxide (DMSO) and added to the medium at a final concentration of 30 µM, then incubated with the cells for 24 h. The concentration of 30 µM was selected on the basis of previous experiments, in which higher concentrations of the tested flavonoids proved in most cases to be toxic to cells. The same concentration of DMSO solution was applied to cells as a control. After incubation, the exposure medium was removed and stored for analysis of the secreted GAGs. The monolayers were washed four times with sterile 10 mM PBS (pH 7.4), and the cell membranes were disrupted using a sonicator. Aliquots of the homogenate were removed for DNA measurement, and the remaining homogenate was used for the analysis of GAG content according to the method of Barbosa *et al* (17).

Briefly, cellular and supernatant proteins were digested in a solution of 50 µg/ml proteinase K in 100 mM K₂HPO₄ (pH 8.0) at 56°C overnight. Proteinase K was then inactivated by heating for 10 min at 90°C. In order to eliminate DNA, which interferes with this assay, digested samples were filtered through an Ultrafree-MC filter (Millipore Corp., USA). 1,9-dimethylmethylene blue (DMMB) (1 ml) was added to 100 µl of the sample and vigorously vortexed for 30 min to promote the complexation of the GAGs with the DMMB. The GAG/DMMB complex was then separated from the solution by centrifugation (12,000 x g, 10 min). The pellet was dissolved by the addition of 1 ml of decomplexation solution (4 M GuHCl, pH 6.8) containing 10% propan-1-ol, and agitated for 30 min. Absorbance was measured at 656 nm.

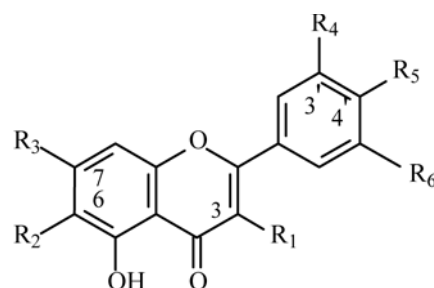


Figure 1. Molecular structure of the flavonoids.

Sulphated GAG quantities were determined by comparison with a calibration curve of CS solutions and expressed as µg of GAGs per µg of DNA. DNA was assayed in the fibroblasts by fluorometric measurement using Hoechst 33258 staining (18).

Statistical analysis. Data are presented as the mean ± SD of three assays. The results were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis rank sum test, with P<0.05 considered significant.

Results

Since the synthesis of sulphated GAGs is dependent on the age of the fibroblast donor, to avoid discrepancies due to age, the concentrations of the GAGs were compared between the patients and the age-matched controls. In the fibroblasts of patients with severe type III and lethal type II OI, the sulphated GAG content was markedly higher than in the controls. This difference was more pronounced in the medium than in the cells (Table II). In a newborn with OI type II and a Gly388→Arg mutation in the *COL1A1* gene (19), the level of sulphated GAGs was 5-fold higher in cells and 8.1-fold higher in medium with respect to the age-matched control. In siblings with OI type III (No. 2a and 2b) and differential expression of the phenotype whose fibroblasts secreted collagen type I with faster electrophoretic mobility, suggesting deletion as a

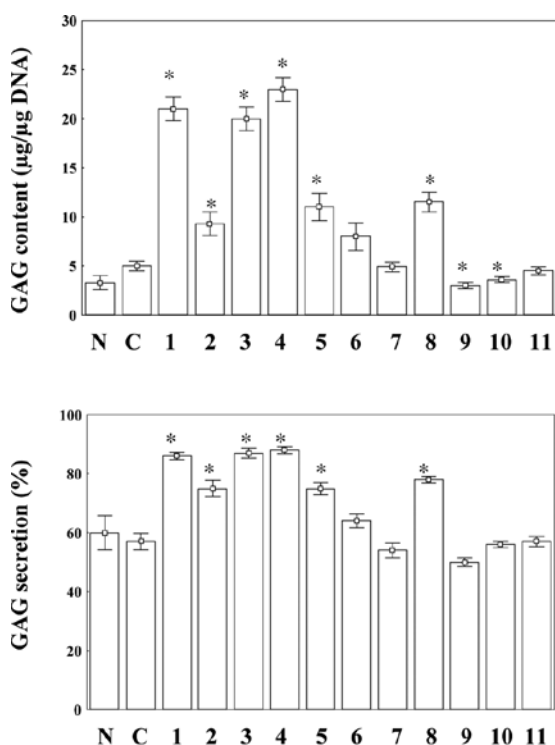


Figure 2. (A) Effect of compounds [1-11] on GAG content when applied at a concentration of 30 μ M. (B) Secretion of compounds [1-11] in OI type I fibroblasts. Mean values \pm SD from three independent experiments performed in duplicate are presented. * $P < 0.05$ in OI-treated compared to -untreated cells (C). N, normal untreated cells.

molecular defect (20), a different GAG level was detected in comparison to their age-matched controls. The most marked discrepancies were detected in the fibroblasts of a more severely affected female patient (no. 2a) compared to her sibling (no. 2b), who had a milder phenotype. These findings suggest that alterations in GAG content, in addition to collagen type I defects, may be among the factors modulating OI phenotype. In the fibroblasts of patients with a mild form of OI type I and a detected 'null allele' in the *COL1A1* gene (21), an approximate 1.5-fold increase in GAG concentration in medium was observed.

Previously, we studied the effect of various flavonoids on collagen biosynthesis in OI fibroblasts (10). Among these, the apigenin glycosides 7-*O*-glucuronide, 7-*O*-methylglucuronide and pectolinarin specifically affected collagen biosynthesis without influencing the overall protein biosynthesis. Since these acted as stimulators of collagen type I biosynthesis, they have the potential to be used in the pharmacotherapy of patients with OI type I having a reduced amount of normal collagen. The names, number [no.] and structure of the compounds investigated in the present study are listed in Table I, with reference to Fig. 1.

Certain of the tested flavonoids used at the concentration of 30 μ M had quite the opposite effect on GAG content compared to their effect on collagen biosynthesis. Treatment of OI cells with the aglycones apigenin [1] and quercetin [4], which inhibited both collagen and non-collagen protein biosynthesis (10), caused the greatest increase in GAG concentration (Fig. 2A). Similarly, a different effect on GAG content was observed in contrast to the essential stimulatory

Table II. Content and distribution of sulphated GAGs in OI skin fibroblasts.

OI type	Molecular defect	OI/C ^a (medium)	OI/C ^a (cell)
I (n=7)	Null allele (<i>COL1A1</i>)	1.5 \pm 0.6	1.0 \pm 0.3
II	Gly388 \rightarrow Arg (<i>COL1A1</i>)	8.1	5.0
III	Gly511 \rightarrow Ser (<i>COL1A1</i>)	3.4	2.9
	2a ^b -deletion	6.9	1.3
	2b ^b -deletion	3.5	1.2

^aRatio of GAG content in OI fibroblasts to GAG content in age-matched controls. ^bSiblings whose fibroblasts secreted collagen type I with a lower molecular mass, suggesting deletion as a molecular defect.

activity of the apigenin glycosides [5] and [6] and pectolinarin [9] in collagen biosynthesis (10). The increase in sulphated GAG content in the presence of apigenin 7-*O*-glucuronide [5] was approximately 2 times lower than that observed in the presence of apigenin [1]. No effect was exerted on GAG levels by apigenin 7-*O*-methylglucuronide [6]. However, pectolinarin [9] caused a decrease in GAG content of ~40%. Of the other tested flavonoids, isokaempferide 7-*O*-glucoside [10] showed a similar effect to pectolinarin [9], decreasing GAG content by ~30% (Fig. 2A). Similarly to apigenin 7-*O*-methylglucuronide [6], the apigenine [7] and quercetine [11] glucosides did not affect GAG content. The effects of the tested flavonoids on GAG concentration were not restricted to the OI cell lines, as we have obtained similar results following the treatment of normal cells.

The effect of the tested compounds on GAG secretion (assayed as the distribution of GAGs between the culture medium and the cell layer) was also examined. In the OI fibroblasts, the distribution of GAGs between the culture medium and the cell layer differed from that in control cultures, except in the cells of patients with OI type I, which secreted ~56% of the sulphated GAGs into the medium (Table II, Fig. 2B). Flavonoids, which at a concentration of 30 μ M caused a \geq 2-fold increase in GAG content [1-5] and [8], appeared to contribute to the significant increase in the secretion of GAGs from the cells (Fig. 2B). Among the compounds, apigenin 7-*O*-methylglucuronide [6] and pectolinarin [9] did not exert any effect on GAG secretion (Fig. 2B).

Discussion

Flavonoids are a large group of naturally occurring polyphenolic compounds with multiple biological functions. The fact that flavonoids interact with reactive oxygen species and influence the activity of a large variety of enzymes, ion channels and transcription factors determines a broad spectrum of their activity (11-13). Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in cancer, cardiovascular diseases and neurodegenerative disorders (12,13), as well as in diseases associated with a disrupted collagen metabolism (22,23).

Previously, we studied the effect of flavonoids isolated from *Cirsium rivulare* (Jacq.) All. flowers and the *Erigeron acris* L. herb (14-16) on collagen biosynthesis in the skin fibroblasts of patients with OI to identify bioactive compounds with the potential for use in the pharmacological therapy of disease (10). Because of structural mutations in patients with OI types II, III and IV, which affect connective tissue through a dominant-negative mechanism, the goal of gene therapy is the selective suppression of mutant allele expression (24,25). By contrast, in OI type I, bone formation is defective due to a deficiency of type I collagen caused by one null allele of the *COL1A1* gene (1,2,4,5), and agents that augment collagen production may have a beneficial effect. The benefits and short term safety of cyclic bisphosphonates have been reported in the literature; however, their long-term effects are still being investigated (25). Of the tested compounds, apigenin 7-*O*-glucuronide [5], apigenin 7-*O*-methylglucuronide [6] and pectolinarin [9], applied at a concentration of 30 μ M, significantly induced collagen type I biosynthesis in the fibroblasts of OI type I patients, without affecting the levels of collagen type III or the overall protein synthesis (10).

With the exception of collagen, GAGs have been shown to participate in the formation of a functional supramolecular complex in the extracellular matrix (9,26); whereas collagen fibers provide tensile strength, GAGs and PGs form a polysaccharide gel that resists compressive forces on the matrix. Specifically, PGs and their spatial arms, GAGs, are proposed to play an important role in the formation of collagen fibres and in the regulation of bone mineralization (26,27). Therefore, it may be postulated that any abnormalities in GAG content and/or composition are implicated in certain clinical aspects of OI. In our research, increases in the concentration of extracellular sulphated GAGs were detected with greater alterations in the fibroblasts of patients with lethal and severe OI phenotypes (types II and III). The increase in GAG content may result from elevated GAG biosynthesis, as reported in OI type II skin fibroblasts (7), or may be due to increases in their sulphation (6). The increase in the level of GAGs was much smaller in the fibroblasts of patients with OI type I in comparison to OI patients with a more severe phenotype. However, an approximate 1.5-fold increase in GAG content in medium and a decrease in collagen type I of approximately 50% (21) resulted in an approximate 3-fold increase in the GAG to collagen type I ratio. Therefore, in the potential pharmacotherapy of OI type I with flavonoids, their effect on GAG level may be of clinical importance.

We found that some of the tested flavonoids influenced GAG content in quite the opposite way than they influenced collagen biosynthesis. For example, the greatest increase in GAG content was observed in OI fibroblasts treated with aglycones, which inhibited collagen biosynthesis (10). The most important factor influencing GAG level was the presence of at least one hydroxyl group at phenyl moiety, as in compounds [1] and [4] (Table I). The presence of methoxyl groups at C-3' and C-5' [2] markedly inhibited the increase in GAGs caused by compound [1], but their presence at C-3 with compound [3] had no effect. Similarly, in contrast to the essential stimulatory activity of the apigenin glycosides [5] and [6] as well as pectolinarin [9] in type I collagen biosynthesis, the flavonoids exerted different effects on GAG content. In cells

treated with apigenin 7-*O*-glucuronide [5], an approximately 2-fold reduction in the increase in GAG content was observed in comparison to apigenin [1]. No significant alterations were detected after treatment of cells with 7-*O*-methylglucuronide [6]. Furthermore, the presence of the *O*-rutinosyl group at C-7 and of two methoxy groups at C-6 and C-4' in pectolinarin [9] caused a decrease in GAG content of approximately 40% at a concentration of 30 μ M.

In a previous study (10), we showed that apigenin 7-*O*-methylglucuronide [6] and pectolinarin [9] at a concentration of 30 μ M were stronger stimulators of collagen biosynthesis than apigenin 7-*O*-glucuronide [5]. Here, taking into account that, in most studied OI type I cell lines, no or little increase in total GAG content was observed, compounds [6] and [9] displayed greater beneficial action than compound [5]. We have also suggested that the stimulatory actions of [5], [6] and [9] on collagen biosynthesis probably involve different mechanisms, with [5] markedly inducing IGF-IR, while compounds [6] and [9] induce β 1-integrin expression (10). It has been suggested that IGF-IR plays an important role in the anabolic modulation of collagen synthesis by altering gene expression and prolydase activity (28). Increases in GAG content in OI cells in the presence of compound [5] could be the result of their increased synthesis. It was reported that IGF-I applied to skin fibroblasts increased the synthesis of PG and markedly stimulated the secretion of small PGs from cells (29). In fact, the treatment of OI fibroblasts with compound [5] caused an increase in GAG secretion in contrast to [6] and [9], which were expected to affect collagen synthesis through signals induced by the β 1-integrin receptor excited by interactions with type I collagen (10). Thus, apigenin 7-*O*-methylglucuronide [6] has the potential to be used in OI type I patients with normal GAG level. However, in those with an increased GAG concentration, pectolinarin [9] may be more beneficial, acting by normalizing the GAG to type I collagen ratio, which plays a key role in fibrogenesis.

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