Stimulation of collagen biosynthesis by flavonoid glycosides in skin fibroblasts of osteogenesis imperfecta type I and the potential mechanism of their action

ANNA GALICKA[1] and JOLANTA NAZARUK[2]

Departments of [1]Medical Chemistry, [2]Pharmacognosy, Medical University of Bialystok, Kilinskiego 1, 15-089 Bialystok, Poland

Received July 24, 2007; Accepted September 5, 2007

Abstract. The aim of the present study was to identify bioactive compounds stimulating collagen biosynthesis with potential for osteogenesis imperfecta (OI) type I pharmacological therapy. Of the compounds tested, apigenin glycosides 7-*O*-glucuronide, 7-*O*-methylglucuronide and pectolinarin at 30 μM were found to significantly induce collagen type I synthesis in OI fibroblasts without an effect on the overall protein synthesis. None of the compounds displayed any toxicity at that concentration. Secretion of collagen into media was not affected by apigenin 7-*O*-glucuronide and was slightly increased in cells treated with apigenin 7-*O*-methylglucuronide and pectolinarin. Furthermore, procollagen secreted by treated cells underwent a more rapid processing into collagen as compared with control untreated cells. In addition, we elucidated the possible mechanism involved in their action. Stimulation of collagen biosynthesis was not due to an increase in cell proliferation, because no differences in DNA content between the compound-treated and untreated cells were observed. Since flavonoids are known as strong inhibitors of metalloproteinases degrading matrix proteins, the increased level of collagen could result from the inhibition of their activity. However, the compounds with a stimulatory effect on collagen synthesis did not influence the activities of collagenase type I, gelatinases A and B, and stromelysin. On the contrary, all compounds stimulated the activity of prolidase which catalyzes the final step of collagen degradation and plays an important role in collagen biosynthesis. Stimulation of collagen synthesis and prolidase activity by apigenin 7-*O*-glucuronide was accompanied by an increase in IGF-I receptor expression. In contrast, the compounds apigenin 7-*O*-methylglucuronide and pectolinarin which normalized collagen synthesis in OI cells may exert their effects through ß1-integrin-mediated signaling.

Correspondence to: Dr Anna Galicka, Department of Medical Chemistry, Medical University of Bialystok, Kilinskiego 1, 15-089 Bialystok, Poland
E-mail: angajko@amb.edu.pl

Key words: osteogenesis imperfecta, flavonoids, collagen biosynthesis, prolidase

Introduction

Osteogenesis imperfecta (OI) is an autosomal dominant disorder characterized by the brittleness of bones and the susceptibility to bone fractures from very mild trauma (1-3). Fractures may be rare or frequent, and bone fragility may be accompanied by various connective tissue abnormalities: short stature, blue sclerae, joint laxity, dentinogenesis imperfecta, easy bruising and hearing loss. OI is currently classified into seven types based on differences in clinical presentation and bone architecture (1,3). In most patients the disease is caused by mutations in one of the two genes encoding type I collagen (2).

Since so far OI has been an incurable genetic disease, cell therapy and gene therapy are being investigated as potential treatments (4). At present bisphosphonates are used in patients who have significant clinical problems, however, the long-term consequences of their use in children are currently unknown (3). Medical therapies other than bisphosphonates play a minor role at present. In the majority of patients with OI type I who are heterozygous for a non-functional *COL1A1* allele, a decreased amount (~50%) of type I collagen is found (2,5,6). Since in these patients the level of collagen is reduced, contrary to more severe cases having normal structure, the aim of the therapy is to increase the biosynthesis of collagen.

The flavonoids are a large group of natural products widely distributed in nature that recently have been the subject of considerable scientific and therapeutic interest (7,8). The group is known for its antioxidant, antiradical, anti-inflammatory, anti-microbial as well as anti-allergic effects. Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in cancer, cardiovascular and neurodegenerative disorders (8) as well as in diseases connected with disrupted collagen metabolism (9-11). The therapeutic usefulness of flavonoids is still increasing.

The purpose of the present study was to identify bioactive compounds isolated from *Cirsium rivulare* (Jacq.) All. flowers (12,13) and from *Erigeron acris* L. herb (14) which

stimulate collagen biosynthesis with potential for OI type I pharmacological therapy.

Materials and methods

Fibroblast cultures. Skin fibroblasts of OI type I patients and age-matched healthy individuals were cultured in DMEM containing 10% fetal bovine serum and maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37˚C. For experiments, cells were plated at a density of $1x10^6$/well in 6-well culture plates (Costar).

Collagen and non-collagen protein synthesis. The confluent cells were preincubated in serum-free medium containing 50 μg/ml of ascorbic acid for 2 h. The flavonoids dissolved in dimethyl sulfoxide (DMSO) were added to the medium to a final concentration of 30 μM and incubated with cells for 24 h in the presence of 5 μCi L-[5-^{3}H]proline (28 Ci/mmol). The concentration was selected on the basis of previous experiments, where higher concentrations of tested flavonoids were in most cases toxic to cells. The control cells were incubated without the compounds, but with the same concentration of DMSO (0.01%). After incubation, the exposure medium was removed and stored for analysis of secreted collagen. The monolayers were washed four times with sterile 10 mM PBS, pH 7.4, and cell membranes were disrupted using a sonicator. Aliquots of the homogenate were removed for protein measurement using the BCA™ Protein Assay Kit (Pierce). The remaining homogenate was used for analysis of collagen synthesis. Incorporation of radioactive precursor into collagen was determined by digesting the proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky *et al* (15). Non-collagen proteins were expressed as the radioactivity of collagenase-resistant proteins. Secretion was estimated as the distribution of collagen and non-collagen proteins between the cell layer and the medium.

Electrophoretic analysis of procollagen. The confluent cells were labeled with 50 μCi/ml L-[5-^{3}H]proline (28 Ci/mmol) for 24 h in serum-free medium containing 50 μg/ml of ascorbic acid and the tested flavonoids in a final concentration of 30 μM. Procollagen was precipitated from medium by 25% saturated $(NH_4)_2SO_4$ and redissolved in 50 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA. The proteinase inhibitors 0.1 mM phenylmethanesulphonyl fluoride, 10 mM N-ethylmaleimide and 25 mM EDTA were added to prevent procollagen degradation during isolation. The samples were resolved on 5% SDS-PAGE with 2 M urea (16) and the gel was processed for fluorography with Amplify.

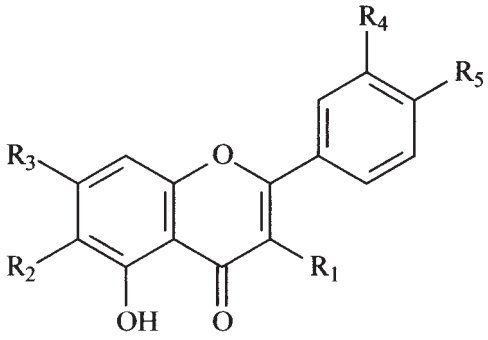
Assay for cell viability. The assay was performed according to the method of Carmichael *et al* (17) using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Confluent cells cultured with the tested compounds for 20 h at 37˚C were washed with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml). The medium was removed and 1 ml of 0.1 M HCl in absolute isopropanol was added to the cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability in the presence of the studied compounds was calculated as a percent of the control cells.

Zymography. Gelatinolytic activity of conditioned medium was determined according to the method of Unemori and Werb (18). Equal amounts (~10 μg) of protein were electrophoresed under non-reducing conditions on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin (Sigma), casein (1 mg/ml) (Sigma) or bovine collagen type I (0.5 mg/ml) (Sigma) as substrates. After electrophoresis, the gels were washed twice for 15 min with 2% Triton X-100 and then incubated overnight at 37˚C in 50 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$. The gels were stained with 0.5% Coomassie Brilliant Blue R-250. Clear bands on the blue background represent areas of substrate-degrading enzymes.

Determination of prolidase activity. The activity of prolidase was determined according to the method of Myara *et al* (19) which is based on the measurement of proline by Chinard's reagent (20). Cells were collected and suspended in 0.15 M NaCl, centrifuged at low speed (200 x g), and the supernatant was discarded. The cell pellet (from six wells) was suspended in 0.3 ml of 0.05 M Tris/HCl, pH 7.8, sonicated and then centrifuged (18,000 x g, 30 min) at 4˚C. The supernatant was used for protein determination and prolidase activity assay. Activation of prolidase requires preincubation with manganese, therefore 0.1 ml of the supernatant was incubated with 0.1 ml of 0.05 M Tris/HCl, pH 7.8, containing 2 mM $MnCl_2$ for 2 h at 37˚C. After preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 94 mM Gly-Pro to a final concentration of 47 mM. After additional incubation for 1 h at 37˚C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes, the reaction was terminated at time 'zero' (without incubation). The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid/Chinard's reagent (25 g of ninhydrin dissolved at 70˚C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90˚C. The amount of proline released was determined colorimetrically (at 515 nm) and calculated by using the proline standards. Enzyme activity was reported as nanomol/min/mg protein.

Assay for DNA content. DNA content was analyzed by fluorometric measurement using Hoechts 33258 (21). Briefly, confluent cells were cultured in serum-free medium with the tested flavonoids in a final concentration of 30 μM for 24 h. After removing media, cells were washed with PBS and then homogenized in phosphate-saline buffer (0.05 M $NaPO_4$, 2.0 M NaCl, pH 7.4), containing 1 μg of Hoechts 33258 (Sigma) per milliliter. Fluorescence was then measured using a fluorocolorimeter. Each assay was performed in duplicate, and a standard curve was constructed using a solution of known DNA concentration.

Analysis of ß1-integrin and IGF-I receptor (IGFR) expression. Samples of the cell supernatants (20 μg of protein) were subjected to electrophoresis on a 7.5% polyacrylamide gel according to the method of Laemmli (16). After SDS-



Compound	Symbol	Substitution pattern				
		R_1	R_2	R_3	R_4	R_5
Apigenin	1	H	H	OH	H	OH
Luteolin	2	H	H	OH	OH	OH
Kaempferol	3	OH	H	OH	H	OH
Quercetin	4	OH	H	OH	OH	OH
Pectolinarigenin	5	H	OCH_3	OH	H	OCH_3
Apigenin 7-*O*-glucoside	6	H	H	*O*-glucosyl	H	OH
Apigenin 7-*O*-glucuronide	7	H	H	*O*-glucuronyl	H	OH
Apigenin 7-*O*-methylglucuronide	8	H	H	*O*-methylglucuronyl	H	OH
Linarin	9	H	H	*O*-rutinosyl	H	OCH_3
Pectolinarin	10	H	OCH_3	*O*-rutinosyl	H	OCH_3
Isokaempferide 7-*O*-methylglucuronide	11	OCH_3	H	*O*-methylglucuronyl	H	OH
Kaempferol 3-*O*-glucoside	12	*O*-glucosyl	H	OH	H	OH

Figure 1. Names and structure of investigated flavonoids.

PAGE, the gels were allowed to equilibrate for 5 min in 25 mM Tris, 0.2 M glycine in 20% methanol. The protein was transferred to 0.2-μm pore-sized nitrocellulose at 100 mA for 1 h using an LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with monoclonal anti-ß1-integrin antibody at a concentration of 1:1000, polyclonal anti-IGFR Ig at a concentration of 1:1000 and polyclonal antibodies against ß-actin at a concentration of 1:3000 in 5% dried milk in TBS-T (20 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20) for 1 h. In order to analyze the ß1-integrin subunit, alkaline phosphatase-conjugated second antibody anti-mouse IgG (whole molecule) was added at a concentration of 1:7500. In order to analyze IGFR and ß-actin, alkaline phosphatase-conjugated second antibody anti-goat IgG (whole molecule) was added at a concentration of 1:1000. Incubation was continued for 30 min with mild shaking. Nitrocellulose was washed with TBS-T (5x5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

Statistical analysis. In all experiments, the mean values for three assays ± SD were calculated. The results were submitted to statistical analysis using one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis ranks test, accepting $p<0.05$ as a significant difference.

Results

Collagen and non-collagen protein synthesis were measured in skin fibroblasts of patients with OI type I treated with the compounds whose structure and names are shown in Fig. 1. The cells of patients with OI type I synthesized a reduced level of collagen by ~50% (Fig. 2) due to the presence of the null allele of collagen type I *COL1A1* gene. Therefore, we were interested in finding stimulators of collagen synthesis in OI cells. The tested flavonoids at a concentration of 30 μM exerted differential effects on collagen and non-collagen protein synthesis in OI cells. Aglycones apigenin [1], luteolin [2], kaempferol [3] and quercetin [4] significantly decreased both collagen and non-collagen proteins with more pronounced inhibitory effects on collagen synthesis (Fig. 2). In contrast, apigenin glycosides 7-*O*-glucoside [6], 7-*O*-glucuronide [7], 7-*O*-methylglucuronide [8] and pectolinarin [10] significantly induced collagen synthesis with little or no effect on the overall protein synthesis. The specific induction of collagen synthesis by [7], [8] and [10] was further demonstrated when collagen synthesis was expressed as a percentage of the total cell protein synthesis (Fig. 2C). At the concentration of 30 μM of [8] and [10], the percentage of synthesized collagen in OI cells was close to that of normal fibroblasts, whereas [7] increased collagen synthesis by ~63% of the control. Treatment of cells with different concentrations of [7] did not cause more effective stimulation; therefore, the concentration of 30 μM for all three compounds was used in further experiments. Furthermore, the compounds at that concentration exerting the most effective stimulatory effect on collagen synthesis did not significantly influence the viability of cells assayed with MTT (Fig. 3). The percentage of collagen secreted into the medium was

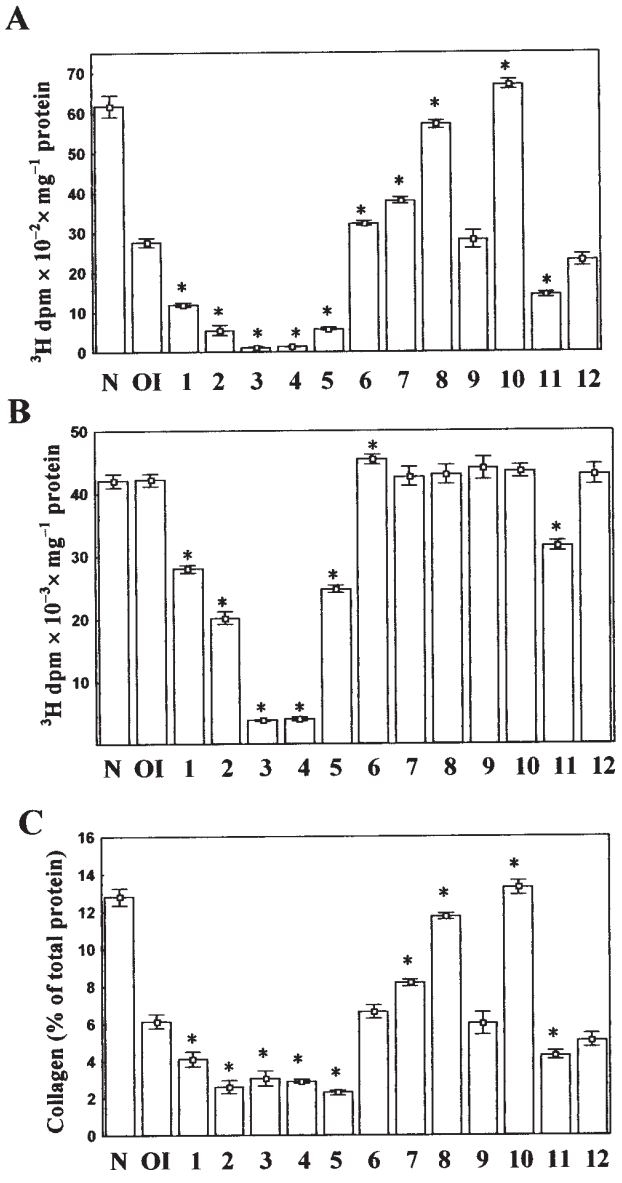


Figure 2. Effect of flavonoids [1-12] on total collagen synthesis (A), total non-collagen protein synthesis (B) and relative collagen synthesis expressed as the percentage of total protein synthesis (C) in OI cells incubated for 24 h in the presence of 5-[^{3}H]proline. Mean values ± SD from three independent experiments performed in duplicate are presented. *P<0.05 in OI-treated cells compared to untreated OI cells (OI). N, normal untreated cells.

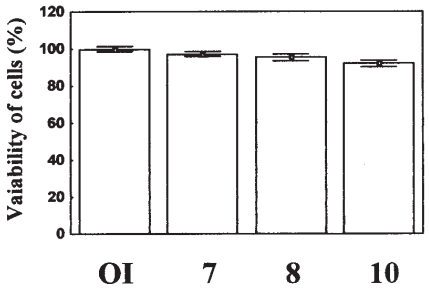


Figure 3. Viability of OI cells treated with the compounds [7], [8] and [10] expressed as a percent of untreated cells (OI). Mean values ± SD from three independent experiments performed in duplicate are presented.

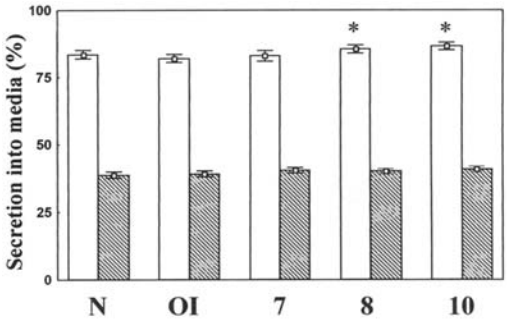


Figure 4. Secretion of collagen (white bars) and non-collagen proteins (grey bars) by normal (N), OI untreated (OI) and OI cells treated with [7], [8] and [10]. Mean values ± SD from three independent experiments performed in duplicate are presented. *P<0.05 compared to the OI untreated control.

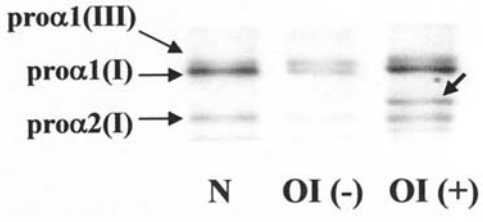


Figure 5. SDS/PAGE of [^{3}H]proline-labeled procollagen synthesized by normal (N), untreated OI cells (-) and the compound-treated OI cells (+). Lane 3 is the representative of [7], [8] and [10] action on collagen. pC-α1(I) is indicated by an arrow.

estimated as ~83% for the normal and 82% for OI cells and it did not change after treatment of OI cells with [7]; in the case of [8] and [10] only a slight increase was observed (Fig. 4). The secretion of non-collagen proteins was estimated approximately as 40% for both the control and the compound-treated fibroblasts.

Electrophoretic analysis of [^{3}H]proline-labeled proteins showed that in OI cells the ratio of procollagen type I to procollagen type III was altered because of type I procollagen deficiency (Fig. 5). Treatment of OI cells with the compounds stimulating collagen synthesis resulted in the increase of type I procollagen level without affecting type III procollagen. The additional band of the intermediate pC-α1(I) form seen on the gel suggests a faster pericellular processing of procollagen into collagen in treated cells as compared with the control untreated cells.

The stimulatory effect on collagen biosynthesis of [7], [8] and [10] was not restricted to the OI cell line, because similar results were obtained following treatment of normal cells. Since these compounds are of special interest for their potential use in the therapy of OI type I, further study was conducted to determine the mechanism of their action.

In view of the fact that some flavonoids are strong inhibitors of metalloproteinases (MMPs) degrading collagen (22), the observed increase in the level of collagen could be a result of inhibition of their activity. Using zymography we detected the presence of collagenase type I (43 kDa), gelatinase A (under non-reducing conditions, 72 kDa progelatinase runs at the slightly lower M_r of 66 kDa), gelatinase B (95-92 kDa) and stromelysin (~50 kDa), but no

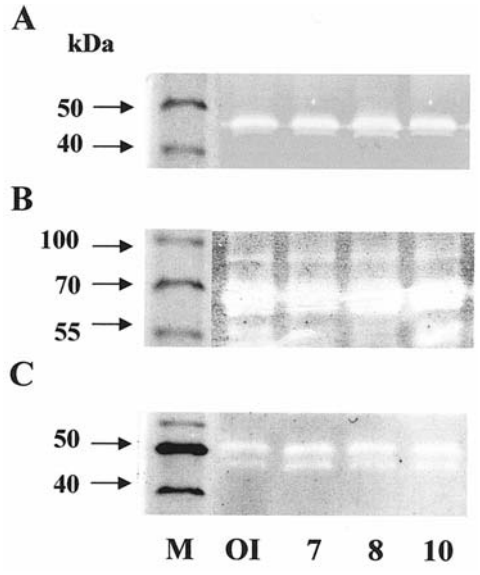


Figure 6. Activity of collagenase type I (A), gelatinases (B) and stromelysin (C) secreted by control OI cells (OI) and OI cells treated with [7], [8] and [10]. The arrows indicate the molecular mass of the standards (M).

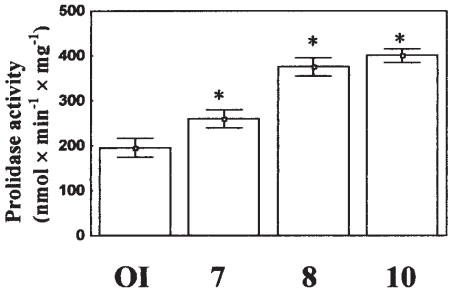


Figure 7. Prolidase activity in the OI control cells (OI) and OI cells incubated for 24 h with [7], [8] and [10]. Mean values ± SD from three independent experiments performed in duplicate are presented. *P<0.05 compared to the control.

differences in their activities between the compound-treated and the control media were observed (Fig. 6). Extracellular collagenases initiate the breakdown of collagen, but the final step of collagen degradation is catalyzed by intracellular prolidase, which plays an important role in collagen biosynthesis (23). In our study, the increase in activity of prolidase was correlated with stimulation of collagen synthesis in OI cells cultured in the presence of the compounds (Fig. 7). To exclude the possibility that the stimulation of collagen biosynthesis and prolidase activity was due to increased cell proliferation, we examined the effect of [7], [8] and [10] on the DNA level in OI cells. Our data showed that there were no differences between the compound-treated and untreated cells (Fig. 8). Previously, it was found that both collagen biosynthesis and prolidase activity are regulated by ß1-integrin and/or IGF-I receptor (IGFR)-mediated signaling (24,25) and that flavonoids can act as modulators of intracellular signaling cascades (10,26). Therefore, we considered the above receptors as a potential target in the compound-induced stimulation of the collagen synthesis.

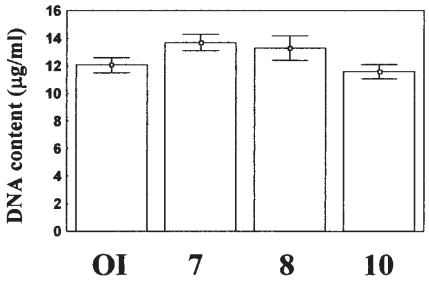


Figure 8. DNA content in control OI cells (OI) and OI cells incubated for 24 h with the compounds [7], [8] and [10]. Mean values ± SD from three independent experiments performed in duplicate are presented.

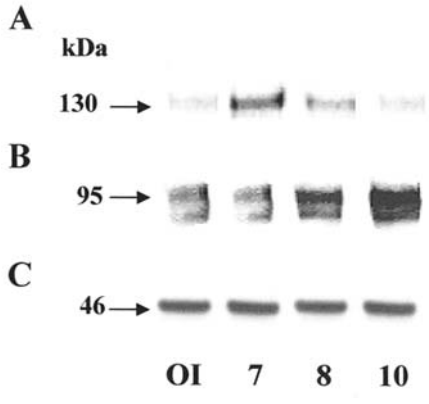


Figure 9. Western immunoblot analysis for IGF-I receptor subunit (A), ß1-integrin subunit (B) and ß-actin (C) in untreated OI cells (OI) and OI cells incubated for 24 h with [7], [8] and [10]. Detection of ß-actin was carried out in order to provide the loading control. The arrows indicate the molecular mass of the standards.

Notably, [7] markedly induced expression of IGFR, but compounds [8] and [10] induced expression of ß1-integrin (Fig. 9).

Discussion

Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in a multitude of disease states most commonly cancer, cardiovascular diseases, and neurodegenerative disorders (8). Studies on the effects of flavonoids on collagen synthesis have suggested their potential therapeutic use in the treatment of various medical conditions centered on disrupted collagen metabolism, like delayed wound healing, excessive scarring or skin aging (9-11).

OI also known as 'brittle bone disease' because of the susceptibility of the affected individuals to fracture from the mildest trauma, exhibits a broad range of clinical severity, ranging from multiple fracturing and perinatal death to a mild form (1-3). The structural mutations in patients with types II, III and IV affect connective tissue through a dominant negative mechanism, in which the presence of the mutant chain in the extracellular matrix directly disorganizes and weakens the matrix (2,3). The goal of gene therapy for dominant negative disorders such as OI is selective suppression of the expression of the mutant allele in

connective tissues (4). On the contrary, in OI type I, where bone formation is defective because of a paucity of type I collagen caused by one null allele of the *COL1A1* gene (2,4-6), agents that augment collagen production could have a beneficial effect.

We found that in OI type I cells, which synthesized about half of the normal level of type I collagen, compounds [8] and [10] at 30 μM specifically increased collagen synthesis 1.9 and 2.1 times, respectively. Compound [7] was a less effective stimulator since it increased collagen synthesis by 63% of the control. It is known that disturbances in proline and lysine hydroxylation as well as lysine glycosylation of procollagen chains result in retarded folding and secretion, and consequently in increased intracellular degradation of procollagen (2,3,27,28). Secretion of procollagen into media by the compound-treated cells was normal or slightly increased which suggests that they did not affect either modification or folding of procollagen chains. Furthermore, pericellular processing of type I procollagen in the compound-treated OI cells yielded an earlier appearance of the intermediate pC-α1(I) form as compared with the control untreated cells, due to the faster secretion of procollagen and/or the faster cleavage by N-proteinase.

Some flavonoids have already been proven to be very effective in chelating ions and thus inhibiting MMPs, which are a family of proteolytic enzymes that can degrade all components of the extracellular matrix (8,22). In our study, the compounds with a stimulating effect on collagen biosynthesis did not influence the activity of collagenase, gelatinases or stromelysin. Additional glycosidic groups present on the flavone skeleton may affect the steric conformation and may result in lack of an inhibiting effect on the proteolytic activity.

Although extracellular collagenases initiate the breakdown of collagen, the final step of collagen degradation is catalyzed by intracellular prolidase. The biological function of prolidase (EC 3.4.13.9) involves the metabolism of collagen degradation products and proline recycling from imidodipeptides (mainly glycyl-proline) for collagen synthesis and cell growth (23). Thus prolidase providing proline for collagen biosynthesis may regulate the turnover of collagen and can be a rate limiting factor in the regulation of collagen production. In our study, the increase in prolidase activity was correlated with stimulation of collagen synthesis in OI cells cultured in the presence of the compounds. Prolidase activity responded to extracellular matrix metabolism through signal mediation by ß1-integrin and IGF-I receptors (24,25). Integrins are transmembrane receptors that are responsible for the recognition and adhesion of cells to extracellular matrix proteins. The interaction between integrin receptors and extracellular matrix proteins, e.g. collagen is implicated in the regulation of cellular gene expression, differentiation and cell growth (29). IGF-I is a peptide-growth factor involved in the regulation of normal and malignant cell growth, differentiation and development, and it is suggested to play an important role in anabolic modulation of collagen synthesis by changing gene expression and prolidase activity (25). IGF-I exerts its action through binding to its own transmembrane tyrosine kinase receptor, which signals by altering the phosphorylation state of target molecules, activating cellular kinases and nuclear transcription factors. We demonstrated that the stimulatory effect on collagen synthesis and prolidase activity of the compounds [8] and [10] was accompanied by a distinct increase in expression of ß1-integrin receptor. Compound [7] increased collagen synthesis and prolidase activity by different mechanism, probably by an increase in IGF-I receptor expression.

There is an emerging view that flavonoids act as modulators of intracellular signaling cascades (8,26). It was reported that in keloid-derived fibroblasts quercetin inhibited the expression of several key proteins in the IGF signaling pathway resulting in the inhibition of collagen synthesis (10). A number of studies have demonstrated that the structure of flavonoids determine whether or not they act as potent inhibitors or stimulators of signaling molecules. Structural comparison between [1] and [7] (Fig. 1) showed that the presence of glucuronic acid in position C-7 [7] is essential for stimulatory activity in collagen synthesis. However, the stimulating effect appeared much greater for [8] when glucuronic acid is methylated. Moreover, differences in just the absence or presence in the molecule of 'small' chemical groups may also confer different biological properties. In particular, the presence in the flavonoid skeleton of [8] of methoxyl group at C-3 [11] resulted in the disappearance of its stimulatory action on collagen. For compound [10], the spatial conformation with the presence of rutinosyl moiety at C-7 and methoxyl at C-6 is essential for its activity, since compound [9] without the metoxyl group at C-6 did not influence collagen synthesis and compound [5] without rutinosyl inhibited collagen synthesis.

The results of the present study suggest that apigenin 7-*O*-methylglucuronide and pectolinarin at the concentration of 30 μM are stronger stimulators of collagen synthesis than apigenin 7-*O*-glucuronide and that their stimulatory actions probably involve different mechanisms. Future study of these flavonoids for their potential therapeutic use in the treatment of medical conditions centered on diminished collagen synthesis such as osteogenesis imperfecta type I is required, and special consideration must be given to their potential toxic actions.

References

1. Sillence DO, Senn A and Danks DM: Genetic heterogeneity in osteogenesis imperfecta. J Med Genet 16: 101-116, 1979.
2. Prockop DJ: Mutations in collagen genes as a cause of connective-tissue diseases. N Engl J Med 326: 540-546, 1992.
3. Rauch F and Glorieux FH: Osteogenesis imperfecta. Lancet 363: 1377-1385, 2004.
4. Millington-Ward S, McMahon HP and Farrar GJ: Emerging therapeutic approaches for osteogenesis imperfecta. Trends Mol Med 11: 299-305, 2005.
5. Willing MC, Pruchno CJ, Atkinson M and Byers PH: Osteogenesis imperfecta type I is commonly due to a COL1A1 null allele of type I collagen. Am J Hum Genet 51: 508-515, 1992.
6. Galicka A, Surazynski A, Wolczynski S, Palka J, Popko J and Gindzienski A: Phenotype variability in a daughter and father with mild osteogenesis imperfecta correlated with collagen and prolidase levels in cultured skin fibroblasts. Ann Clin Biochem 42: 80-84, 2005.
7. Harborne JB and Baxter H (eds): The Handbook of Natural Flavonoids. Wiley & Sons, Chichester, 1999.
8. Havsteen BH: The biochemistry and medical significance of the flavonoids. Pharmacol Ther 96: 67-202, 2002.

9. Phan TT, Sun L, Bay BH, Chan SY and Lee ST: Dietary compounds inhibit proliferation and contraction of keloid and hypertrophic scar-derived fibroblasts *in vitro*: therapeutic implication for excessive scarring. J Trauma 54: 1212-1224, 2003.
10. Phan TT, See P, Tran E, Nguyen TT, Chan SY, Lee ST and Huynh H: Suppression of insulin-like growth factor signaling pathway and collagen expression in keloid-derived fibroblasts by quercetin: its therapeutic potential use in the treatment and/or prevention of keloids. Br J Dermatol 148: 544-552, 2003.
11. Kapoor M, Howard R, Hall I and Appleton I: Effects of epicatechin gallate on wound healing and scar formation in a full thickness incisional wound healing model in rats. Am J Pathol 165: 299-307, 2004.
12. Nazaruk J and Gudej J: Flavonoid compounds from the flowers of *Cirsium rivulare* (Jacq.) All. Acta Pol Pharm 60: 87-89, 2003.
13. Nazaruk J and Jakoniuk P: Flavonoid composition and antimicrobial activity of *Cirsium rivulare* (Jacq.) All. flowers. J Ethnopharmacol 102: 208-212, 2005.
14. Nazaruk J: Flavonoid aglycones and phytosterols from the *Erigeron acris* L. herb. Acta Pol Pharm 63: 317-319, 2006.
15. Peterkofsky B, Chojkier M and Bateman J: Determination of collagen synthesis in tissue and cell culture system. In: Immunochemistry of the Extracellular Matrix. Furthmay M (ed). CRC Press, Boca Raton, FL, pp19-47, 1982.
16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
17. Carmichael J, Degraff W, Gazdar A, Minna J and Mitchell J: Evaluation of a tetrazolinium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936-942, 1987.
18. Unemori EN and Werb Z: Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. J Cell Biol 103: 1021-1031, 1986.
19. Myara I, Charpentier C and Lemonnier A: Optimal conditions for prolidase assay by proline colorimetric determination: application to imidodipeptiduria. Clin Chim Acta 125: 193-205, 1982.
20. Chinard FP: Photometric estimation of proline and ornithine. J Biol Chem 199: 91-95, 1952.
21. Labarca C and Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102: 344-352, 1980.
22. Sartor L, Pezzato E, Dell'Aica I, Caniato R, Biggin S and Garbisa S: Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design. Biochem Pharmacol 64: 229-237, 2002.
23. Phang JM and Scriver CR: Disorders of proline and hydroxyproline metabolism. In: The Metabolic Basis of Inherited Disease. Scriver CR, Beaudet AL, Sly WS and Valle D (eds). McGraw Hill, New York, pp577-597, 1989.
24. Palka JA and Phang J: Prolidase activity in fibroblasts is regulated by interaction of extracellular matrix with cell surface integrin receptors. J Cell Biochem 67: 166-175, 1997.
25. Miltyk W, Karna E, Wolczynski S and Palka J: Insulin-like growth factor-I-dependent regulation of prolidase activity in cultured human skin fibroblasts. Mol Cell Biochem 189: 177-184, 1998.
26. Williams RJ, Spencer JP and Rice-Evans C: Flavonoids: Antioxidants or signaling molecules? Free Radic Biol Med 36: 838-849, 2004.
27. Galicka A, Wolczynski S, Anchim T, Surazynski A, Lesniewicz R and Palka J: Defects of type I procollagen metabolism correlated with decrease of prolidase activity in a case of lethal osteogenesis imperfecta. Eur J Biochem 268: 2172-2178, 2001.
28. Galicka A, Wolczynski S, Gindzienski A, Surazynski A and Palka J: Gly511 to Ser substitution in the COL1A1 gene in osteogenesis imperfecta type III patients with increased turnover of collagen. Mol Cel Biochem 248: 49-56, 2003.
29. Labat-Robert J and Robert L: Interaction between cells and extracellular matrix: signaling by integrins and the elastin-laminin receptor. Prog Mol Subcell Biol 25: 57-70, 2000.