

## *Panax ginseng* induces human Type I collagen synthesis through activation of Smad signaling

Jongsung Lee<sup>a,b</sup>, Eunsun Jung<sup>a</sup>, Jiyoung Lee<sup>a</sup>, Sungran Huh<sup>a</sup>, Jieun Kim<sup>a</sup>, Mijung Park<sup>a</sup>, Jungwoon So<sup>a</sup>, Younggeun Ham<sup>a</sup>, Kwangseon Jung<sup>a</sup>, Chang-Gu Hyun<sup>a</sup>, Yeong Shik Kim<sup>b</sup>, Deokhoon Park<sup>a,\*</sup>

<sup>a</sup> Biospectrum Life Science Institute, 101-701 SK VENTUM, 522 Dangjung Dong, Gunpo City, 435-833 Gyunggi Do, Republic of Korea

<sup>b</sup> Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul, Republic of Korea

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### Abstract

Skin aging appears to be principally related to a decrease in levels of Type I collagen, the primary component of the dermal layer of skin. It is important to introduce an efficient agent for effective management of skin aging; this agent should have the fewest possible side effects and the greatest wrinkle-reducing effect. In the course of screening collagen production-promoting agents, we obtained *Panax ginseng* C.A. Meyer. This study was designed to investigate the possible collagen production-promoting activities of *Panax ginseng* C.A. Meyer root extract (PGRE) in human dermal fibroblast cells. As a first step to this end, human COL1A2 promoter luciferase assay was performed in human dermal fibroblast cells. In this assay, PGRE activated human COL1A2 promoter activity in a concentration-dependent manner. Human Type I procollagen synthesis was also induced by PGRE. These results suggest that PGRE promotes collagen production in human dermal fibroblast cells. Additionally, we have attempted to characterize the mechanism of action of PGRE in Type I procollagen synthesis. PGRE was found to induce the phosphorylation of Smad2, an important transcription factor in the production of Type I procollagen. When applied topically in a human skin primary irritation test, PGRE did not induce any adverse reactions. Therefore, based on these results, we suggest the possibility that PGRE may be considered as an attractive, wrinkle-reducing candidate for topical application.

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**Keywords:** Human Type I procollagen; COL1A2 promoter; Smad2; *Panax ginseng*; Irritation

### 1. Introduction

Aging of the skin is fundamentally related to reductions in the levels of Type I collagen, the principal component of the dermal layer of the skin. Type I collagen is the main structural component of the extracellular matrix (ECM), which is known to perform a pivotal function in maintaining the structure of the dermis. Several molecules have been reported to augment Type I collagen synthesis, namely, transforming growth factor- $\beta$  (TGF- $\beta$ ), asiaticoside, and sphingosine 1-phosphate (Markus et al., 2000; Cuiyan et al., 2004; Lu et al., 2004).

Collagen synthesis is regulated both transcriptionally and post-translationally. Several studies have reported that the Smad

pathway functions in the activation of Type I collagen gene expression. The Smads are a series of proteins that perform downstream functions from the serine/threonine kinase receptors of the TGF- $\beta$  family, thereby transducing signals to the nucleus (Piek et al., 1999; Attisano and Wrana, 2000; Massagué and Wotton, 2000).

In Asia, ginseng has a long history of traditional medicinal use as a general health-promoting tonic. There are extensive reports which have determined that ginseng has many pharmacological effects on the immune, cardiovascular, endocrine, and central nervous systems (Nah et al., 1995; Attele et al., 1999). A blood glucose-lowering effect of ginseng root has also been found (Sotaniemi et al., 1995; Chung et al., 2001). However, despite the various reported functions of ginseng, no studies have yet reported the effects of ginseng on skin aging. Chemically, the constituents of ginseng can be divided into saponin and non-saponin fractions. The saponins can be further classified

\* Corresponding author. Tel.: +82 31 436 2090; fax: +82 31 436 0605.  
E-mail address: [pdh@biospectrum.com](mailto:pdh@biospectrum.com) (D. Park).

into three major groups: protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanane saponins. The PPD group includes Rb1, Rb2, Rc, Rd, and Rh2, while the PPT group includes Re, Rf, Rg1, Rg2, and Rh1. The oleanane saponins include the Ro. 20(S)-PPT group, which is an aglycone of the PPT group, and the 20(S)-PPD group, an aglycone of the PPD group (Han et al., 2006).

In this study, in order to assess the anti-wrinkle effects of PGRE, we investigated the collagen production-inducing effect of PGRE and its action mechanism in *in vitro* experiments and the possible use of PGRE as a topical agent for the management of skin wrinkling.

## 2. Materials and methods

### 2.1. Materials

Anti-phospho-Smad2 (Ser465/467) antibody was obtained from Cell Signaling Technology Inc. (Beverly, MA). Smad2 (S-20) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). *Panax ginseng* C.A. Meyer (6 years) was kindly supplied by the Korea Ginseng and Tobacco Research Institute. The COL1A2 luciferase reporter (COL1A2-Luc) plasmid was constructed through the fusion of the human COL1A2 promoter region into pLuc vector (Stratagene). The COL1A2-Luc contains 376 bp of the  $\alpha 2(I)$  collagen (COL1A2) promoter and 58 bp of the transcribed sequence.

### 2.2. Preparation of *Panax ginseng* C.A. Meyer root extract (PGRE)

A 1.000 g sample of the powered root of *Panax ginseng* C.A. Meyer was weighed in a cellulose cartridge and extracted in a Soxtec HT2 apparatus (Tecator, Hoganas, Sweden) with 95 ml of dichloromethane (boiling period: 60 min; rinsing period: 15 min; drying period: 45 min). The dichloromethane extract was discarded. During a second step, the ginsenosides were then extracted from the powder in the same cartridge with 95 ml of 96% ethanol (boiling period: 90 min; rinsing period: 90 min). The ethanol extract was evaporated to dryness under reduced pressure. The residue was added to 25 ml of water and sonicated for 5 min. The resulting aqueous solution or suspension was introduced into a separating funnel and extracted three times with 8 ml of 1-butanol; a centrifugation step was sometimes required. The butanol extracts were combined and adjusted to 50 ml with methanol. The isolation of ginsenosides from *Panax ginseng* involved either liquid or dry extracts, according to their composition, ethanol distillation under reduced pressure, after dissolution in water or dilution with water, the partition with 1-butanol.

### 2.3. Determination of ginsenosides

The freeze-dried extract was dissolved in 20% (v/v) acetonitrile/water and filtrated through a 0.45 mm membrane filter for analysis of ginsenosides by HPLC (Li et al., 1996).

### 2.4. Cell lines and cell culture

Human dermal fibroblast cells (derived from neonatal foreskin) were acquired from the Amore-Pacific Corporation R&D Center, which is located in the Republic of Korea. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (GibCo, MD) and penicillin–streptomycin at 37 °C in a humidified atmosphere containing 95% air/5% CO<sub>2</sub>.

### 2.5. Transfection and luciferase reporter gene assay

Human dermal fibroblast cells were transiently transfected with 2  $\mu$ g of firefly luciferase reporter gene under the control of COL1A2 responsible elements and 0.2  $\mu$ g of Renilla luciferase expression vector driven by thymidine kinase promoter (Promega) and Superfect reagent (Invitrogen) (Lee et al., 2005). The transfected cells were transferred to 6-well plates and incubated for 24 h at a density of  $8 \times 10^5$  cells/ml. After 24 h, the cells were further cultured in the presence or absence of PGRE for 5 h. Luciferase activity was determined using a Dual Luciferase Assay system (Promega) and an LB953 luminometer (Berthold, Germany) and was expressed as a ratio of COL1A2-dependent firefly luciferase activity divided by control thymidine kinase Renilla luciferase activity (relative luciferase unit). Results were confirmed by three independent transfections.

### 2.6. Quantitative detection of Type I collagen

The quantity of Type I collagen in the cells was determined using a commercially available kit (Takara Bio Inc., Japan). This kit is capable of detecting procollagen Type I carboxy-terminal peptide (PIP) using polyclonal antibodies, rather than through the direct measurement of collagen. Human dermal fibroblast cells were then incubated in either the presence or absence of PGRE or TGF- $\beta$  for 24 h. The culture supernatants were then harvested and measured with a sandwich immunoassay kit, which was utilized in accordance with instructions of the manufacturer (Takara Bio Inc., Japan). The measurement was performed with a microplate at 450 nm (Lee et al., 2006).

### 2.7. Cytotoxicity assay

Human dermal fibroblast cells were cultured in DMEM containing 10% fetal bovine serum and  $1 \times$  antibiotic solution. Cells were incubated at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were seeded on 24-well plates and drug treatment was initiated 24 h after seeding. General viability of cultured cells was determined by the reduction of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazam (Mosmann, 1983). After the incubation of human dermal fibroblast cells treated with various concentrations of PGRE for 24 h at 37 °C in 5% CO<sub>2</sub> atmosphere, the MTT assay was performed. MTT (1 mg/ml in PBS) was added to each well at 1/10 of the volume of the media. Cells were incubated at 37 °C for 3 h and harvested by centrifugation. Dimethyl sulfoxide (DMSO) was then added in

order to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a spectrophotometer (Power Wave, Bio-Tek Inc.).

### 2.8. Immunoprecipitation of Smad proteins

Smad immunoprecipitation and blotting were conducted as previously described (Lee et al., 2006). In brief, the fibroblast cells were seeded in 6-well plates and cultured for 24 h. The medium was then replaced with HEPES buffer (1 M) for 2 h, and the cells were subsequently treated with TGF- $\beta$ , asiaticoside, or PGRE for 30 min. The fibroblast cells were rinsed twice in ice-cold PBS and harvested in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM NaF, and 40 mM  $\beta$ -glycerophosphate). The lysates were centrifuged at 14,000  $\times$  g for 30 min. About 100  $\mu$ g of lysate proteins were immunoprecipitated overnight at 4 °C with 0.2  $\mu$ g of either of the anti-Smad2 antibodies, followed by precipitation using 10  $\mu$ l of protein G plus agarose at 4 °C for 90 min. After four washings with complete RIPA buffer, the immunoprecipitates were eluted by 5 min of boiling in 60  $\mu$ l of SDS sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol).

### 2.9. Western blot analysis

In the Western blot analysis performed in this study, cell lysates were separated via SDS-PAGE. The gels were blotted overnight on polyvinylidene difluoride membranes and then exposed to the appropriate antibodies. Proteins were visualized with the ECL system from Amersham Biosciences using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody.

### 2.10. Human skin primary irritation test

Thirty healthy Korean subjects were studied, and written informed consent was obtained in each case. The average age was 25.1 years (range 21–29; all females). The subjects had no skin diseases, nor had they used topical or systemic irritant preparations during the previous month. All volunteers were non-smokers. The subjects received test materials that were formulated with petrolatum. The primary skin irritation test was performed using a Finn Chamber<sup>®</sup> secured to the back site with Scanpore tape. These chambers are made of inflexible aluminum, with a diameter of 8 mm and a depth of 0.5 mm. The round border of the chamber was placed firmly against the skin, causing a tight occlusion of the test materials. PGRE formulated with petrolatum was prepared and applied. The patches (chambers) stayed in place for 48 h. During this period, the subjects abstained from showering or performing any work or exercise that might wet or loosen the patches. After the patches were removed, a reading was done after 30 min and 1 day later; read-

ings were scored according to the criteria of the International Contact Dermatitis Research Group (ICDRG) (Park et al., 2004).

### 2.11. Statistical analysis

Non-parametric one-way analysis of variance (Kruskal–Wallis test) and Mann–Whitney tests were used for statistical analysis. Differences between groups were considered significant at \* $p < 0.05$ .

## 3. Results

### 3.1. Composition of ginsenosides of *Panax ginseng* C.A. Meyer root extract (PGRE)

As shown in Table 1, nine different types of ginsenoside, representing 45.14% of PGRE, were identified. Ginsenoside Rb1 (11.13%), ginsenoside Rb2 (13.39%), and ginsenoside Rc (10.01%) were the most abundant ginsenosides of PGRE. The other ginsenosides were Rd (3.64%), Re (1.05%), Rf (2.16%), Rg1 (0.35%), Rg2 (2.25%), and Rg3 (1.16%).

### 3.2. *Panax ginseng* C.A. Meyer root extract (PGRE) has anti-oxidant activity

Siwik et al. (2001) reported that oxidative stress is involved in the regulation of the quantity and quality of the extracellular matrix. To be precise, oxidative stress decreases collagen synthesis through the activation of matrix metalloproteinases (MMP). Therefore, in order to investigate whether *Panax ginseng* C.A. Meyer root extract (PGRE) has anti-oxidant properties, we carried out *in vitro* testing through a diphenyl-*p*-picrylhydrazyl (DPPH) scavenging assay. The DPPH test showed that PGRE has significant anti-oxidant activity (Fig. 1). Ascorbic acid (100  $\mu$ M) was employed as a positive control.

### 3.3. COL1A2 promoter activation is induced by PGRE

Significant progress has recently been made toward a better understanding of the expression of the human  $\alpha$ 2(I) collagen

Table 1  
Composition of *Panax ginseng* C.A. Meyer root extract (PGRE) used in this study

Types of ginsenosides	Content (%)
Rb1 <sup>a</sup>	11.13
Rb2 <sup>a</sup>	13.39
Rc <sup>a</sup>	10.01
Rd <sup>a</sup>	3.64
Re <sup>b</sup>	1.05
Rf <sup>b</sup>	2.16
Rg1 <sup>b</sup>	0.35
Rg2 <sup>b</sup>	2.25
Rg3	1.16
Total content of ginsenosides	45.14
PD/PT	6.76

<sup>a</sup> Protopanaxadiol (PPD).

<sup>b</sup> Protopanaxatriol (PPT).

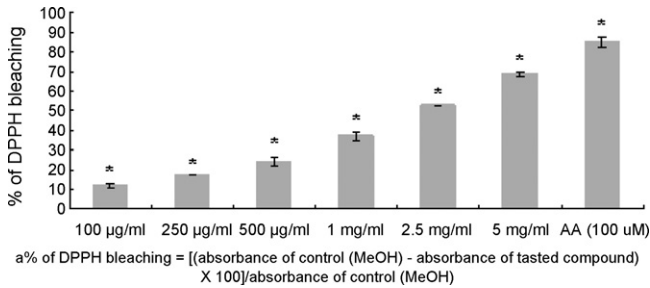


Fig. 1. *In vitro* anti-oxidant activities of *Panax ginseng* C.A. Meyer root extract (PGRE), as determined by DPPH assay. Data are expressed as means  $\pm$  S.D. \* $p$  < 0.05 compared with a control. Results were confirmed by an experiment that was repeated four times in triplicate; A.A, ascorbic acid.

(COL1A2) gene and its transcriptional regulation by cytokines and growth factors. The Smads are a series of transcription factors that function to enhance the transcription of human  $\alpha$ 2(I) collagen through the activation of COL1A2 promoter. As a preliminary step to determine whether PGRE affects collagen production, we performed a COL1A2 luciferase assay. As shown in Fig. 2, PGRE increased COL1A2 reporter activity by three-fold in a concentration-dependent manner. This result suggests the possibility that PGRE may be involved in the production of human  $\alpha$ 2(I) collagen.

#### 3.4. Effects of PGRE on Type I procollagen synthesis

We further studied the effect of PGRE on the production of Type I procollagen synthesis. As shown in Fig. 3, consistent with the finding in Fig. 2, PGRE significantly increased the production of Type I procollagen in a concentration-dependent manner, confirming that PGRE induces the production of Type I procollagen and also suggesting a collagen production-inducing function of PGRE through activation of COL1A2 promoter. TGF- $\beta$  was employed as a positive control.

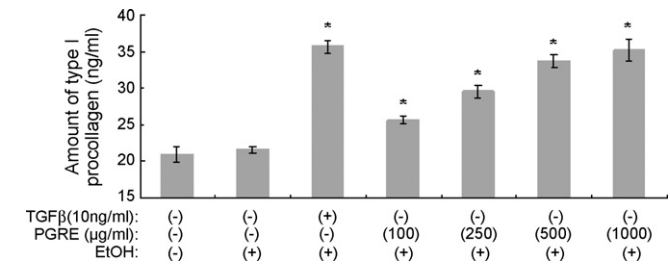
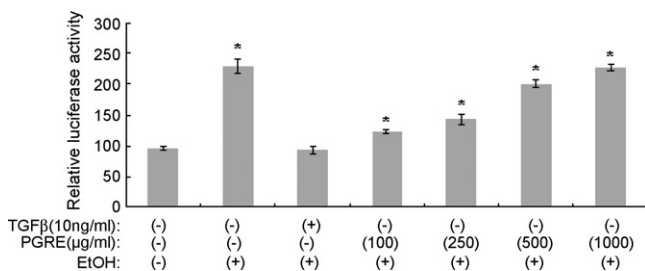


Fig. 3. Effects of PGRE on Type I procollagen synthesis, as determined using a sandwich immunoassay kit (Takara Bio Inc., Japan). Data are expressed as the means  $\pm$  S.D. \* $p$  < 0.05 compared with controls. The results were verified by the repetition of three experiments, each in triplicate.

#### 3.5. Effects of PGRE on the phosphorylation of Smad2 in human dermal fibroblast cells

The Smad signaling cascade is known to perform an important function in the human collagen production events associated with TGF- $\beta$  or asiaticoside (Cuiyan et al., 2004; Lee et al., 2006). Therefore, as an initial step toward the elucidation of the action mechanism of PGRE on Type I collagen synthesis, we examined its effects on the phosphorylation of Smad2. As shown in Fig. 4, Smad2 phosphorylation could be induced by treatment with PGRE. TGF- $\beta$  and asiaticoside were employed as positive controls.

#### 3.6. PGRE has relatively low cytotoxic effects in human dermal fibroblast cells

We examined the cytotoxic effects of PGRE on human dermal fibroblast cells (Fig. 5). While PGRE at concentrations lower than 100  $\mu$ g/ml showed almost 100% cell viability, PGRE at 1 mg/ml showed over 95% viability of human dermal fibroblast cells. As in human dermal fibroblast cells, PGRE did not show significant cytotoxic effects in HaCaT cells, which represent a human keratinocyte cell line (data not shown). These data suggest that PGRE has low cytotoxic properties against mammalian cells.

#### 3.7. Human skin primary irritation test of PGRE

To evaluate the irritating effect of PGRE for clinical applications to human skin, a patch test was performed. In our study, as

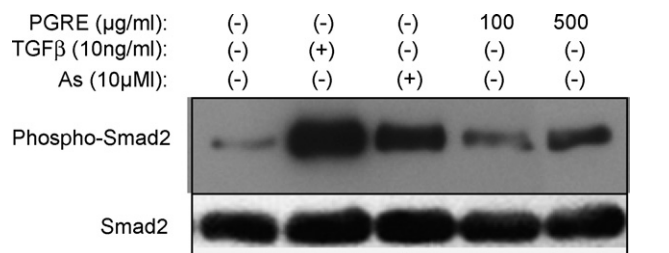


Fig. 4. Effects of PGRE on the phosphorylation of Smad2 in human dermal fibroblast cells. Cells were treated with TGF- $\beta$  (10 ng/ml), asiaticoside (10  $\mu$ M), or the indicated concentrations of PGRE for 30 min. The cells were subjected to immunoprecipitation with anti-Smad2 antibodies, followed by Western blot with anti-phosphoserine antibodies. \* $p$  < 0.05 compared with untreated control; As, asiaticoside.

Fig. 2. *Panax ginseng* C.A. Meyer root extract (PGRE) activates human COL1A2 promoter activation. Human dermal fibroblast cells were transfected with COL1A2-Luc using Superfect<sup>TM</sup>. After incubation for 24 h, cells were stimulated for 14 h by indicated concentrations of PGRE, harvested, and lysed. Supernatants were assayed for luciferase activity, which is determined by the amount of light produced through the conversion of luciferin substrate into oxyluciferin by luciferase. Relative luciferase activity, calculated as the relationship between luciferase activity and the quantity of cells or the efficiency of the transfection, was determined three times in duplicate for each experiment, and the standard deviation is indicated as a bar. All values were significant (\* $p$  < 0.05) compared with values for untreated control.

Table 2  
The results of human skin primary irritation test ( $n = 30$ )

No.	Test material	48 h					72 h					Reaction grade <sup>a</sup>		
		±	1+	2+	3+	4+	±	1+	2+	3+	4+	48 h	72 h	Mean
1	Petroleum	– <sup>b</sup>	–	–	–	–	–	–	–	–	–	0	0	0
2	PGRE (100 mg/ml) <sup>c</sup>	–	–	–	–	–	–	–	–	–	–	0	0	0
3	PGRE (1 mg/ml)	–	–	–	–	–	–	–	–	–	–	0	0	0
4	PGRE (10 mg/ml)	–	–	–	–	–	–	–	–	–	–	0	0	0
5	PGRE (20 mg/ml)	–	–	–	–	–	–	–	–	–	–	0	0	0

<sup>a</sup> Reaction grade =  $\sum[(\text{grade} \times \text{number of responders}) / \{4 (\text{maximum grade}) \times 30 (\text{total subjects})\}] \times 100 \times (1/2)$ .

<sup>b</sup> No reaction.

<sup>c</sup> PGRE, *Panax ginseng* C.A. Meyer root extract.

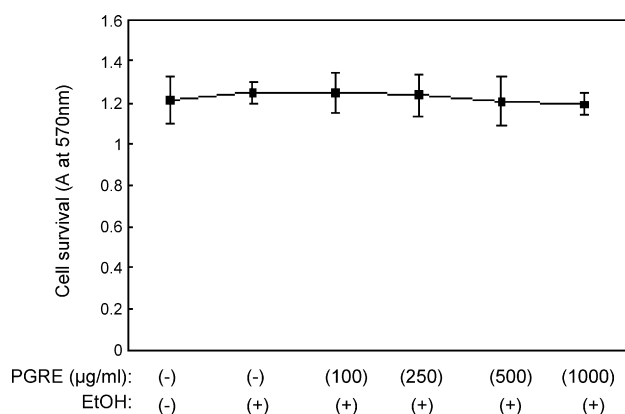


Fig. 5. Cytotoxicity of PGRE against human dermal fibroblast cells. Human dermal fibroblast cells were cultured for 24 h in medium in either the presence or absence of PGRE. Cellular cytotoxicity was determined according to a rapid colorimetric MTT assay. Data are expressed as means  $\pm$  S.D. \* $p < 0.05$  compared with controls. The results were verified by the repetition of three experiments, each in triplicate.

shown in Table 2, none of the 30 subjects experienced reactions based on the 30 min and 1 day readings. More specifically, no adverse reactions related to the topical treatment of PGRE, such as erythema, burning, or pruritus, were observed in the study subjects.

#### 4. Discussion and conclusions

To the best of our knowledge, this study is the first to attempt to elucidate the collagen synthesis-inducing effect of *Panax ginseng* root extract (PGRE) and the action mechanisms underlying PGRE-induced Type I collagen synthesis.

Although *Panax ginseng* has been extensively reported to have many pharmacological effects on the immune, cardiovascular, endocrine, and central nervous systems (Nah et al., 1995; Atte et al., 1999), its effect on skin aging has never been reported. Therefore, we have attempted to elucidate the effect of PGRE on Type I collagen, which is the primary component of the dermis and is fundamentally related to skin aging. As an initial step, we have attempted to determine whether or not PGRE can activate COL1A2 promoter in human dermal fibroblast cells, as well as whether it has an anti-oxidant effect. In this study, we found that PGRE has anti-oxidant activity and induces COL1A2 promoter activation. In addition, after PGRE

treatment, we observed that human Type I procollagen synthesis was induced by PGRE. These results indicate that PGRE induces human collagen production through the activation of COL1A2 promoter.

Smad2 phosphorylation is an initial molecular event of Smad signaling. It is well known that Smad signaling is involved with the collagen synthesis induced by TGF- $\beta$  or sphingosine 1-phosphate. In this study, we determined that Smad2 was phosphorylated by treatment with PGRE. This indicates that PGRE does, indeed, induce Type I collagen synthesis through the activation of Smad signaling.

TGF- $\beta$ -induced Smad phosphorylation is known to be mediated by TGF- $\beta$  receptor I kinase (T $\beta$ RI kinase) (Markus et al., 2000). Recently, signals derived from growth factor receptors that exhibited tyrosine kinase activity were also determined to modulate Smad-dependent effects. This may occur as the result of the activation of a kinase located downstream of MEK-1, and upstream of the MAPK/ERK kinase pathway, resulting in the phosphorylation of Smad2 (Brown et al., 1999). In addition, a host of other kinases have been implicated in Smad signaling, including TAK-1 and TAB, although their precise functions have yet to be elucidated (Yamaguchi et al., 1995; Shibuya et al., 1996; Shirakabe et al., 1997). When taken together, these data suggest that PGRE-induced Smad signaling may be mediated by TGF- $\beta$  receptor I kinase (T $\beta$ RI kinase) or other kinases that play the same role as T $\beta$ RI kinase.

Both the quantity and quality of extracellular collagen are determined by the balance that exists between degradation and synthesis (Dhalla et al., 1996). Degradation appears to be mediated by matrix metalloproteinases (MMPs), as well as by endogenous tissue inhibitors (TIMPs). Therefore, we conducted an MMP-1 activity assay to investigate the effect of PGRE on TNF- $\alpha$ -induced MMP-1 secretion in human dermal fibroblast cells. Our MMP-1 activity test revealed that PGRE significantly inhibits TNF- $\alpha$ -induced MMP-1 secretion (data not shown).

In conclusion, the data acquired in this study demonstrate that PGRE can induce the synthesis of Type I collagen, and the mechanisms underlying its action may be mediated via the Smad activation pathway.

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