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Ginsenoside Rb_1 induces type I collagen expression through peroxisome proliferator-activated receptor-delta

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ABSTRACT

Wrinkle formation is one of the primary characteristics of skin aging, the major cause of wrinkle is the loss of structural protein type I collagen in dermal layer of skin. Topical application of natural substances to reduce wrinkle is gaining attention in recent years. Although a number of polyphenoic compounds are suggested to prevent ultraviolet-induced wrinkle, very few of them are able to increase type I collagen synthesis directly. Ginseng has been known in folk medicine of its beneficial effect to skin. The present study investigate the effect of ginsenoside on type I collagen induction in human dermal fibroblasts. Ginsenoside Rb_1 was shown to induce type I collagen expression in dermal fibroblasts in a dose- and time-dependent manner. Recent studies suggest the important post-transcriptional regulatory role of microRNAs; here we demonstrated that miR-25 can directly inhibit type I collagen protein expression, and treatment of fibroblasts with Rb_1 can reduce the inhibition by decreasing miR-25 level. Furthermore, we identified that the nuclear receptor, peroxisome proliferator-activated receptor-delta (PPARδ) is the key mediator of Rb_1-induced type I collagen expression. Knockdown of PPARδ by small-interference RNA abolished the Rb_1-induced type I collagen production and reversed the Rb_1-suppressed miR-25 expression. These results demonstrated that ginsenoside Rb_1 can increase target gene expression through transcriptional pathway, at the same time, inhibit the corresponding miRNA expression to minimize the translation repression. Furthermore, this study provide solid support of ginsenoside Rb_1-induced type I collagen expression, which warrant further study in the dermatological application of ginsenosides in skin disorders.

1. Introduction

Type I collagen is the most abundant protein of the extracellular matrix, which provides structural mechanical support to surrounding tissues. Progressive decline of type I collagen synthesis in skin dermis attributes to the formation of wrinkles and aging skin [1]. Dermal fibroblast is responsible for the production of heterotrimeric type I procollagen, which consists two proα1(I) and one proα2(I) chains. Proα1(I) and proα2(I) are encoded by two different genes which are located on different chromosomes. Gene expression of type I collagen is highly regulated in both transcriptional and post-transcriptional manner. A number of well-characterized transcription factors such as SMADS, AP-1, SP-1 [2] and nuclear receptors including peroxisome proliferator-activated receptors (PPARs) [3] have been shown to interact with the regulatory elements of collagen genes.

PPARs are ligand-inducible transcription factors which regulate gene expression by forming heterodimer with retinoid X receptor (RXR) and binding to PPAR response elements (PPRE) of the target genes. PPARs control diverse metabolic functions and inflammatory processes, but recent studies demonstrated that PPARs also play an important role in skin homeostasis. Among the three identified PPAR isoforms, PPARδ is ubiquitously expressed in many tissues including dermal fibroblasts and a variety of skin cells. It has been shown that PPARδ controls keratinocyte proliferation, adhesion, and migration during wound healing [4]. PPARδ also regulates transformation growth factor-β1 (TGF-β1) gene expression [5] and promotes wound healing by up-regulating extracellular matrix proteins synthesis including type I collagen [6].

MicroRNAs (miRNAs) are small noncoding RNAs (~22 nucleotides) that coordinate diverse cellular functions. MiRNA functions primary by imperfect base pairing to the 3′-untranslated region (3′-UTR) of target mRNAs. The imperfect base pairing also implicated that a single miRNA is potentially capable of binding to multiple targets. After binding to target mRNA, miRNAs can promote the decay of mRNA or translation inhibition; the post-translational regulation by miRNA is serving as a fine-tune mechanism of gene expression. Recent studies revealed the role of miRNAs on maintaining skin homeostasis [7]. In particular, it was shown that miR-29 family are negative regulators of type I

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collagen in both hepatic stellate cells and cardiac fibroblasts [8,9]. However, the upstream regulation of miRNAs is largely unknown.

Panax ginseng C. A. Meyer is widely used as general tonic to improve vitality. Large number of ginseng-based skin care products can also be found in the market. Ginsenosides are the pharmacological active phytochemicals of ginseng. They are classified into three categories: protopanaxadiols, protopanaxatriols and oleanolic acid derivatives [10]. Beneficial effects of ginseng on skin have been previously described; ginseng saponins isolated from red ginseng roots particularly Rb_1, increases neovascularization by up-regulation of vascular endothelial growth factor and interleukin-1β surrounding the burn area [11]. Also, it was also shown that Korean ginseng extract can inhibit collagen degradation after ultraviolet irradiation on mice skin [12]. Moreover, it was also demonstrated that *Panax ginseng* extract can induce human type I collagen synthesis through activation of SMAD signaling [13]. Therefore, in this study, we attempted to further elucidate the molecular mechanism of ginsenoside-induced type I collagen expression, and our data demonstrated that ginsenoside Rb_1 is a potent type I collagen inducer which is associated with PPARδ activation and suppression of miR-25 expression.

2. Materials and methods

2.1. Materials

Cell culture medium DMEM was purchased from Invitrogen (Carlsbad, CA, USA). Anti-COL1A2 (C-19) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ginsenosides with approximately 98% purity were obtained from Fleton (Chengdu, China); the purities of all ginsenosides are verified by high-performance liquid chromatography and thin-layer chromatography.

2.2. Cell culture and treatment

Human neonatal dermal fibroblasts (HDFs) were obtained from Lonza (Walkersville, MD, USA). Cells with cumulative population doubling between 20 and 35 were used. Cells were cultured in DMEM supplemented with fetal bovine serum (FBS) (10%, v/v) and penicillin/steptomycin (1%, v/v), and maintained at 37 °C in a humidified 5% CO_2 incubator. Cells were incubated with serum-free DMEM for 24 h before drug treatment.

2.3. Procollagen type I enzyme immunoassay (EIA)

Human dermal fibroblasts (2×10^4 cells) were seeded onto 96-well plates overnight. Cells were then treated with ginsenosides for another 24 h. The collagen content in the medium was determined by procollagen type I EIA (Takara, Shiga, Otsu, Japan). Absorbance was measured at 450 nm using a microplate reader (Infinite F200, Tecan, Männedorf, Switzerland). The actual amount of type I collagen was determined by comparing the absorbance of sample with the procollagen type I carboxy-terminal peptide standard. Experiments were performed in triplicate.

2.4. Western blot analysis

To study the effect of ginsenosides on type I collagen expression, supernatant and lysate of HDFs were harvested after drug treatment. Cells were washed with ice-cold PBS twice, and lysed by ice-cold cell lysis buffer (Novagen, USA) supplemented with protease inhibitor cocktail (0.5%, v/v) and phosphatase inhibitor cocktail (0.5%, v/v) (Calbiochem, San Diego, CA, USA). The cells were scraped off by rubber policeman, and the protein in cell lysate was collected after centrifugation. The concentration of protein was determined by Dc protein assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein was separated by 10% SDS-PAGE followed by electroblotting onto nitrocellulose membrane. The membrane was blocked with blocking buffer and then incubated with primary antibody overnight. The washed membrane was then further incubated with horseradish peroxidase-conjugated rabbit-anti-goat IgG secondary antibody (Invitrogen). The protein band was visualized by Chemiluminescent Western Detection Kit (Bio-Rad).

2.5. Cell viability assay

HDFs (1×10^4 cells/well) were seeded onto 96-well plates overnight. After drug treatment for the indicated time, cell proliferation was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incubated with MTT solution (0.5 mg/ml) in DMEM for 4 h. The formazan product was solubilized by DMSO after complete removal of medium; absorbance at wavelength 540 nm and 690 nm (reference) was measured using the microplate reader (Infinite F200, Tecan, Männedorf, Switzerland).

2.6. Real-time PCR for mRNA quantitation

Total RNA was extracted from HDFs using TRIzol (Invitrogen) according to the manufacturer's protocol. The RNA was reverse transcribed to complementary DNA (cDNA) using Superscript III first-strand synthesis system (Invitrogen). mRNA levels were quantified by real-time RT-PCR using Brilliant SYBR green QPCR master mix (Stratagene, La Jolla, CA, USA) and Mx3000P real-time PCR machine (Stratagene). The relative expression of target gene was calculated by ΔΔCt method, normalized with housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared with negative control.

2.7. TaqMan miRNA assay

For miRNA measurement, cDNA was synthesized using 10 ng of total RNA and TaqMan MicroRNA reverse transcription stem-loop primer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using TagMan universal PCR master mix reagent from Applied Biosystems with Mx3000P PCR machine (Stratagene). The level of U6 RNA was determined in parallel and used to normalize the relative expression of mature hsa-miR-25.

2.8. PPARδ time-resolved fluorescence resonance energy transfer (TM-FRET) competitive binding assay

PPARδ LanthaScreen™ TR-FRET competitive binding assay (Invitrogen) was used to study the binding affinity of ginsenoside Rb_1 to PPARδ. This assay allowed screening of ligands for PPARδ. The principle of this method is that when two suitable fluorophores are brought into close proximity, excitation of the donor fluorophore can result in energy transfer to the acceptor second fluorophore. This energy transfer resulted in an increase in the fluorescence emission of the acceptor and a decrease in fluorescence emission of the donor. In this assay, the GST-tagged PPARδ ligand-binding domain (LBD) was combined with terbium-labeled anti-GST antibody. When a green fluorescent pan-PPAR ligand (tracer) is bound to the receptor, energy transfer from the antibody to the tracer occurs, and a high TR-FRET ratio is observed. The ability of GW0742 (1 pM–100 μM), a PPARδ specific agonist, and ginsenoside Rb_1 (10 nM–100 μM) to displace the tracer from the LBD was tested, which results in a loss of FRET ratio. The