Effect of phlorotannins on myofibroblast differentiation and ECM protein expression in transforming growth factor β1-induced nasal polyv-derived fibroblasts

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Abstract. Phlorotannins (PTNs), a group of phenolic compounds from seaweeds, have diverse bioactivities. However, there has been no report on their antifibrotic effects during nasal polyp (NP) formation. In the present study, the effect of PTNs on transforming growth factor (TGF)-β1-induced profibrotic responses in nasal polyp-derived fibroblasts (NPDFs) were determined and the relevant signaling pathways were investigated. The expression levels of collagen type-1 (Col-1) and fibronectin in NP tissues were measured by western blot analysis and immunohistochemistry. The NPDFs were treated with TGF-β1 (1 ng/ml) in the presence or absence of PTNs (5-30 µg/ml). The expression levels of α-smooth muscle actin (α-SMA), Col-1, fibronectin, and phosphorylated small mothers against decapentaplegic 1/3 in NPDFs were measured by western blot analysis. The contractile activity of the NPDFs was determined by a collagen gel contraction assay. Col-1 and fibronectin proteins were found to be expressed in NP tissues. PTNs had no significant cytotoxic effect on TGF-β1-induced NPDFs. TGF-β1 induced the expression of α-SMA, Col-1 and fibronectin, and stimulated fibroblast-mediated contraction of collagen gel. However, pre-treatment with PTNs inhibited the expression of these proteins. The inhibitory effects were mediated through the suppression of Smad2/3 signaling pathways in TGF-β1-induced NPDFs. These resulted suggested that PTNs may be important in inhibiting myofibroblast differentiation and extracellular matrix protein accumulation in NP formation through the Smad2/3 signaling pathway.

Introduction

Nasal polyps (NPs) are pink-colored, tear-drop shaped outgrowths, which form either in the nose or in the paranasal sinuses. NPs are characterized by tissue remodeling, consisting of stromal and epithelial cell proliferation, inflammatory cell infiltration, goblet cell hyperplasia, pseudocyst formation, focal fibrosis, edema, and basement membrane thickening, with a high recurrence rate in the nose and paranasal sinuses (1,2). The majority of individuals with NPs exhibit morbidities, including nasal congestion, rhinorrhea, decreased taste, anosmia and headaches, which reduce the individual's quality of life (3). NPs typically embody a chronic infiltration of inflammatory cells. The recurrence of NPs, following surgical procedure, is common; therefore, many patients require additional procedures. The etiology and pathophysiology of NPs formation remain to be fully elucidated. However, according to reports by other research groups, a remodeling process is considered to be involved; damage to the mucosal epithelium is accompanied by extracellular matrix (ECM) protein accumulation and inflammatory cell infiltration (4).
Several types of cells, including epithelial cells, T cells, mast cells and fibroblasts, are involved in the pathogenesis of NPs (5). Among these, fibroblasts are the major structural components of NP architecture and are actively involved in NP formation (6). Fibroblasts are found in the stroma and are the cellular source of ECM components, including collagen and fibronectin, the excessive deposition of which is characteristic of the majority of fibrotic responses (7).

Marine algae have been traditionally used in folk medicine and as ingredients in food in Asian countries. Marine algae are rich sources of vitamins, minerals, dietary fibers, proteins, polyunsaturated fatty acids, essential amino acids and bioactive substances (8), which have antioxidant, anti-inflammatory, antiviral, anticoagulant, anticancer, immunomodulatory and antibacterial activities (9,10). Marine algae also form a potential resource for bioactive secondary metabolites, which may provide antibacterial activities (9,10). Marine algae of the majority of fibrotic responses (7).

Individuals were diagnosed with NPs based on the minimal criteria for chronic rhinosinusitis with NPs. A total of 15 subjects (male:female, 9:6; median age, 43) with NPs and 15 subjects with deviated nasal septa were recruited from the Department of Otorhinolaryngology, Inje University Pusan Paik Hospital (Pusan, Korea) between July 2017 and September 2017. Written informed consent was obtained from each patient and the study was approved by the Ethics Committees of Inje University Pusan Paik Hospital (Pusan, Korea). A NP was defined as the presence of endoscopically visible bilateral polyps growing from the middle nasal meatus into the nasal cavities, and affecting the ethmoid and maxillary sinuses on computed tomography (CT) of the paranasal sinus. NPs were obtained from the region of the middle meatus at the beginning of the surgical procedure. As a control, nasal mucosal tissue was also obtained from the inferior turbinate (IT) of patients who underwent a septoturbinoplasty. The subjects had no history of nasal allergy, asthma, or aspirin sensitivity. The diagnosis of allergy was based on both a history of allergy and the results of ImmunoCAP or skin prick tests. No patient had received steroids (systemic or topical), nonsteroidal anti-inflammatory drugs, antihistamines, or macrolide antibiotics during the 4 weeks before the biopsy. NPdFs were isolated from surgical tissues by enzymatic digestion with collagenase (500 U/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), hyaluronidase (30 U/ml; Sigma-Aldrich; Merck KGaA), and DNase (10 U/ml; Sigma-Aldrich; Merck KGaA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1,000 U/ml penicillin, and 1,000 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂. The purity of the NPdFs was confirmed by flow cytometry and characteristic spindle-shaped cell morphology. Experimental cells were used in the fourth to six cell passages.

**Materials and methods**

**Reagents.** The PTNs were provided by Professor W. K. Jung (Pukyong National University, Busan, Korea). TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Antibodies against α-smooth muscle actin (α-SMA; cat. no. ab5694) and Col-1 (cat. no. ab81847) were purchased from Abcam (Cambridge, MA, USA). Antibodies against actin (cat. no. 612656) and fibronectin (cat. no. 610077) were purchased from BD Biosciences (San Jose, CA, USA). Antibodies against goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (cat. no. LF-SA8001) and GAPDH (cat. no. LF-PA0018) were purchased from Young In Frontier Co., Ltd. (Seoul, Korea). Antibodies against phosphorylated (p-) small mothers against decapentaplegic (Smad)2 (cat. no. 3101) and p-Smad3 (cat. no. 9520) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Smad2/3-specific small interfering (si)RNAs (cat. no. sc-37238) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rat tail type-1 collagen was purchased from BD Biosciences.

**NP-derived fibroblast culture.** Patients with NPs were recruited and NPdFs were cultured as in our previous report (16). Individually were diagnosed with NPs based on the minimal criteria for chronic rhinosinusitis with NPs. A total of 15 subjects (male:female, 9:6; median age, 43) with NPs and 15 subjects with deviated nasal septa were recruited from the Department of Otorhinolaryngology, Inje University Pusan Paik Hospital (Pusan, Korea) between July 2017 and September 2017. Written informed consent was obtained from each patient and the study was approved by the Ethics Committees of Inje University Pusan Paik Hospital (Pusan, Korea). A NP was defined as the presence of endoscopically visible bilateral polyps growing from the middle nasal meatus into the nasal cavities, and affecting the ethmoid and maxillary sinuses on computed tomography (CT) of the paranasal sinus. NPs were obtained from the region of the middle meatus at the beginning of the surgical procedure. As a control, nasal mucosal tissue was also obtained from the inferior turbinate (IT) of patients who underwent a septoturbinoplasty. The subjects had no history of nasal allergy, asthma, or aspirin sensitivity. The diagnosis of allergy was based on both a history of allergy and the results of ImmunoCAP or skin prick tests. No patient had received steroids (systemic or topical), nonsteroidal anti-inflammatory drugs, antihistamines, or macrolide antibiotics during the 4 weeks before the biopsy. NPdFs were isolated from surgical tissues by enzymatic digestion with collagenase (500 U/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), hyaluronidase (30 U/ml; Sigma-Aldrich; Merck KGaA), and DNase (10 U/ml; Sigma-Aldrich; Merck KGaA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1,000 U/ml penicillin, and 1,000 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂. The purity of the NPdFs was confirmed by flow cytometry and characteristic spindle-shaped cell morphology. Experimental cells were used in the fourth to six cell passages.

**Immunohistochemistry.** To detect Col-1 and fibronectin, immunohistochemistry was performed as previously reported (16). Briefly, 5-µm-thick NP sections were prepared from formalin-fixed paraffin-embedded tissues. The sections were incubated overnight with Col-1 (1:100) and fibronectin (1:300) antibodies at 4°C overnight. The slides were then incubated with anti-mouse IgG-HRP at a 1:2,000 dilution for 1 h at room temperature in the dark. DAB was used as a chromogen, and Mayer’s hematoxylin was used for counterstaining. The expression levels of Col-1 and fibronectin were evaluated under a digital slide scanner (NanoZoomer 2.0-RS; Hamamatsu, Shizuoka, Japan).

**Cell viability assay.** Cellular viability was assessed using the CCK-8 (Dojindo Molecular Technologies, Inc.). In a 96-well microplate, NPdFs (1x10³ cells/well) were treated with PTNs (5, 10 and 30 µM). Following incubation for 24 h at 37°C in an atmosphere of 5% CO₂, the cells were washed twice with PBS. CCK-8 solution was then added to each well and incubated at 37°C for 1 h, followed by an absorbance analysis at 450 nm using a microplate reader (SpectraMax M2e; Molecular Devices LLC, Sunnyvale, CA, USA). All assays were performed in triplicate.

**Western blot analysis.** The cells were lysed with lysis buffer (Mammalian Cell-PE LB; G-Biosciences, St. Louis, MO, USA). Protein concentration was quantified using the
Bradford method (Bio-Rad protein assay dye reagent concentration; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal quantities of protein (20 mg) were separated on 10% SDS-polyacrylamide mini-gels and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont, UK). Membranes were blocked in 5% non-fat milk diluted in Tris-buffered saline/0.1% Tween-20 (TBST) at room temperature for 1 h. Following incubation with the appropriate primary antibody (α-SMA, Col-1, fibronectin, p-Smad2 and p-Smad3) at a dilution of 1:1,000 overnight at 4˚C, the membranes were incubated for 1 h at room temperature with secondary antibody conjugated to HRP (goat anti-mouse IgG; 1:1,000). Following three washes with TBST, the immunoreactive bands were visualized using an ECL detection system (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and band intensities were evaluated quantitatively using the Multi gauge version 2.2 software (Fuji Film, Tokyo, Japan).

Silencing of Smad2/3 by synthetic siRNAs. At 16 h following plating, the cells were transfected with Smad2/3-siRNAs (40, 80 and 100 nM) using the siRNA transfection reagent (Santa Cruz Biotechnology, Inc.), in accordance with the manufacturer's protocol. Following 6 h of incubation, an equal volume of fibroblast growth medium 2 (cat. no. C-23020; PromoCell, Heidelberg, Germany) was added. The cells were then used for estimating the expression of α-SMA, fibronectin and Col-1 at 16 h post-transfection. The transfection efficiency was evaluated by western blot analysis of the protein expression of Smad2/3.

Rat tail type-1 collagen gel contraction assay. The rat tail type-1 collagen gel contraction assay was performed as previously described (16). Briefly, type-1 collagen from the rat tail was diluted with fibroblast basal medium (CC-3131; Lonza Group, Ltd., Basel, Switzerland) to a concentration of 1 mg/ml and mixed with NPDFs to reach a final concentration of 1x10⁵ cells/ml. Following the addition of 1 N NaOH as per the manufacturer's protocol, 500 µl of the cell-collagen mixture was added into each well of a 24-well cell culture plate. The plate was incubated at 37˚C for 30 min. The cells were then incubated in fibroblast growth medium 2 overnight, and treated with PTNs and TGF-β₁, as indicated. The gel sizes were measured using ImageJ software (version 1.51j8; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean. All statistical analyses were performed with GraphPad Prism software 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between groups were performed by Dunnett's multiple range tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Col-1 and fibronectin in NP tissues. To examine whether Col-1 and fibronectin were expressed in NP tissues, immunohistochemistry was performed in IT tissues and NP tissues. In the NP tissues, Col-1 and fibronectin immunoreactivity was detected in lesions in which overall stroma was observed (Fig. 1A). In addition, Col-1 and fibronectin proteins were found to be expressed in the NP tissue lysates via western blot analysis (Fig. 1B). However, minimal expression signals of the Col-1 and fibronectin proteins were detected in the IT tissues.
Effects of PTNs on the viability of NPDFs. The viability of the NPDFs treated with PTNs was examined using the CCK-8 assay. There was no cytotoxicity towards NPDFs at PTN doses up to 30 µg/ml (Fig. 2). On the basis of these results, a PTN concentration range of 5-30 µg/ml was selected for the subsequent experiments.

Effect of PTNs on protein expression levels of α-SMA, Col-1, and fibronectin in TGF-β1-induced NPDFs. To determine whether PTNs attenuated the TGF-β1-induced expression of α-SMA, Col-1 and fibronectin in TGF-β1-stimulated NPDFs, the cells were treated with various concentrations of PTNs (5-30 µg/ml) for 30 min, prior to TGF-β1 stimulation for 24 h. It was found that the expression levels of α-SMA, Col-1 and fibronectin were significantly attenuated in a PTN concentration-dependent manner (Fig. 3).

PTNs inhibits TGF-β1-stimulated Smad2/3 signaling pathways. The phosphorylation of Smad2 and Smad3 in NPDFs was markedly enhanced by TGF-β1 induction (Fig. 4A). However, when the cells were pretreated for 30 min with PTNs, particularly at 30 µg/ml, prior to TGF-β1 stimulation for 24 h, the phosphorylation of Smad2 and Smad3 was significantly reduced. Therefore, the antifibrotic effects of PTNs may be mediated by the inhibition of TGF-β1-induced Smad2/3 signaling pathways.

Silencing of Smad2/3 inhibits the TGF-β1-induced expression of α-SMA, Col-1 and fibronectin in NPDFs. To confirm whether Smad2/3 are critical to the TGF-β1-induced expression of α-SMA, Col-1 and fibronectin in TGF-β1-stimulated NPDFs, siRNAs were used to knock down the Smad2/3 genes in the NPDFs, and the expression levels of α-SMA, Col-1 and fibronectin were examined. As expected, the siRNA-mediated
silencing of Smad2/3 resulted in significantly reduced the expression levels of α-SMA, Col-1 and fibronectin (Fig. 4B).

PTNs inhibits TGF-β1-induced fibroblast contractile activity.

The cells were cultured in type-1 collagen gel, as described above. The cells were then pretreated with PTNs (5, 10 or 30 µg/ml) for 30 min, followed by TGF-β1 (1 ng/ml) stimulation for 24 h. Stimulation with TGF-β1 resulted in a decrease in the size of the collagen gel (73.71% vs. TGF-β1-untreated group; P<0.05), whereas pretreatment with the PTNs was observed to inhibit this contraction effect at PTN concentrations of 5, 10 and 30 µg/ml (100.86, 106.90 and 156.46% vs. TGF-β1-untreated group, respectively), as shown in Fig. 5 (P<0.05 and P<0.001).

Discussion

The present study investigated the antifibrotic effect and signaling mechanisms involved in the regulation by PTNs, which are well known anti-inflammatory agents. Accumulating evidence suggests that PTNs have a protective effect against inflammatory diseases (17,18). NPs are associated with chronic inflammation and are characterized by structural abnormalities, including stromal fibrosis in the sinus, that cause them to grow. Therefore, inhibition of the inflammatory process and attenuation of the fibrotic process is considered to be a promising strategy for the therapy of NPs.

The present study investigated the association between the morbidity and expression levels of ECM proteins in the NPs, using immunohistochemical and western blot assays. As shown in Fig. 1, the expression levels of Col-1 and fibronectin were higher in the NP tissues and lysates than those in the IT tissues used as a control. Therefore, the high expression levels of Col-1 and fibronectin were correlated with the morbidity of NPs. On the basis of this result, the antifibrogenic effect and inhibitory signaling mechanism in vitro were investigated using PTNs in TGF-β1-induced NPDFs.

Although diverse factors have been implicated in the development and progression of fibrosis, TGF-β, one of the most potent fibrogenic factors, is considered to be crucial in the pathogenesis of NP disease.
of fibrosis, TGF-β is a secreted homodimeric protein that regulates multiple biological processes, including cell proliferation, differentiation, migration, extracellular matrix production, angiogenesis and apoptosis (19,20). The excessive elevation of TGF-β correlates with diverse fibrotic disorders, including pulmonary fibrosis, cardiac fibrosis, cirrhosis, glomerulosclerosis, diabetic nephropathy, Crohn’s disease, rheumatoid arthritis, radiation-induced fibrosis and myocarditis in various human organs (21). Enhanced TGF-β levels have been observed in NPs, suggesting that TGF-β is also involved in the pathogenesis of NPs (22,23). It is well known that TGF-β induces fibroblast activation, proliferation and differentiation. Fibroblasts are found in the stroma of NPs and are considered to be important in development of fibrosis. Previous investigations on the fibroblasts of NPs showed that exposure of TGF-β1 stimulated myofibroblast differentiation, induced collagen production and increased α-SMA (24,25). To elucidate the antifibrotic activity of PTNs, the present study investigated myofibroblast differentiation and profibrotic protein expression, in addition to the mechanism underlying the effect of PTNs in TGF-β1-stimulated NPdFs.

Fibroblasts can be activated by various chemical signals, which promote their proliferation and differentiation into myofibroblasts (7). Myofibroblasts are characterized by their morphology, functional properties and gene expression. They are the principal effector cells, which synthesize profibrotic proteins, including α-SMA, and high quantities of ECM proteins, particularly Col-1 and fibronectin. Myofibroblasts are important in ECM remodeling in several pathological conditions of the human airway, including asthma, chronic rhinosinusitis and NPs (26). In NPs, myofibroblasts are considered to originate via the differentiation of resident NP fibroblasts. The expression of α-SMA is the hallmark of myofibroblast differentiation and is critical for its function. Fibronectin, a multifunctional glycoprotein involved in tissue remodeling, is known to be a chemoattractant for fibroblasts and can be released in increased quantities by the fibroblasts in response to various cytokines (27). Compared with that in the normal control IT tissues, Col-1 was found to be increased in all NPs. Collagen deposition was most abundant in the sub-mucosal connective tissue and in the basement membrane zone (28). TGF-β1 induces fibroblast-to-myofibroblast differentiation, and increases the expression of α-SMA, Col-1 and fibronectin. Therefore, the approaches to reduce the conversion of fibroblasts to myofibroblasts and ECM proteins may be beneficial therapeutic strategies for NPs. In the present study, it was found that the expression levels of α-SMA, fibronectin and Col-1 were significantly induced in TGF-β1-stimulated NPdFs. However, the results showed that PTNs inhibited the expression of α-SMA, fibronectin and Col-1 in response to TGF-β1 in the absence of cytotoxic concentrations. These results suggested that PTNs suppressed TGF-β1-induced myofibroblast differentiation and ECM protein accumulation in NPdFs.

The present study also investigated the signal pathways underlying the inhibition of α-SMA and ECM levels by PTN treatment. TGF-β is recognized by two heterodimeric membrane receptors, type-1 and type-II TGF-β receptors, which are transmembrane serine/threonine kinases (29). The Smad-dependent signal transduction system is necessary for
TGF-β signaling, and the TGF-β/Smad signaling pathway is one of the most common pathways in fibrosis. When TGF-β1 binds to its receptor, Smad2/3 is phosphorylated and binds with Smad4, consequently translocating to the nucleus, where these complexes activate the transcription of profibrotic genes and induce fibrogenesis (30). In the present study, PTN treatment was observed to attenuate TGF-β1-induced Smad2 and Smad3 phosphorylation in the nucleus (Fig. 4A). To further confirm the role of Smad2/3 in the inhibitory effect of PTNs, siRNAs were used to knock down the Smad2/3 genes prior to TGF-β1 treatment in NPDs; the levels of α-SMA, Col-1 and fibronectin were then measured. As expected, the siRNA-mediated silencing of Smad2/3 resulted in significant inhibition of the production of TGF-β1-induced α-SMA and ECM proteins (Fig. 4B). These data demonstrated that PTNs inhibited myofibroblast differentiation and ECM protein accumulation by inhibiting the phosphorylation of Smad2/3 pathways in TGF-β1-stimulated NPDs.

Finally, the present study assessed the effect of PTN treatment on type-I collagen gel contraction mediated by TGF-β1-stimulated NPDs. Myofibroblasts have increased contractile activity owing to their elevated expression levels of α-SMA with increasing mechanical load (31). Simulation with TGF-β1 resulted in a decrease in the size of the collagen gel, indicating an increase in contractility, whereas pretreatment with PTNs was observed to inhibit the collagen gel contraction (Fig. 5). Collectively, these results confirmed that PTNs suppressed the TGF-β1-mediated fibrotic process in vitro.

In conclusion, the results of the present study demonstrated that PTNs effectively suppressed TGF-β1-augmented myofibroblast differentiation, ECM protein accumulation, and collagen gel contraction in vitro, by inhibiting the phosphorylation of Smad2/3 signaling pathways in NPDs. These results suggested that PTNs may be potential therapeutic agents for treating NP formation. Furthermore, this possibility has important implications in the development of novel therapeutic approaches for managing any fibrotic disorder in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JP, GC, DL and IC conceived and designed the project and prepared the manuscript. MY, JL, JY, WP and TK performed the experiments. SeP and SS performed statistical analysis and data interpretation. SaP, DL and IC analyzed the data. DL and IC wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Local Ethics Committee of Busan Paik Hospital, Inje University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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