Oligomeric Procyanidins (OPCs) Inhibit Procollagen Type I Secretion of Fibroblasts

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Abstract Wound healing is composed of a complex process that requires harmonies of various cell populations where fibroblasts play the main role. Oligomeric procyanidins (OPC) are main components of grape (*Vitis vinifera*) seed extracts, and recent studies showed OPC's effects on inflammation, cell migration, and proliferation. We investigated the effect of OPC on fibroblasts to regulate wound healing process. Human dermal fibroblast known as Hs27 cells were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 µg/µl). Cell cytotoxicity was evaluated by the Cell Counting Kit assay, and the expression levels of secreted procollagen were analyzed. Procollagen levels in OPC treated cells exposed to transforming growth factor beta 1 (TGF-β1) or ascorbic acid were evaluated using Western blot and immunocytochemistry. Relative mRNA expressions of procollagen, molecular chaperone such as HSP47, P4H were determined by real-time PCR in OPC treated cells. OPC showed no cytotoxicity on Hs27 cells at every concentration but inhibited procollagen secretion in a dose-dependent manner. The inhibitory effect also appeared under TGF-β1 induced collagen overproduction. Immunocytochemistry showed that higher levels of intracytoplasmic procollagen were accumulated in TGF-β1 treatment group, whereas ascorbic acid induced a release of accumulated procollagen under OPC treatment. The mRNA expressions of procollagen, molecular chaperone were not affected by OPC, but procollagen level was increased when exposed to TGF-β1. OPC inhibits procollagen secretion from fibroblasts with no effects on cell proliferations even under the environment of TGF-β1-induced collagen overproduction. OPC could regulate the diseases and symptoms of abnormal overabundant collagen production.

Keywords Oligomeric procyanidins · Fibroblasts · Procollagen · Ascorbic acid · *Vitis vinifera*

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1 Introduction

Wound healing is a complex and dynamic biochemical events to repair the tissue damage. It consists of three highly integrated phases: inflammation, proliferation, and

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remodeling. During these processes, collagen plays a key role in regenerating tissue defect.

Collagen is the main protein that constitutes the extracellular matrix and is the most abundant protein in animal tissues [1]. The overall process for collagen formation is well understood through previous studies. Briefly, collagen production initiates with turning on genes that are associated with collagen formation. After transcription, the final mRNA exits from the nucleolus links with the ribosome in the cytoplasm to produce pre-pro-collagen. These peptides undergo post-translational structural modification in the endoplasmic reticulum (ER) to become procollagen which has a triple helix structure. Heat shock protein (Hsp) 47, a collagen-specific molecular chaperone in the ER, involves in the maturation of collagen molecule. Prolyl 4-hydroxylase (P4H) is a key enzyme that involves the hydroxylation with the essential cofactors such as ascorbic acid. Procollagen is packed into vesicle in Golgi apparatus, and released into the extracellular space. With some additional processes in the extracellular space, procollagen eventually turns into collagen fibril. Transforming growth factor beta 1 (TGF- β 1) is a potent cytokine stimulating procollagen deposition.

However, 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 [2], and the hypertrophic scar is generated from excessive dermal fibrosis and scarring. Kai Zhang showed that fibroblasts producing about seven times more collagen were found in hypertrophic scar patients [3].

Oligomeric procyanidins (OPCs) are the main components of grape (*Vitis vinifera*) seed extracts and also called proanthocyanidins. Procyanidins, a group of flavonoids, 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 [4]. OPCs are well known for its antioxidant, anti-bacterial, and anti-viral properties [5–7], and recent studies suggested that OPC can also regulate wound healing process. OPC showed the anti-inflammatory effect by inhibiting nitric oxide and prostaglandin E2 production and suppressing iNOS expression [8]. García-Conesa and coworkers [9] reported that OPC inhibits cell migration and modulates proliferation of human umbilical vascular endothelial cells by inducing significant gene expression change. They also reported that OPC has cardioprotective activity in myocardial infarction model [10], and gives regulatory effect on proliferation and migration of endothelial cells [11].

However, there has not been any research regarding the effect of OPC on fibroblasts which are one of the most abundant cell populations of the human body, especially in the skin. Also, the mechanism of OPC regulating collagen production has not been fully understood. The purpose of this study is to evaluate the effect of OPC on fibroblasts and to elucidate the regulatory mechanism of OPC focusing on collagen production. We hypothesized that OPC inhibits procollagen production in fibroblasts by modulating gene expression.

2 Materials and methods

2.1 Cell culture

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

2.2 Cell cytotoxicity assay

Cell cytotoxicity assay was performed based on the previous article [12]. To determine cytotoxicity of OPC, Hs27 cells in the exponential phase were seeded at 2×10^4 cells/well in 24-well plates and were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 μ g/ μ l) for 24, 48 and 72 h. Cell cytotoxicity was then determined by the Cell Counting Kit (CCK) assay. Briefly, the CCK-8 solution of 10 μ l was added to each well and incubated for 1–4 h. Viable cells were measured with the absorbance at 450 nm using a microplate reader.

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2.3 OPC treatment

The Hs27 cells were seeded at 2×10^5 cells per well in 6-well plates. After serum starvation for 24 h, cells were treated with various concentrations of OPC (0, 2.5, 5, 10, and 20 $\mu\text{g}/\mu\text{l}$). The cell culture supernatants were collected from each well at 24 or 48 h and procollagen expression level was determined by Western blot.

To evaluate the mechanism of procollagen secretion, we performed an additional experiment using TGF- β 1. Non-treatment group served as control group (Group 1), and three experimental groups were set—OPC (10 $\mu\text{g}/\mu\text{l}$) treatment group (Group 2), TGF- β 1 (10 ng/ml) treatment group (Group 3), and combination of OPC pretreatment (10 $\mu\text{g}/\mu\text{l}$ for 1 h) and TGF- β 1 (10 ng/ml) treatment (Group 4). Cell supernatants were collected at 6, 12, and 24 h and were analyzed by Western blot.

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

2.4 Ascorbic acid treatment

The Hs27 cells were seeded 2×10^5 cells per well in 6-well plate. After serum starvation, cells were pretreated with 10 $\mu\text{g}/\text{ml}$ OPC for 1 h. Then, 0.1 mM of ascorbic acid (Sigma-Aldrich, St. Louis, MO) was added and incubated for additional 24 h. Cell supernatant, lysate, and fixed cells were analyzed by Western blot and immunocytochemistry, respectively.

2.5 Real-time PCR

Total RNA was isolated with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Total RNA concentrations were determined by absorbance at 260 nm. For RT-PCR, 1 μg of mRNA was reverse-transcribed with oligo (dT) 18 using SuperScript III (Invitrogen, Carlsbad, CA). 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, Shiga, Japan) and the appropriate primers: COL1A1 forward, 5'-CCCGGGTTTCAGAGACAACCTTC-3'; COL1A1 reverse, 5'-TCCACATGCTTTATTCCAGCAATC-3'; beta-actin forward, 5'-TGGCAGCCAGCACAATGAA-3'; beta-actin reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; P4H α (I) forward, 5'-CGTCTCCAGGATACCTACAA-3'; P4H α (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'-CTTGTCATGGCCTCAGTCA-3'.

Cycling parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Melting curve stage was performed at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The level of gene expression was analyzed according to the comparative threshold cycle (Ct) method and calculated by $2^{-\Delta\Delta\text{Ct}}$.

2.6 Western blot analysis of procollagen type I

The Hs27 cells 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). The cell supernatants were separated by 10% SDS-PAGE, followed by electrophoretic transfer to PVDF membrane (Bio-Rad, Hercules, CA). For protein detection, the membranes were incubated with anti-procollagen type I antibodies (SP1.D8) at 1:20 concentration with 1% skim milk in phosphate-buffered saline (PBS)-T for overnight. Peroxidase-conjugated anti-mouse immunoglobulin (Kirkgaard & Perry Lab, Gaithersburg, MD) was used as a secondary antibody. Protein expression levels were detected using the ECL Western blotting system (Amersham Pharmacia Biotech., Piscataway, NJ). The densitometric analysis was carried out using an Image J Data Analyzer (National Institutes of Health, Bethesda, MD).

2.7 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 1.0×10^4 cells/well. After serum starvation for 24 h, cells were treated with 10 $\mu\text{g}/\mu\text{l}$ OPC, and either 0.1 mM ascorbic acid or 10 ng/ml TGF- β 1 (R&D systems, Minneapolis, MN). Control group cultured in DMEM containing 1% FBS. One set of experiment included control, OPC, TGF- β 1, and OPC + TGF- β 1 (OPC 1-h pretreatment + 24-h TGF- β 1 treatment) groups. Another set of the experiment included control, OPC, ascorbic acid, and OPC + ascorbic acid (OPC 24-h pretreatment + 3-h ascorbic acid treatment) groups. The ascorbic acid group was cultured in DMEM containing 1% FBS for 24 h, followed by treatment with ascorbic acid for 3 h.

After treatments, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized with 0.1% tween-20 in PBS for 10 min and incubated with normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. After the solution was removed, cells were

incubated with anti-procollagen type I antibodies (SP1.D8) for 30 min 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 min at 1:100 concentrations. Cell nucleus was stained with 4', 6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). Immunofluorescence images were taken and merged using a BX-61 fluorescent microscope and its digital imaging system (Olympus, Melville, NY).

2.8 Statistical analysis

A Kruskal–Wallis test was used to compare more than two groups. Nonparametric analysis of covariance method was used in performing multiple comparisons 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.

3 Results

3.1 OPC has no cytotoxic effect on cells

There was no statistically significant difference in OD₄₅₀ between groups at every indicated time points (24, 48 and 72 h). The proliferation of cells was observed in groups of 72-h treatment but was not affected by various concentrations of OPC treatment (Fig. 1).

3.2 OPC inhibits procollagen type I secretion of Hs27 cells

After 24 h of treatment, OPC showed inhibitory effects to procollagen secretion of Hs27 cells in a dose-dependent manner. Densitometry data showed that procollagen

Fig. 1 Cytotoxicity of OPC on human fibroblasts according to OPC concentrations at 24, 48 and 72 h. Cytotoxicity of OPC was evaluated by CCK assay. Cell proliferation rate was expressed as OD₄₅₀ value (N = 23, nonparametric repeated measure of ANOVA, *p* value < 0.05)

secretion in 2.5 and 5 µg/µl 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 µg/µl concentration of OPC (Fig. 2A). Dose-dependent inhibitory effect of OPC was maintained for 48 h but did not show statistically significant difference at 48 h (Fig. 2B).

The stimulatory effect of TGF-β1 for procollagen secretion did not show until 12 h. When compared to control group, procollagen secretion was increased by TGF-β1 by 1.84-fold and 1.72-fold at 12 and 24 h, respectively although no significance was found. Procollagen secretion of OPC treated group was completely inhibited at 6 h and showed a statistically significant decrease at 12 h compared to control group (*p* < 0.05). Procollagen secretion of co-treatment group (OPC with TGF-β1) was similar to control group after 12 h. 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 (Fig. 3).

3.3 OPC has no effect on procollagen gene expression by Hs27 cells

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 the 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 (Fig. 4).

3.4 OPC induces accumulation of procollagen in cytoplasm and inhibits secretion from cytoplasm

Quantified data showed TGF-β1-stimulated procollagen secretion was significantly decreased by OPC treatment in a dose-dependent manner. When compared to control group, procollagen secretion was markedly increased to 6.39-fold by TGF-β1 treatment but was decreased by adding OPC into 2.12-fold and 0.38-fold in 2.5 and 5 µg/µl-treated group, respectively. Secreted procollagen was not shown with more than 10 µg/µl concentration of OPC. In contrast, intracellular level of procollagen increased in a dose-dependent manner (Fig. 5A, B).

Immunocytochemistry was performed to localize the procollagen. 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 the cytoplasm in OPC and TGF-β1 co-treatment group (Fig. 5C).

Fig. 2 Effects of OPC on procollagen type I secretion from Hs27 cells. Hs27 cells were treated with 0, 2.5, 5, 10, and 20 μg/μl of OPC for 24 and 48 h. **A** Cell supernatants were collected and procollagen type I production was determined by Western blot analysis. MMP-1 proteins served as the internal control. (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 Image J densitometry software. Data represent the relative procollagen expression compared to control group (N = 12, Kruskal–Wallis test, Spearman rank correlation coefficient, **p*-value < 0.05)

3.5 OPC has no effect on mRNA expression of molecular chaperones and enzymes of collagen biosynthesis

Gene expressions of P4H α , P4H β , and HSP47 were statistically not changed after OPC treatment (Fig. 6).

3.6 Ascorbic acid promotes the secretion of accumulated procollagen from cytoplasm

After treatment with ascorbic acid, 2.62 times more procollagen was secreted than that from the control group. OPC group showed very low secretion level (0.28-fold) of procollagen, and co-treatment group had a similar level (2.30-fold) compared to ascorbic acid-treated group (Fig. 7A).

Immunocytochemical localization showed that ascorbic acid promoted collagen secretion and intracellular procollagen was stained very weakly in the ascorbic acid treatment group. On the other hand, accumulated procollagen by OPC 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 (Fig. 7B).

4 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 the remodeling phase. Among those cell populations, fibroblasts form dominant population and have the most important role in collagen production [13].

Several studies reported that the OPC had inhibitory effects on cell proliferation. Exposure to 10 μg/μl of OPC arrested the human umbilical vascular endothelial cells at the G0/G1 phase and also cell migration was inhibited [9, 14]. Zhang demonstrated that OPC induced G2/M stage arrest when treated with more than 5 μg/μl of OPC in U-87 cells, U251 cells, Hep3B cells, HL-60 cells, primary rat microglial cells, and human A549 cells [3].

In our experiment, human fibroblast cell line Hs27 showed a different response to OPC treatment. A direct effect of OPC on cells was examined and showed that various concentration of OPC had no effect on cell proliferation and necrosis (Fig. 1). The increased values of all groups at 72 h can be explained by cell proliferation, as

Fig. 3 Effects of OPC on time-dependent changes in procollagen secretion under TGF- β 1 stimulation. Hs27 cells were incubated with combinations of OPC and TGF- β 1 for 24 h. Procollagen in the cell supernatants were detected at 6, 12, and 24 h

Fig. 4 Effects of OPC on procollagen gene expression of Hs27 cells. OPC pretreated Hs27 cells were exposed to TGF- β 1 for 24 h. Isolated mRNA from cell lysates was subjected to real-time PCR analysis. Gene expression level was quantified by Image J densitometry software. Data represent the relative procollagen mRNA expression compared to control group (N = 4, Wilcoxon signed rank test, p -value < 0.05)

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 a dose-dependent manner and nearly

complete suppression was observed in the concentration of more than 10 μ g/ml (Fig. 2). As inhibitory effects were shown most strongly at 24 h, additional experiments were performed to evaluate time-dependent effects during first 24 h of OPC treatment.

It has been shown that TGF- β is capable of stimulating types I and III collagen biosynthesis [15], and keloid, hypertrophic scar, and fibrosis have common cause, overabundant collagen deposition, related with collagen overproduction by TGF- β [3, 16]. Younai et al. treated anti-TGF- β antibody to keloid fibroblast and reduced collagen synthesis by 40% [3, 16]. We treated TGF- β 1 to human fibroblast to mimic the environment of keloid, hypertrophic scar, and post-traumatic scar fibrosis. Procollagen secretion was promoted after TGF- β 1 treatment to 1.84-fold and 1.72-fold at 12 and 24 h, respectively (Fig. 3). However, OPC suppressed the effect of TGF- β 1 stimulation and dropped procollagen secretion level down similar to the 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- β 1 treated group showed significantly increased mRNA expression level (2.07-fold) compared to that in the control group and this stimulatory effect of TGF- β 1 was not affected by OPC co-treatment (Fig. 4). These conflicting results could be explained through investigation of intracellular level of procollagen and localization of procollagen (Fig. 5). Although OPC had no statistically significant effect on mRNA expression for procollagen production, there could be inhibitory effects to procollagen type I secretion, resulting in cytoplasmic accumulation.

Ascorbic acid is well known as an essential cofactor to catalyze the 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 [17].

In a parallel experiment, ascorbic acid promoted procollagen secretion not only in control Hs27 cells (2.62-fold) but also in OPC treated group. The inhibition of procollagen secretion and consequent accumulation of procollagen by OPC treatment was effectively released from the cytoplasm by adding of ascorbic acid (Fig. 7). This phenomenon is similar to *in vitro* culture condition of ascorbic acid deficiency, leading to accumulation of unstable collagen structure and delayed collagen secretion [18]. We have postulated the condition of OPC treatment was much like that of ascorbic deficiency.

Fig. 5 Effect of OPC on procollagen secretion from Hs27. The Hs27 cells were treated with various concentrations of OPC with 10 ng/ml of TGF- β 1 for 24 h. **A, B** The expression level of procollagen in cell supernatants **A** and lysates **B** were determined by Western blot analysis (N = 12, Kruskal–Wallis test, Spearman rank correlation coefficient, p -value < 0.05). **C, D** Bands from the Western blot were quantified in cell supernatants **C** and lysates **D** by Image J densitometry software and represent the relative expression level

compared to control group (N = 12, Kruskal–Wallis test, Spearman rank correlation coefficient, p -value < 0.05). **E–H** Cells of other experimental sets with 10 μ g/ml OPC and 10 ng/ml TGF- β 1 to immunocytochemistry for procollagen type I (green) (control **E**, treatment with OPC only **F**, treatment with TGF- β 1 only **G**, treatment with both OPC and TGF- β 1 **H**). The nucleus was counterstained with DAPI (blue). (Color figure online)

The post-translational processing of collagen has been well studied. The signal peptide of translated pre-pro-collagen is recognized and transported into the ER for post-translational modification such as N-terminal elimination, hydroxylation, and glycosylation (Fig. 8). In the ER, procollagen undergoes the series of events like C-propeptide globular domain folding, trimerization and triple helix forming by at least nine ER-resident enzymes. Hsp 47 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]. P4H plays a critical role in hydroxylation of proline residues [21], and only hydroxylated trimers can fold and form the triple helix.

Among those, we have investigated P4H α and P4H β , and HSP47. They are known as molecular chaperones mediating the retention of procollagen within the cells [22]. Especially, as hydroxylation of proline by P4H is related to ascorbic acid, we assumed that adding of OPC might influence on these enzymes. However,

Fig. 6 Effects of OPC on endoplasmic reticulum proteins involving collagen biosynthesis. The mRNA expressions of P4Hβ **A**, P4Hα **B**, and Hsp47 **C** 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)

Fig. 7 Effects of ascorbic acid on inhibited procollagen secretion. The 0.1 mM of ascorbic acid was added to OPC pretreated cells for 3 h. **A**, **B** The expression level of procollagen in cell supernatants **A** and cytoplasm **B** 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). **C–F** Immunocytochemical localization of procollagen type I (green) (control **C**, treatment with OPC only **D**, treatment with AsA only **E**, treatment with both OPC and AsA **F**). The nucleus was counterstained with DAPI (blue). Scale bar = 50 μm. (Color figure online)

quantitative PCR results (Fig. 6) revealed that inhibitory effects of OPC to procollagen secretion were not related to mRNA expression alteration of Hsp47 and P4Hα, P4Hβ.

From the results, we concluded that OPC inhibits procollagen secretion into the ECM space, although OPC does not influence on the biosynthesis of procollagen or

key elements related to post-translational modification such as Hsp47 and P4H. We assume that OPC treatment induced the state of ascorbic acid deficiency and influenced on the hydroxylation during post-translational modification (Fig. 8).

It is possible that other molecular chaperones or enzymes, such as procollagen glucosyltransferase and

Fig. 8 Diagrams for the postulated effects of TGF- β 1, OPCs, and ascorbic acid on procollagen secretion from fibroblasts. ① Procollagen mRNA level is not changed by OPC treatment but increased when exposed to TGF- β 1. ② Post-translational modification is needed for proper synthesis and secretion of procollagen. Hydroxylation of proline by P4H, and assistance of other molecular enzymes and chaperones are essential for post-translational modification. Ascorbic

acid has effects on P4H at the post-translational level. ③ OPC is thought to inhibit procollagen secretion by blocking post-translational modification by regulating the activities of P4H and HSP47 or levels of other enzymes. ④ We assumed that OPC inhibits procollagen secretion and consequently, causes accumulation of procollagen within the cytoplasm (OPC oligomeric procyanidin, TGF- β 1 transforming growth factor beta 1, ER endoplasmic reticulum)

lysyl hydroxylase, are related to OPC treatment. Also, the activities of P4H and HSP47 might be of greater consequence than we thought. A definite biological mechanism should be investigated in future studies regarding the post-translational regulation of OPC on the procollagen.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical statement This paper does not include any ethical issues for human and animal rights.

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