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지도교수 김 석 화

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2013년 8월

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The Inhibitory Effect of Oligomeric Procyanidins(OPCs) on Procollagen Type I Secretion of Fibroblasts – In Vitro Study.

by

Jung-keun Park, M.D.

A Thesis Submitted to the Department of Plastic Surgery
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Department of Plastic Surgery
at the Seoul National University College of Medicine

August, 2013

Doctoral committee:
Professor _____, Chairman
Professor _____, Vice chairman
Professor _____
Professor _____
Professor _____

Abstract

The Inhibitory Effect of Oligomeric Procyanidins(OPCs) on Procollagen Type I Secretion of Fibroblasts – In Vitro Study.

Jung-Keun Park

College of Medicine, Plastic Surgery

The Graduate School

Seoul National University

Purpose: Wound healing is a very complex process that requires harmonies of various cell populations. It consists of complex series of events including inflammatory, proliferative, and remodeling phases. In final remodeling phase, fibroblasts play an important role of returning the wound to pre-injury state through collagen degradation. Oligomeric procyanidins (OPC) are main components of grape (Vitis vinifera) seed extracts and also found in wine and green tea. Recent studies showed OPC's effects on inflammation, cell migration and proliferation. From the results we can assume OPC can have effects in regulating wound healing process. The purpose of this study is to evaluate the effect of OPC on fibroblasts to regulate wound healing process

Materials and Methods: Human dermal fibroblast known as Hs27 cells, were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 ㎍/㎖) for 24, 48 and 72 hours. To determine cytotoxicity, cell viability was evaluated by the Cell Counting Kit assay (CCK-8 ; Dojindo Molecular Technologies, Rockville, MD, USA). The expression levels of intra and extra-cellular procollagen were analyzed by Western blot and real-time PCR. Gene

expression of molecular chaperone and enzymes regulating collagen synthesis were determined by real-time PCR, and also intracellular localization of procollagen was determined by immunocytochemistry in OPC treated cells exposed to TGF-β1 or ascorbic acid.

Results: OPC had no proliferative effect or cytotoxicity on Hs27 cells at every concentration. The mRNA expression of procollagen, molecular chaperone and enzymes were not affected by OPC. However, OPC inhibited procollagen secretion in dose-dependent manner. The inhibitory effect also appeared under TGF-β1 induced collagen overproduction. Immunocytochemistry showed that ascorbic acid, essential cofactor in catalyzing post-translational hydroxylation of the procollagen, induced release of accumulated procollagen under OPC treatment.

Conclusion: In conclusion, OPC inhibits procollagen secretion from fibroblasts with no effects on cell proliferations. OPC has no effects on cell proliferation, and can regulate the diseases and symptoms of abnormal overabundant collagen production.

Key words: Oligomeric procyanidins, fibroblasts, procollagen, ascorbic acid, Vitis vinifera seed extract, Collagen

Student number: 2009-30517

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Introduction

The wound healing process consists of complex series of events including inflammatory, proliferative, and remodeling phases. During these processes, various kinds of cells and factors participate in generating new blood vessels, promoting granulation tissue formation, and enhancing re-epithelialization (1, 2). In final remodeling phase, dermis returns to its pre-injury states with production of collagen and extracellular matrix proteins (3). Collagen is the most abundant protein in animal tissues (4) and forms the major part of the extracellular dermal matrix of skin. Abnormal overproduction of collagen can cause keloid, hypertrophic scar or fibrosis during wound healing process. Keloids are characterized by overproduction of types I and III collagen by fibroblasts (5), and hypertrophic scar is generated from excessive dermal fibrosis and scarring. Kai Zhang (6) showed that fibroblasts producing about seven times more collagen were found in hypertrophic scar patients.

Oligomeric procyanidins (OPCs) are the main components of grape (Vitis vinifera) seed extracts and also called proanthocyanidins. Procyanidins, a group of flavonoids (Figure 1A), are oligomeric forms of catechins that are abundant in red wine, grapes, cocoa, tea and apples and are thought to have beneficial effects on human health. Grape seed extract contains OPCs made up of dimers or trimers of (+)-catechin and (-)-epicatechin (Figure 1B) (7). From class of nutrients belonging to the flavonoid family, it also can be found in apples, maritime pine bark, cinnamon, cocoa beans. OPCs are well known for its antioxidant, anti-bacterial, and anti-viral properties (8-10), and recent studies suggested that OPC can also regulate wound healing process. OPC showed anti-inflammatory effect by inhibiting nitric oxide and prostaglandin E2 production, and suppressing iNOS expression (11). García-Conesa and coworkers (12) reported that OPC inhibits cell migration and modulates proliferation of human umbilical vascular endothelial cells. They also reported that OPC has cardioprotective activity in myocardial infarction model (13), and gives regulatory effect on proliferation and migration of endothelial

cells (14).

However, there has not been any research regarding the effect of OPC on fibroblasts which are one of the most abundant cell populations of human body, especially in the skin. On the other hand, we found that wound healing was delayed by OPC treatment in the full-thickness skin defect animal model. Its histological analysis showed more immature epithelialization with decreased collagen deposition and increased inflammation compared to the control group. The purpose of this study is to evaluate the regulatory mechanisms of OPC focusing on collagen production.

Materials and Methods

1) Cell culture

Normal human fibroblasts cell line, Hs27, was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured at 37° C in a humidified atmosphere containing 5% CO2, with Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 100 units penicillin and 100 ㎍ streptomycin (all from Gibco BRL, CA, USA). Cells in passages between 25 and 28 were used for all experiments.

2) Cell viability assay

To determine cytotoxicity of OPC, Hs27 cells in the exponential phase were seeded at 2 × 104 cells/well in 24-well plates and were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 ㎍/㎖) for 24, 48 and 72 hours. Cell viability was then determined by the CCK assay.

3) OPC treatment

The Hs27 cells were seeded at 2×105 cells per well in 6-well plates. After serum starvation for 24 hours, cells were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 ㎍/㎖). The cell culture supernatants were collected from each well at 24 or 48 hours and procollagen expression level was determined by Western blot.

To evaluate the time course of procollagen secretion, we performed additional experiment using TGF-β1. Non-treatment group served as control group (Group 1), and three experimental

groups were set - OPC(10 ㎍/㎖) treatment group (Group 2), TGF-β1 (10 ng/㎖) treatment group (Group 3), and combination of OPC pretreatment (10 ㎍/㎖for 1 hour) and TGF-β1 (10 ng/㎖) treatment (Group 4). . Cell supernatants were collected at 6, 12, and 24 hours and were analyzed by Western blot.

For evaluation of accumulation of procollagen in cytoplasm, we obtained cell lysate or Hs27 cells by 10-minute 4% paraformaldehyde fixation at room temperature after 24 hours of OPC treatment.

4) Ascorbic acid treatment

The Hs27 cells were seeded 2×105 cells per well in 6-well plate. After serum starvation, cells were pretreated with 10 μg/㎖ OPC for 1 hour. Then, 0.1 mM of ascorbic acid (Sigma-Aldrich, USA) was added and incubated for additional 24 hours. Cell supernatant, lysate, and fixed cells were analyzed by Western blot and immunocytochemistry, respectively.

5) Real-time PCR

Total RNA were isolated with RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instruction. Total RNA concentrations were determined by absorbance at 260 nm. For RT-PCR, 1 ㎍ of mRNA was reverse-transcribed with oligo (dT) 18 using SuperScript III (Invitrogen, CA, USA). Then, real-time PCR was carried out with Applied Biosystems 7500 Real-time PCR system. The PCR mixture contained SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan) and the appropriate primers: COL1A1 forward, 5′-CCCGGGTTTCAGAGACAACTTC-3′; COL1A1 reverse, 5′-TCCACATGCTTTATTCCAGCAATC-3′; beta actin forward, 5′-

TGGCACCCAGCACAATGAA-3 ´ ; beta actin reverse, 5 ´ -CTAAGTCATAGTCCGCCTAGAAGCA-3 ´ ; P4H α (I) forward, 5'-CGTCTCCAGGATACCTACAA-3'; P4Hα(I) reverse, 5'-AAGCAGTCCTCAGCCGTTAG-3'; P4H β /PDI forward, 5'-CTCGACAAAGATGGGGTTGT-3' ; P4H β /PDI reverse 5'-GCAAGAACAGCAGGATGTGA-3' ; HSP47 forward, 5'-TGAAGATCTGGATGG GGAAG-3' ; HSP47 reverse, 5'-CTTGTCAATGGCCTCAGTCA-3' . Cycling parameters were 95° C for 30 seconds, followed by 40 cycles of 95° C for 5 seconds and 60° C for 34 seconds. Melting curve stage was performed at 95° C for 15 seconds, 60° C for 1 minute and 95° C for 15 seconds. The level of gene expression was analyzed according to the comparative threshold cycle (Ct) method and calculated by 2- Δ Δ Ct.

6) Western blot analysis of procollagen type I

The Hs27cells culture supernatants and cell pellets were collected at indicated time from each well in order to measure procollagen secretion. Monoclonal procollagen type I N-terminal extension peptide (SP1.D8) antibody was obtained from culture media of hybridoma cell line (Developmental Studies Hybridoma Bank; Iowa City, IA, USA). The cell supernatants were separated by 10% SDS-PAGE, followed by electrophoretical transfer to PVDF membrane (Bio-Rad, Hercules, CA, USA). For protein detection, the membranes were incubated with anti-procollagen type I antibodies (SP1.D8) at 1:20 concentration with 1% skim milk in PBS-T for overnight. Peroxidase-conjugated anti-mouse immunoglobulin (KPL, Gaitherburg, MD, USA) was used as secondary antibody. Protein expression levels were detected using the ECL Western blotting system (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The densitometric analysis was carried out using an Image J Data Analyzer (Macintosh, MD, National Institutes of Health, USA).

7) Immunocytochemistry for localization of procollagen type I

For localization of procollagen type I, immunocytochemistry was done to two different experimental set groups. The Hs27 cells were seeded on 4-well chamber slides (SPL Lifesciences, Pocheon, Korea) at a density of 1.0×104 cells/well. After serum starvation for 24 hours, cells were treated with 10 ㎍/㎖ OPC, and either 0.1 mM ascorbic acid or 10 ng/㎖ TGF-β1 (R&D systems, MN, USA). Control group cultured in DMEM containing 1% FBS. One set of experiment included control, OPC, TGF-β1 and OPC + TGF-β1 (OPC 1-hour pretreatment + 24-hour TGF-β1 treatment) groups. Another set of experiment included control, OPC, ascorbic acid, and OPC + ascorbic acid (OPC 24-hour pretreatment + 3-hour ascorbic acid treatment) groups. Ascorbic acid group was cultured in DMEM containing 1% FBS for 24 hours, followed by treatment with ascorbic acid for 3 hours.

After treatments, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were permeabilized with 0.1% tween-20 in PBS for 10 minutes and incubated with normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 20 minutes at room temperature. After the solution was removed, cells were incubated with anti-procollagen type I antibodies (SP1.D8) for 30 minutes at 1:100 concentration. And then, cells were washed with PBS and incubated with Alexafluor 488 anti-mouse IgG (Molecular Probes Eugene, OR) for 30 minutes at 1:100 concentration. Cell nucleus was stained with 6-diamidino-2-phenylindole (DAPI; Invitrogen, CA, USA). Immunofluorescence images were taken and merged using a BX-61 fluorescent microscope and its digital imaging system (Olympus, Melville, NY, USA).

8) Statistical analysis

A Kruskal-Wallis test was used to compare more than two groups. Nonparametric analysis of covariance method was used in performing multiple comparison of time-dependent changes in procollagen secretion. Spearman rank correlation coefficient and Wilcoxon signed rank test was also performed for statistical analysis of extracellular and intracellular procollagen expression. Means and standard deviations were calculated and presented in the text and figures.

Results

1) OPC has no effect on cell viability and proliferation (Figure 2)

There was no statistically significant difference in OD450 between groups at every indicated time points (24, 48 and 72 hours). Proliferation of cells were observed in groups of 72-hour treatment but was not affected by various concentrations of OPC treatment.

2) OPC inhibits procollagen type I secretion of Hs27 cells (Figure 3, 4)

After 24 hours of treatment, OPC showed inhibitory effects to procollagen secretion of Hs27 cells in dose-dependent manner. Densitrometric data showed that procollagen secretion in 2.5 and 5 ㎍/㎖ OPC treated groups decreased by 0.77-fold and 0.48-fold compared to the control group, respectively. Especially, secretion of procollagen is completely inhibited in more than 10 ㎍/㎖ concentration of OPC (Figure 3A). Dose-dependent inhibitory effect of OPC was maintained for 48 hours but did not show statistically significant difference at 48 hours (Figure 3B).

Stimulatory effect of TGF-β1 for procollagen secretion did not show until 12 hours. When compared to control group, procollagen secretion was increased by TGF-β1 by 1.84-fold and 1.72-fold at 12 and 24 hours, respectively. Procollagen secretion of OPC treated group was completely inhibited at 6 hours and showed statistically significant decrease at 12 hours compared to control group. Procollagen secretion of co-treatment group (OPC with TGF-β1) was similar to control group after 12 hours. As showed in time-dependent changes, stimulatory effects of TGF-β1 to secrete procollagen in human fibroblasts was actively inhibited by OPC to control level, although not statistically significant(Figure 4, Table 1).

3) OPC has no effect on procollagen gene expression by Hs27 cells (Figure 5)

Gene expression of procollagen type I was not changed in OPC treatment group. TGF-β1 treated group showed significantly increased mRNA expression level (2.07-fold) compared to that in control group and this stimulatory effect of TGF-β1 was not affected by OPC co-treatment. The results showed no alteration of mRNA expression by OPC treatments.

4) OPC induces accumulation of procollagen in cytoplasm and inhibits secretion from cytoplasm (Figure 6)

Quantified data showed TGF-β1-stimulated procollagen secretion was significantly decreased by OPC treatment in a dose-dependent manner (Figure 6A). When compared to control group, procollagen secretion was markedly increased to 6.39-fold by TGF-β1 treatment, but was decreased with adding OPC into 2.12-fold and 0.38-fold in 2.5 and 5㎍/㎖-treated group, respectively. In contrast, intracellular level of procollagen increased in a dose-dependent manner, but was not statistically significant (Figure 6B).

Immunocytochemistry was performed to localize the procollagen (Figure 6C). Higher levels of intracytoplasmic procollagen were detected in OPC treatment group, and also in TGF-β1 stimulated group compared to control group. Likewise, intense procollagen expression was observed in cytoplasm in OPC and TGF-β1 co-treatment group.

5) OPC has no effect on mRNA expression of molecular chaperones and enzymes of collagen biosynthesis (Figure 7)

Gene expressions of P4Hα, P4Hβ, and HSP47 were statistically not changed after OPC treatment.

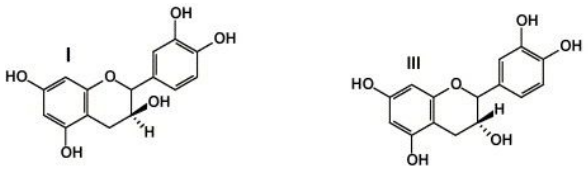
6) Ascorbic acid promotes the secretion of accumulated procollagen from cytoplasm (Figure 8)

After treatment with ascorbic acid, 2.62 times more procollagen was secreted than that from control group. OPC group showed very low secretion level (0.28-fold) of procollagen, and co-treatment group had similar level (2.30-fold) compared to ascorbic acid-treated group (Figure 8A).

Immunocytochemical localization showed that ascorbic acid promoted collagen secretion and intracellular procollagen was stained very weakly in ascorbic acid treatment group (Figure 8B). On the other hand, accumulated procollagen by OCP was completely released from cytosol to extracellular space after ascorbic acid treatment. Western blot data of intracellular level of procollagen showed corresponding results with immunocytochemistry.

Legends

A



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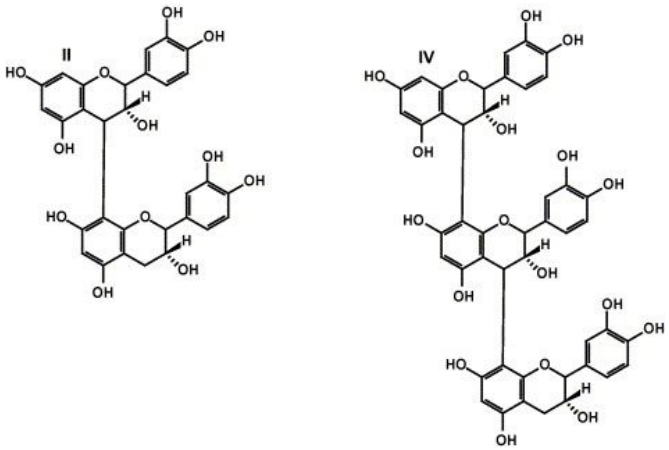


Figure 1. Structure of flavonoids and oligomeric procyanidins(OPCs). A, The structures of monomeric flavonoids are (+)-catechin (I) and (-)-epicatechin (III). B, The structures of the oligomeric flavonoids are procyaninin dimer (II), procyanidin trimer (IV).

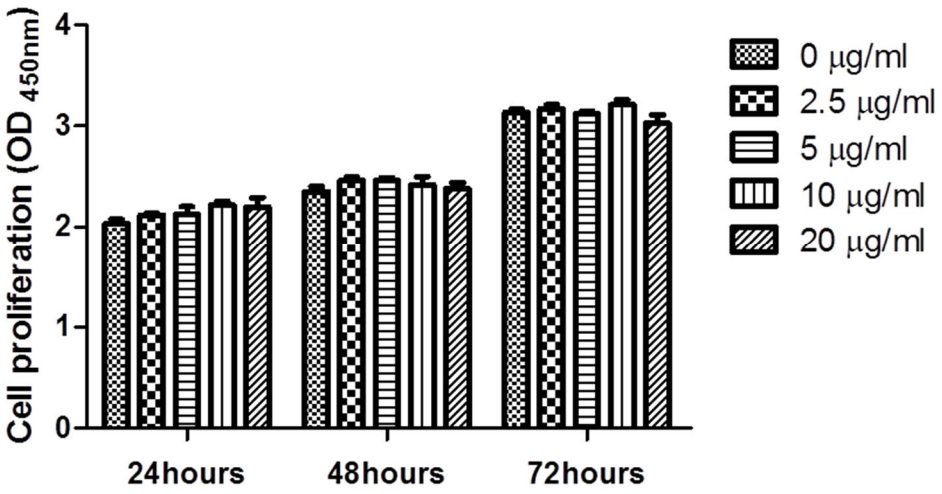
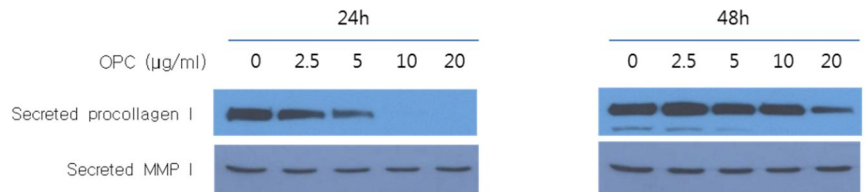


Figure 2. Cytotoxicity of OPC on human fibroblasts at various concentrations and times. Hs27 cells were treated with various concentrations of OPC for 24, 48 and 72 hours. Cytotoxicity of OPC was evaluated by CCK assay. Cell proliferation rate was expressed as OD_{450} value. (N=23, nonparametric repeated measure of ANOVA, P-value<0.05)

A



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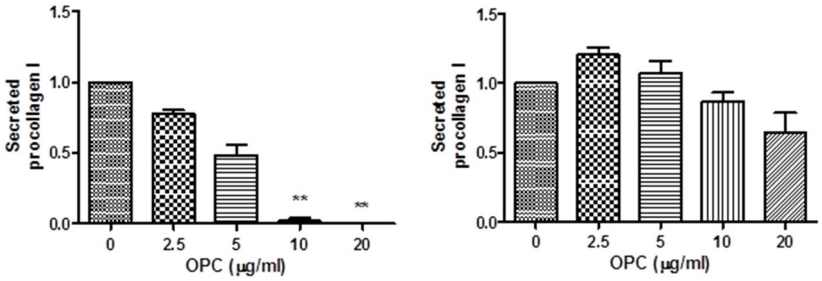


Figure 3. Effects of OCP on procollagen type I secretion from Hs27 cells. Hs27 cells were treated with 0, 2.5, 5, 10,and 20 ㎍/㎖ of OPC for 24 and 48 hours. A, Cell supernatants were collected and procollagen type I production was determined by Western blot analysis.(N=16, Kruskal-Wallis test, Spearman rank correlation coefficient, *p-value<0.05, **p-value<0.01) B, Bands on Western blots were quantified using ImageJ densitometry software. MMP-1 proteins served as the internal control. Data represent the relative procollagen expression compared to control group(N=12, Kruskal-Wallis test, Spearman rank correlation coefficient, *p-value<0.05)

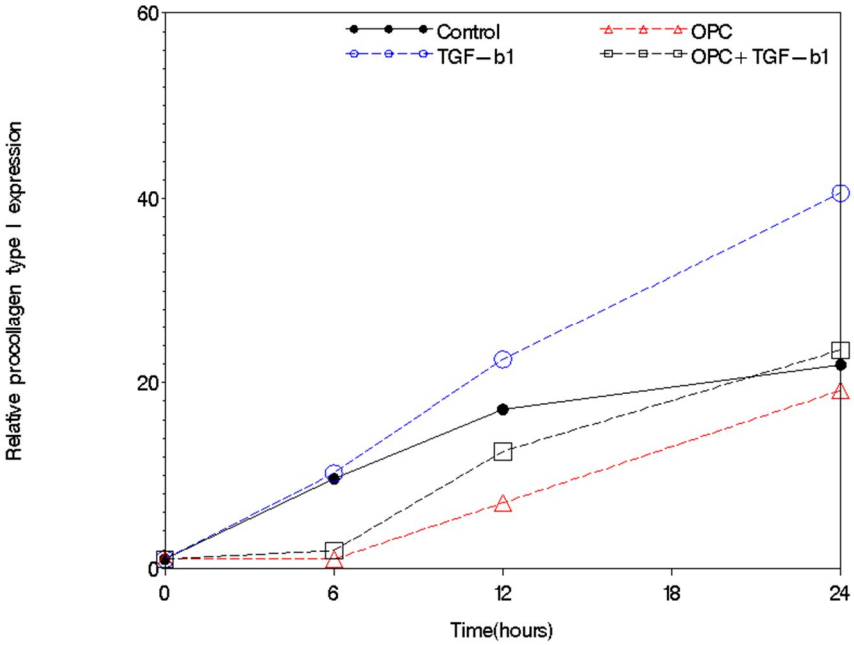


Figure 4. Effects of OPC on time-dependent changes in procollagen secretion under TGF-β1 stimulation. Hs27 cells were incubated with combinations of OPC and TGF-β1 for 24 hours. Procollagen in the cell supernatants were detected at 6, 12 and 24 hours.

Table 1. nonparametric Analysis of Covariance

	Statistics	Degree of freedom	Adjusted P-value[a]
OPC group vs. control	34.971	1	<0.0001*
TGF-β1 group vs. control	2.307	1	0.1821
OPC+TGF-β1 group vs. control	2.856	1	0.1821

a: P-value adjusted by Step-down Bonferroni method

(*: p-value<0.05)

Compared to control group, OPC group showed statistically significant decrease of procollagen expression(N=12, nonparametric repeated measure of ANOVA, P-value<0.05).

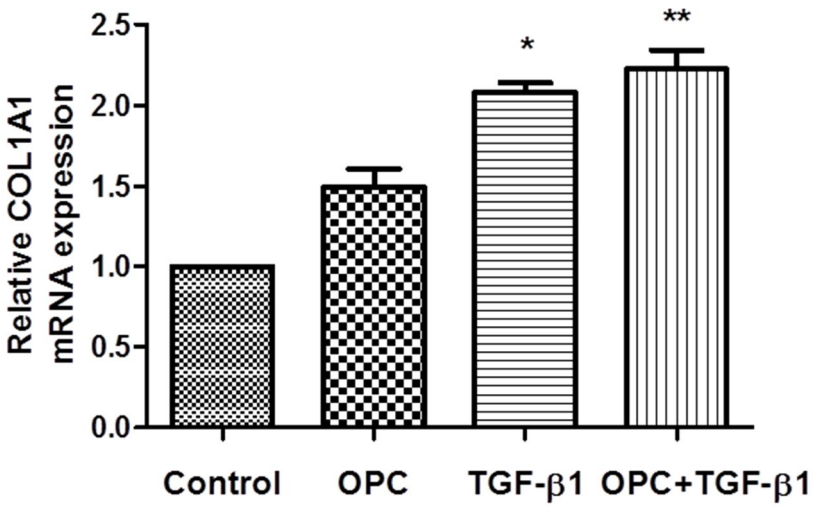
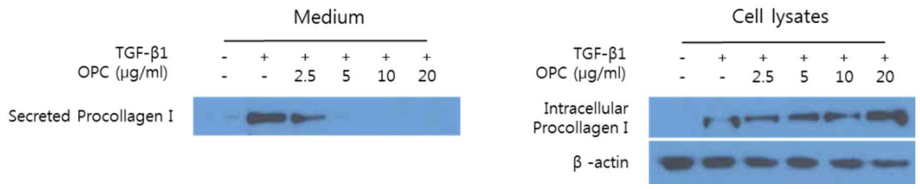


Figure 5. Effects of OPC on procollagen gene expression of Hs27 cells. OPC pretreated Hs27 cells were exposed to TGF-β1 for 24 hours. Isolated mRNA from cell lysates was subjected to real-time PCR analysis. Gene expression level was quantified by ImageJ densitrometry software. Data represent the relative procollagen mRNA expression compared to control group(N=4, Wilcoxom signed rank test, p-value<0.05).

A



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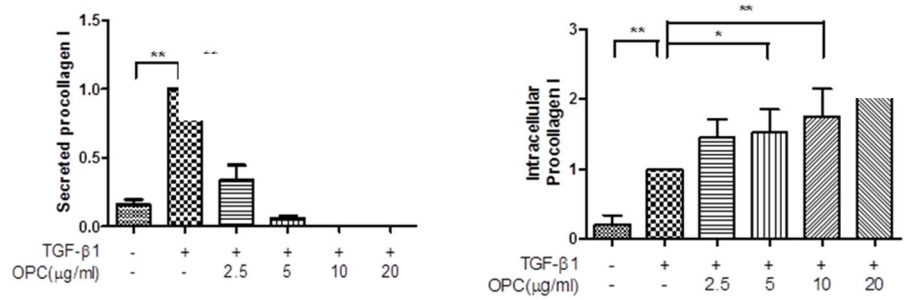

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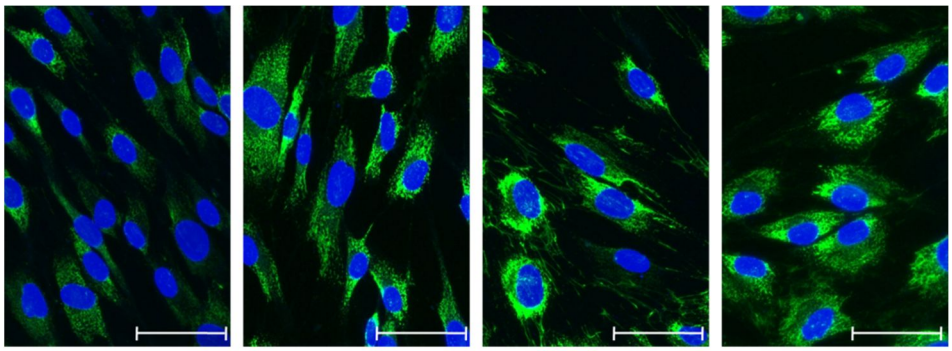


Figure 6. Effect of OPC on procollagen secretion from Hs27. The Hs27 cells were treated with various concentrations of OPC with 10 ng/㎖ of TGF-β1 for 24 hours. A, Expression level of procollagen in cell supernatants and lysates were determined by Western blot analysis(N=12, Kruskal-Wallis test, Spearman rank correlation coefficient, p-value<0.05). B, Bands were quantified by ImageJ densitrometry software and represent the relative expression level

compared to control group(N=12, Kruskal-Wallis test, Spearman rank correlation coefficient, p-value<0.05). C, Cells of another experimental sets were treated with 10 ㎍/㎖ OPC and 10 ng/㎖ TGF-β1 to immunocytochemistry for procollagen type I (green). The nucleus was counterstained by DAPI (blue)

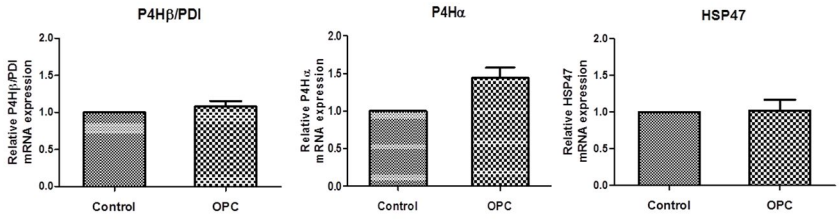
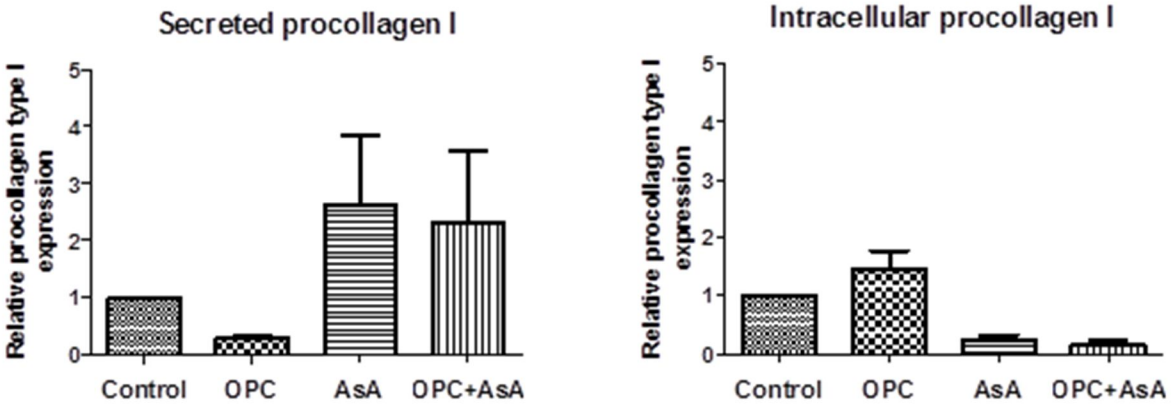


Figure 7. Effects of OPC on endoplasmic reticulum proteins involving collagen biosynthesis. The mRNA expressions of P4Hα, P4Hβ, and Hsp47 were analyzed after OPC treatment by quantitative PCR. Data represent the relative expression compared to control group(N=6, Kruskal-Wallis test, p-value<0.05).

A



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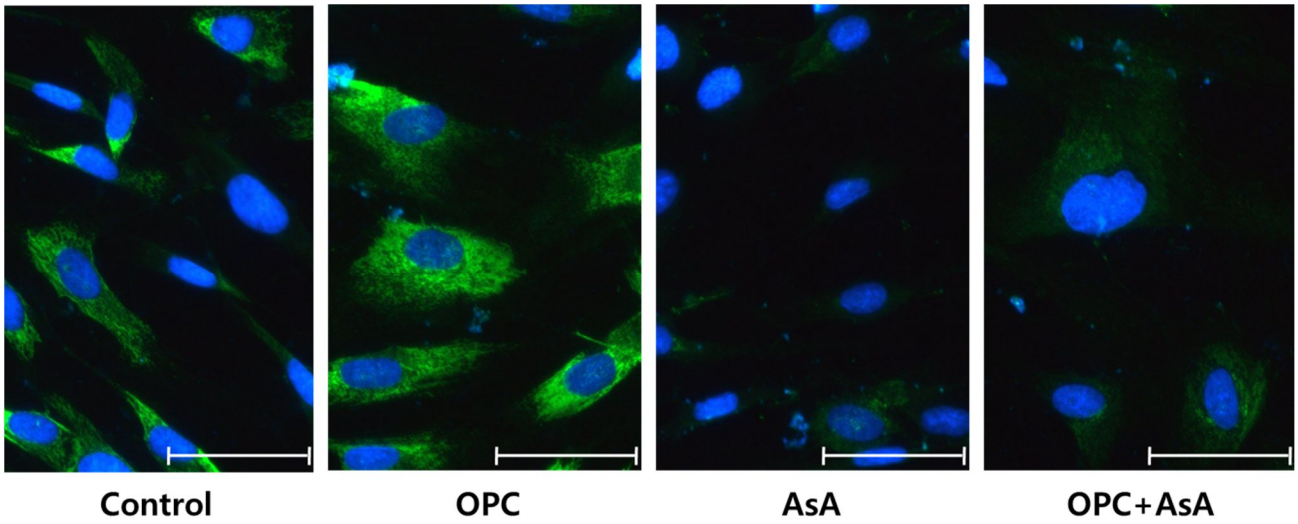
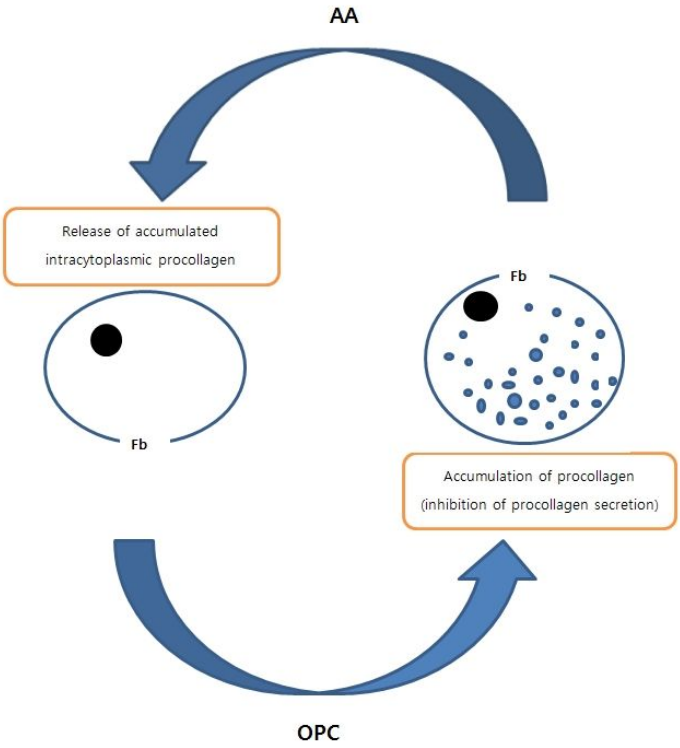
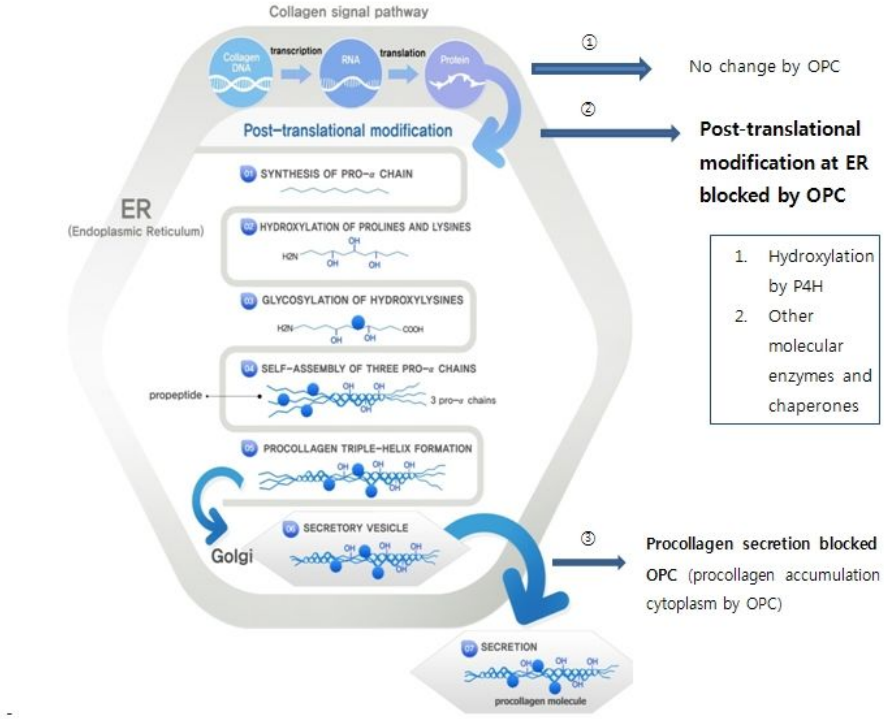


Figure 8. Effects of ascorbic acid on inhibited procollagen secretion. The 0.1mM of ascorbic acid were added to OPC pretreated cells for 3 hours. A, Expression level of procollagen in cell supernatants and cytoplasm were determined by Western blot analysis. Bands were quantified and represent the relative expression level compared to control group (N=12, Kruskal-Wallis test, p-value<0.05). B, Immunocytochemical localization of procollagen type I (green). The nucleus was counterstained by DAPI (blue). Scale bar = 50 μm.

A


AA
Release of accumulated
intracytoplasmic procollagen
Fb
Fb
Accumulation of procollagen
(inhibition of procollagen secretion)
OPC

B


Collagen signal pathway
Collagen DNA
transcription
RNA
translation
Protein
Post-translational modification
ER
(Endoplasmic Reticulum)
01 SYNTHESIS OF PRO-α CHAIN
02 HYDROXYLATION OF PROLINES AND LYSINES
03 GLYCOSYLATION OF HYDROXYLYSINES
04 SELF-ASSEMBLY OF THREE PRO-α CHAINS
propeptide
3 pro-α chains
05 PROCOLLAGEN TRIPLE-HELIX FORMATION
Golgi
06 SECRETORY VESICLE
07 SECRETION
procollagen molecule
①
No change by OPC
②
Post-translational modification at ER blocked by OPC
1. Hydroxylation by P4H
2. Other molecular enzymes and chaperones
③
Procollagen secretion blocked
OPC (procollagen accumulation cytoplasm by OPC)

C

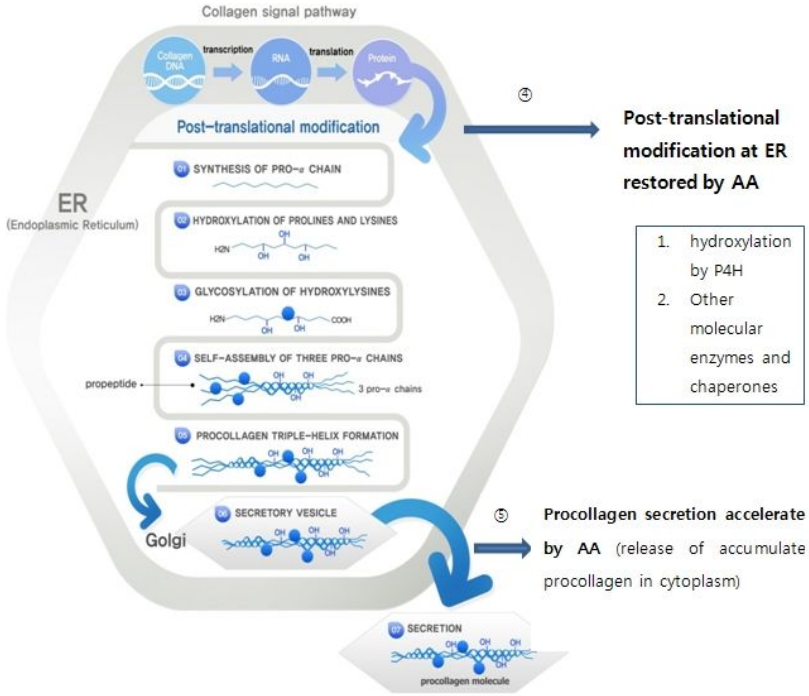


Figure 9. Diagrams for the postulated effects of oligomeric procyanidins(OPCs) and ascorbic acid on procollagen secretion from fibroblasts.

A. Diagram grossly depicted for the effects of oligomeric procyanidins and ascorbic acid. Procollagen secretion from fibroblasts is inhibited by OPC treatment. Eventually procollagen is accumulated within the cytoplasm. Conversely, accumulated procollagen is released from within the cytoplasm by ascorbic acid treatment.

B. Oligomeric procyanidin inhibits procollagen secretion and consequently, causes accumulation of procollagen within the cytoplasm.

㉠Procollgen mRNA level is not changed by oligomeric procyanidin treatment. ㉡Post-translational modification is needed for proper synthesis and secretion of procollagen. Hydroxylation of proline by Proline-4-hydroxylase, and assistance of other molecular enzymes and chaperones are essential for post-translational modification. ㉢Oligomeric procyanidin inhibits procollagen secretion by blocking post-translational modification.

C. Ascorbic acid releases accumulated procollagen from within the cytoplasm and consequently, restores procollagen secretion.

㉣Ascorbic acid has effects on prolyl-4-hydroxylase at post-translational level. ㉤Secretion of accumulated procollagen is accelerated by ascorbic acid.

(**OPC**: oligomeric procyanidin, **AA**: ascorbic acid, **Fb**: fibroblast, **P4H**: prolyl-4-hydroxylase, **ER**: endoplasmic reticulum.)

Discussion

Collagen synthesis is very important in remodeling phases of wound healing process. Abnormal overproduction of collagen leads to keloid, hypertrophic scar, and fibrosis. There are many cell populations participating in remodeling phase. Among those cell populations, fibroblasts form dominant population (15) and have the most important role in collagen production. In a previous study, we found that wound healing was delayed by OPC treatment with retarded collagen deposition in the full-thickness skin defect rat model. To further investigate the mechanism of delayed wound healing by OPC, we examined the effect of OPC on collagen production of human fibroblast cell line, Hs27.

Several studies reported that the OPC had inhibitory effects on cell proliferation. Exposure to 10 ㎍/㎖ of OPC arrested the human umbilical vascular endothelial cells at the G0/G1 phase and also cell migration was inhibited (12, 16). Zhang (6) demonstrated that OPC induced G2/M stage arrest when treated with more than 5 ㎍/㎖ of OPC in U-87 cells, U251 cells, Hep3B cells, HL-60 cells, primary rat microglial cells, and human A549 cells. In our experiment, human fibroblast cell line Hs27, showed different response to OPC treatment. Direct effect of OPC on cells was examined and showed that various concentration of OPC had no effect on cell proliferation and necrosis (Figure 2). The increased values of all groups at 72 hours can be explained by cell proliferation, as more metabolites such as yellow-colored formazan production were found.

Little is known of the effect of OPC on collagen synthesis. In this study, we showed that OPC inhibited procollagen secretion in dose-dependent manner and nearly complete suppression was observed in concentration of more than 10 ㎍/㎖ (Figure 3). As inhibitory effects were shown most strongly at 24 hours, additional experiments were performed to evaluate time-dependent effects during first 24 hours of OPC treatment.

It has been shown that TGF-β is capable of stimulating types I and III collagen biosynthesis (17), and keloid, hypertrophic scar, and fibrosis have common cause, overabundant collagen deposition, related with collagen overproduction by TGF-β (6, 18). Younai et al. (6, 18) treated anti-TGF-β antibody to keloid fibroblast and reduced collagen synthesis by 40%. We treated TGF-β1 to human fibroblast to mimic the environment of keloid, hypertrophic scar, and post-traumatic scar fibrosis. Procollagen secretion was promoted after TGF-β1 treatment to 1.84-fold and 1.72-fold at 12 and 24 hours, respectively (Figure 4). However, OPC suppressed the effect of TGF-β1 stimulation and dropped procollagen secretion level down similar to control group. And interesting results were found from our additional experiments. Gene expression of procollagen type I was not changed in OPC treatment group. TGF-β1 treated group showed significantly increased mRNA expression level (2.07-fold) compared to that in control group and this stimulatory effect of TGF-β1 was not affected by OPC co-treatment(Figure 5). These conflicting results could be explained through investigation of intracellular level of procollagen (Figure 6A, 6B) and localization of procollagen (Figure 6C). Although OPC had no statistically significant effect on mRNA expression, there could be inhibitory effects to procollagen type I secretion, resulting in cytoplasmic accumulation.

We tried to investigate how OPC suppressed the procollagen secretion at the post-translation level. The post-translational processing of collagen has been well studied. The signal peptide of translated pre-pro-collagen is recognized and transported into endoplasmic reticulum (ER) for post-translational modification. There are four processes in modification. First, signal peptide on the N-terminal is eliminated, called propeptide. Second, lysines and prolines are hydroxylated by prolyl and lysyl hydroxylases. Third, glycosylation of hydroxylated lysines, and finally, three propeptides form a triple helix, procollagen (Fig. 8). In case of collagen type I, procollagen is composed of two α-I propeptides and one α-II propeptide. In this study, monoclonal anti-α-I procollagen aminoterminal extension peptide (SP1.D8) antibody was used for tracing at the post-transcriptional level.

In endoplasmic reticulum (ER), procollagen undergoes the series of event like C-propeptide globular domain folding, trimerization and triple helix forming. Hsp47 acts as a molecular chaperone and interacts with procollagen in ER during folding and assembly (19). Hsp47 is found in collagen synthesizing cells, such as primary fibroblasts including human dermal fibroblast (20). Prolyl 4-hydroxylase (P4H) plays a critical role in hydroxylation of proline residues (21), and only hydroxylated trimmers can fold and form the triple helix. Quantitative PCR results (Figure 7) showed inhibitory effects of OPC to procollagen secretion was not related to mRNA expression alteration of Hsp47 and P4H.

In parallel experiment, ascorbic acid promoted procollagen secretion not only in control Hs27 cells (2.62-fold), but also in OPC treated group (Figure 8A). Accumulated procollagen by OPC treatment was effectively released from cytoplasm by ascorbic acid (Figure 8A). This phenomenon is similar to in vitro culture condition of ascorbic acid deficiency, leading to accumulation of unstable collagen structure and delayed collagen secretion (22).

In this study, we found that OPC inhibited procollagen secretion and immunohistochemistry showed that procollagen, when treated with OPC, is accumulated within the cytoplasm (Figure 6C). Quantified data also showed procollagen secretion stimulated by TGF-β1 was significantly decreased by OPC treatment in a dose-dependent manner(Figure 6A, B) and intense procollagen expression was observed in cytoplasm in OPC and TGF-β1 co-treatment group(Figure 6C).

These results suggest that OPC has effects on procollagen accumulation within the cytoplasm and we have found that this condition was similar to that of ascorbic acid deficiency. So we attempted to add ascorbic acid to the OPC-treated fibroblasts to find out what would happen. And finally, it showed that accumulated procollagen was released from within the cytoplasm of OPC-treated fibroblasts after we added ascorbic acid to OPC-treated fibroblasts. In other words, the inhibition of procollagen secretion and consequent accumulation of procollagen by OPC treatment was resolved by adding of ascorbic acid (figure 9A).

So we postulated the condition of OPC treatment was much like that of ascorbic deficiency.

Then we attempted to learn how the condition of ascorbic deficiency impacted on procollagen secretion from human fibroblasts, as the attempt could elucidate the regulatory mechanisms of OPC focusing on procollagen production.

Ascorbic acid is well known as essential cofactor to catalyze post-translational hydroxylation of procollagen. The accumulation of procollagen within cytoplasm occurs during ascorbic acid deficiency due to incomplete hydroxylation of proline. In addition to its cofactor function, ascorbic acid independently regulates the activities of proline hydroxylase and lysine hydroxylase. Prolonged exposure of human fibroblasts to ascorbic acid increases collagen synthesis with lysine hydroxylase activity increased 3-fold and proline hydroxylase activity decreased (23) .

As we have postulated the condition of OPC treatment was much like that of ascorbic deficiency, we assumed that OPC treatment might influence on the hydroxylation during the procollagen biosynthesis (figure 9B, 9C).

Biosynthesis of procollagen involves post-translational modification by at least nine ER-resident enzymes. Some studies have demonstrated procollagen biosynthesis is associated ER-resident molecular chaperones like HSP47, BiP, GRP94 and protein disulphide isomerase(PDI). Among those, we have investigated P4Hα and P4Hβ, associated with hydroxylation of proline residue, and HSP47. They are known as molecular chaperones mediating the retention of procollagen within the cells (24). Especially, as hydroxylation of proline by prolyl-4-hydroxylase is related to ascorbic acid, we assumed that adding of OPC might influence on these enzymes.

Gene expressions of P4Hα, P4Hβ, and HSP47 were statistically not changed after OPC treatment(Figure 7). Although this study could not elucidate the post-translational mechanisms of OPC, results from ascorbic acid experiments suggest indirect evidences. It is possible that OPC induced the state of ascorbic acid deficiency and eventually resulted in incomplete hydroxylation of proline during post-translational modification. Otherwise, there are

possibilities that other enzymes or molecular chaperones are related to OPC treatment or that the activities of P4H and HSP47 are of greater consequence. So we need to study further other related enzymes or the activities of P4H and HSP47. And also in the next study we need to investigate whether the accumulated procollagen has hydroxylated-prolines or not.

Conclusion

OPC inhibits procollagen secretion from fibroblasts without cytotoxicity. This inhibitory effect was also valid in the presence of TGF-β1, which is a key stimulator of collagen deposition. According to the results, we can assume OPC will have its part in treating collagen related symptoms and diseases, such as keloid, hypertrophic scar, and post-traumatic scar fibrosis, although additional studies on detailed mechanism and in vivo studies will be needed in the future.

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국문 초록

목 적: 상처치유 기전은 다양한 세포군의 조화가 요구되는 복잡한 과정으로 inflammatory, proliferative 그리고 remodeling phase의 세 단계로 구성되어있다. 마지막 단계인 remodeling phase에서는 콜라겐을 재배열하여 상처이전의 상태로 돌아가는 단계로 섬유아세포가 중요한 역할을 한다.
Oligomeric procyanidins (OPC)는 포도 (Vitis vinifera) 씨의 추출 성분으로 와인이나 녹차에서도 찾아볼 수 있다. 최근 연구에서 OPC가 면역, 세포 이동 그리고 세포 증식을 조절함을 보여주었는데 이는 OPC가 상처치유 과정을 조절할 수 있음을 시사한다. 본 연구는 상처 치유 과정을 조절하고자 섬유아세포에 미치는 OPC의 영향을 조사하였다.

대상 및 방법: 사람 진피 유래의 섬유아세포인 Hs27 세포에 다양한 농도 (0, 2.5, 5, 10, and 20 ㎍/㎖)의 OPC를 24, 28 그리고 72 시간 동안 처리하였다. OPC가 세포 독성을 가지는지 알아보기 위하여 세포의 생존률은 CCK 분석 법으로 측정하였다. 세포내와 세포외의 프로콜라겐의 발현은 western blot 과 real-time PCR을 이용하여 분석 하였다. 콜라겐 합성 조절에 관여하는 molecular chaperone 과 효소의 유전자 수준의 발현 분석은 real-time PCR을 통하여 이루어 졌다. 세포내 콜라겐을 관찰하고자OPC를 전처리한 세포에 TGF-β1 또는 ascorbic acid를 처리한 후 면역화학염색을 하였다.

결과: 시험한 모든 농도에서 OPC는 Hs27에 대하여 세포증식 효과나 세포독성이 나타나지 않았다. 프로콜라겐, molecular chaperones 과 효소의 유전자 발현 수준 또한 OPC에 의하여 영향을 받지 않았다. 그러나 OPC는 농도의존적으로 프로콜라겐의 분비를 방해하였다. 이러한 분비 효과는 TGF-β1 처리에 의한 콜라겐 과생산 유도 상황에서도 확인 할 수 있었다. 면역화학 염색결과OPC에 의하여 세포내에 축적된 프로콜라겐이 프로콜라겐의 번역 후 hydroxylation을 촉진하는 필수적인 cofactor인 ascorbic acid의 처리에 의하여 세포질으로 부터 분비 됨을 관찰하였다.

결론: OPC는 섬유아세포에서의 프로콜라겐의 분비를 저해하여 세포외기질에 프로콜

라겐 축적을 감소시킨다. 따라서 사람 섬유아세포에 대하여 직접적인 세포 억제효과를 가지지는 않지만 비정상적인 콜라겐 과다생산의 경우 OPC를 통한 조절이 가능할 것으로 사료된다.

색인 단어: 저중합체 프로시아니딘, 섬유아세포, 프로콜라겐, 아스코빅산, 비티스 비니페라 씨 추출물, 콜라겐

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