

Effect of simvastatin on transforming growth factor beta-1-induced myofibroblast differentiation and collagen production in nasal polyp-derived fibroblasts

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ABSTRACT

Background: Statins are the most commonly prescribed drugs for the treatment of hypercholesterolemia. Statins exert not only lipid-lowering but also other cellular effects, including antifibrotic properties. The purpose of this study was to determine the effect of simvastatin on transforming growth factor (TGF)-beta-1-induced myofibroblast differentiation and collagen production in nasal polyp-derived fibroblasts (NPDFs) and to verify the mechanism of the effect of simvastatin in TGF-beta-1-induced myofibroblast differentiation in NPDFs.

Methods: NPDFs were pretreated with simvastatin with or without mevalonate or Y-27643 for 2 hours before induction by TGF-beta-1. The expression of alpha-smooth muscle actin (SMA) and collagen type IV mRNA was determined by a reverse transcription-polymerase chain reaction, and the expression of alpha-SMA protein was determined by immunofluorescent cytochemical staining. Total soluble collagen production was analyzed by the SirCol collagen dye-binding assay (Biocolor, Belfast, U.K.). Phosphorylation of Smad 2/3 was evaluated by Western blot analysis.

Results: In TGF-beta-1-induced NPDFs, simvastatin significantly inhibited the expressions of α -SMA and collagen type IV mRNA and reduced alpha-SMA and collagen protein levels. Pretreatment with mevalonate reversed the effect of simvastatin. The expression of alpha-SMA mRNA and protein was significantly decreased by pretreatment with Y-27632. The TGF-beta-1-induced expression of pSmad 2/3 protein was notably decreased by pretreatment with simvastatin.

Conclusion: We showed that simvastatin inhibits TGF-beta-1-induced myofibroblast differentiation (expression of alpha-SMA) and collagen production in NPDFs and Rho/Rock and TGF- β /Smad signaling is involved as an underlying mechanism. The results of our study suggest that simvastatin is a possible candidate for the suppression of nasal polyp formation.

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Nasal polyps are a chronic inflammatory condition of the paranasal sinuses, the overall prevalence of which ranges from 1 to 4% in the general population. Nasal polyps cause considerable morbidity, such as nasal obstruction, rhinorrhea, and anosmia. Despite considerable effort to identify the important factors underlying nasal polyp formation, the pathogenesis of nasal polyps is still not fully elucidated. Among many observed phenomena for nasal polyp formation, extracellular matrix (ECM) accumulation has been proposed as one of the important factors based on animal studies.^{1,2} Fibroblastic cells are the cellular source of ECM. With respect to fibroblastic cells, proliferation of fibroblasts is increased and correlated with fibronectin accumulation in nasal polyps.³ It has also been shown that the number of myofibroblasts observed in nasal polyps and myofibroblast accumulation is induced by transforming growth factor (TGF) β 1.⁴ Myofibroblasts are the activated cell phenotype of fibroblasts and have a high capacity for ECM protein secretion.

Statins, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are the most commonly prescribed drugs for the treatment of hypercholesterolemia.⁵ Statins inhibit the rate-limiting enzyme, HMG-CoA reductase, in the mevalonate pathway for the biosynthesis of cholesterol in cells. The lowering of blood cholesterol by statins can influence a number of pathogenic mechanisms that are

thought to contribute to coronary artery disease. Statins exert not only lipid-lowering but also other cellular effects, including antifibrotic properties.^{6,7} In several fibrotic diseases involving other organs, statins inhibit the differentiation of fibroblasts and production of ECM.

These findings led us to theorize that statins have effects on nasal polyp formation. The purpose of this study was to determine the effect of simvastatin in TGF- β 1-induced myofibroblast differentiation (α -smooth muscle actin [α -SMA]) and collagen production (collagen type IV) in nasal polyp-derived fibroblasts and to verify the mechanism of the effect underlying simvastatin in TGF- β 1-induced myofibroblast differentiation.

MATERIALS AND METHODS

Reagents

Human recombinant TGF- β 1 was obtained from R & D Systems (Minneapolis, MN). Simvastatin (Sigma, St. Louis, MO) was dissolved in ethyl alcohol and Y-27632 (Sigma). Mevalonate (Sigma) was dissolved in distilled water and then diluted to the desired concentrations with complete medium for use in the experiments.

Induction of Fibroblasts and Cell Culture

To induce fibroblasts from nasal polyps, six patients (three women and three men; mean age, 38.3 ± 5.2 years) were recruited from the Department of Otorhinolaryngology-Head and Neck Surgery of Korea University Guro Hospital. The patients were nonsmokers and not treated with oral antiallergic agents for at least 2 months. Nasal polyps were obtained intraoperatively after written informed consent was obtained. The study was approved by the Ethics Committee of Korea University (KUGGR-2010-025).

Nasal polyp-derived fibroblasts (NPDFs) were isolated from surgical tissues by enzymic digestion with collagenase (500 U/mL; Sigma), hyaluronidase (30 U/mL; Sigma), and DNase (10 U/mL;

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Sigma). Briefly, after a 2-hour incubation in 5% CO₂ at 37°C in a culture plate, the cells were collected by centrifugation, washed twice, resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY) containing 10% (v/v) heat-inactivated fetal bovine serum and antibiotics, 2-glutamate (Invitrogen), 100 µg/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen). Cells were allowed to attach the culture plate for 4 days. Nonadherent cells were removed by changing the medium. Fibroblasts were detached with trypsin-EDTA solution (Invitrogen). After the cells were washed, the cells were resuspended in medium and used for subsequent experiments. Fibroblast purity was >99% and used for NPDFs. Cells were used at passage 5.

Cytotoxicity Assay

The cytotoxic action of simvastatin was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide method. In a 96-well microstate plate, NPDFs (5×10^3 cells/well) were cultivated in DMEM medium supplemented as described previously. Simvastatin was evaluated at various concentrations within physiologically relevant levels of drug, as used in clinical practice (0.1–50 µM) for 24 hours at 37°C in a 5% CO₂ and 95% air humid atmosphere. After this culture period, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (Sigma) for 4 hours, and the reaction was interrupted by the addition of acidified isopropanol. A fluorescence microplate reader (F2000; Hitachi, Ltd., Tokyo, Japan) was used to obtain the results (570 nm). All assays were performed in triplicate.

Reverse Transcription-Polymerase Chain Reaction

NPDFs (5×10^6 cells/mL) were pretreated with simvastatin, Y-27632, and mevalonate for 2 hours before TGF-β1 stimulation for 24 hours. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed for α-SMA mRNA. Total RNA was extracted using a commercially available reagent (RNAiso Plus; Takara Bio, Inc., Otsu, Japan). Total RNA from each sample was reverse transcribed in 20 µL of reaction mixture containing 2.5U of Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and 2.5 mmol of dNTP at 37°C for 60 minutes. PCR was performed using the following primers: α-SMA (sense sequence, 5-GGT GCT GTC TCT CTA TGC CTC TGG A-3; antisense sequence, 5-CCC ATC AGG CAA CTC GAT ACT CTT C-3; 321 bp), collagen type IV (sense sequence, 5-CCA GGA CCA AGT GGA AGA G-3; antisense sequence, 5-CTG CAA CAC CAT CTC TGC C-3; 318 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense sequence, 5-GTG GAT ATT GTT GCC ATC AAT GAC C-3; antisense sequence, 5-GCC CCA GCC TTC TTC ATG GTG GT-3; 270 bp) were purchased from Bioneer (Daejeon, Korea). The GAPDH was used to verify that equal amounts of RNA were used for RT and PCR amplification from different experimental conditions. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The gels were certificated using a Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA).

Western Blotting of pSmad 2/3

The cells were lysed in protein extraction solution (Pro-Prep; Intrin Biotechnology, Inc., Sung-nam, Korea) and centrifuged for 1 minute at 14,000 rpm. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were then blocked for 60 minutes with 5% nonfat dry milk and incubated at 21°C for 2 hours with polyclonal pSmad antibody (Santa Cruz Biothechnology, Inc., Santa Cruz, CA). After incubation, the membrane was washed three times for 5 minutes in Tris-buffered saline and Tween 20 buffer and then treated with peroxidase-conjugated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA)

at a dilution of 1:5000 for 1 hour at room temperature. The membrane was washed, and substrate was added from an enhanced chemiluminescence reagent kit (Intron Biotechnology, Inc.) and exposed to x-ray film for 10 seconds. As an internal control, β-actin expression was analyzed using β-actin antibody (Sigma).

Immunofluorescence of α-SMA Protein

NPDFs (2×10^3 cells/mL) were plated on coverslips. Fibroblasts were pretreated with simvastatin and Y-27632 for 2 hours before TGF-β1 stimulation for 24 hours. Then, the cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 30 minutes, blocked with 3% bovine serum albumin, and incubated with an anti-α-SMA antibody (1: 200) for 2 hours, and washed three times with PBS for 5 minutes. The cells were then incubated in goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) at a dilution of 1:200 for 1 hour, and washed three times with PBS for 5 minutes. Then, the cells were mounted in Vectashield (Vector Laboratories, Inc.) with 4',6-diamidino-2-phenylindole. The cells were visualized on an immunofluorescence microscope (IX71; Olympus, Tokyo, Japan) under identical settings.

Collagen Measurements

Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor, Belfast, U.K.) according to the manufacturer's instruction. For these experiments, confluent cells in 25-cm² culture dishes were incubated for 24 hours with 1 mL of DMEM-5% fetal bovine serum. One milliliter of Sirius red dye, an anionic dye that reacts specifically with basic side chain groups of collagens under assay conditions, was added to 400 µL of supernatant and incubated with gentle rotation for 30 minutes at room temperature. After centrifugation at $12,000 \times g$ for 10 minutes, the collagen-bound dye was redissolved with 1 mL of 0.5M NaOH, and absorbance at 540 nm was measured by an enzyme-linked immunosorbent assay (MRX; Dynex, Chantilly, VA). The absorbance was directly proportional to the amount of newly formed collagen in the cell culture supernatant.

Statistical Analysis

The statistical significance of the difference between the control and experimental data was analyzed using an ANOVA test. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS

Effect of Simvastatin on α-SMA Expression in TGF-β1-Induced NPDFs

First, to evaluate the ability of simvastatin to inhibit TGF-β1-induced α-SMA gene expression, we examined the cytotoxicity of simvastatin on NPDFs using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide method. Simvastatin did not show cytotoxic effects for 48 hours up to a concentration of 50 µM. Next, cells were pretreated with simvastatin (0.1, 0.5, 1, 5, and 10 µM) for 2 hours and stimulated with TGF-β1 (5 ng/mL) for 24 hours. TGF-β1 significantly increased the level of expression of α-SMA mRNA. Pretreatment with simvastatin significantly inhibited TGF-β1-induced α-SMA mRNA expression in a dose-dependent manner (Fig. 1, A and B). Immunofluorescence microscope showed that treatment of simvastatin resulted in reduction in the number of α-SMA protein-positive cells (Fig. 1 C).

Effect of Simvastatin on Collagen Production in TGF-β1-Induced NPDFs

To determine the inhibitory effect of simvastatin in TGF-β1-induced collagen type IV mRNA expression and total soluble collagen

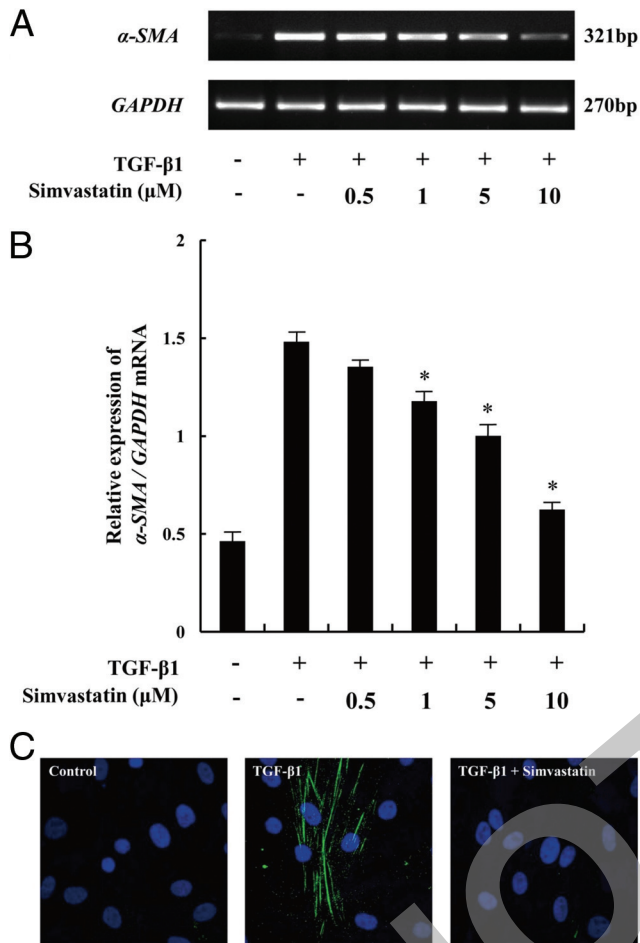


Figure 1. Simvastatin inhibited phenotype change (α -smooth muscle actin [α -SMA]) of transforming growth factor (TGF) β 1-induced nasal polyp-derived fibroblasts (NPDFs). Expression of α -SMA mRNA was determined by (A) reverse transcription-polymerase chain reaction (RT-PCR) and (B) densitometric analysis. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown as an internal control. Values are expressed as the means $\pm$ SD of six separate experiments (* p < 0.05 versus TGF- β 1 alone). (C) The expression of simvastatin α -SMA protein was determined in immunofluorescence microphotography. Nuclei were stained with 4',6-diamidino-2-phenylindole (original magnification, $\times$ 200). Representative of six independent experiments.

production, we pretreated cells with simvastatin for 2 hours before stimulating the cells with TGF- β 1 (5 ng/mL) for 24 hours. The expression of collagen type IV mRNA and production of total soluble collagen were increased by stimulation with TGF- β 1 and was notably decreased by pretreatment with simvastatin in a dose-dependent manner (Fig. 2).

Effect of Rho-Associated Serine-Threonine Protein Kinase Inhibitor on α -SMA Expression in TGF- β 1-Induced NPFDs

Beneficial effects of statins could be attributed to their ability to suppress the synthesis of downstream products of cholesterol such as isoprenoid intermediates, which is needed for prenylation of Rho and it is known that Rho inhibition by simvastatin abrogates the differentiation of myofibroblasts in other tissues.⁸ So, we hypothesized that the effect of simvastatin on modulation of TGF- β 1 signaling in NPFDs would also occur by Rho/ROCK pathway inhibition. Y-27632 ([+]-[R]-*trans*-4-1[1-

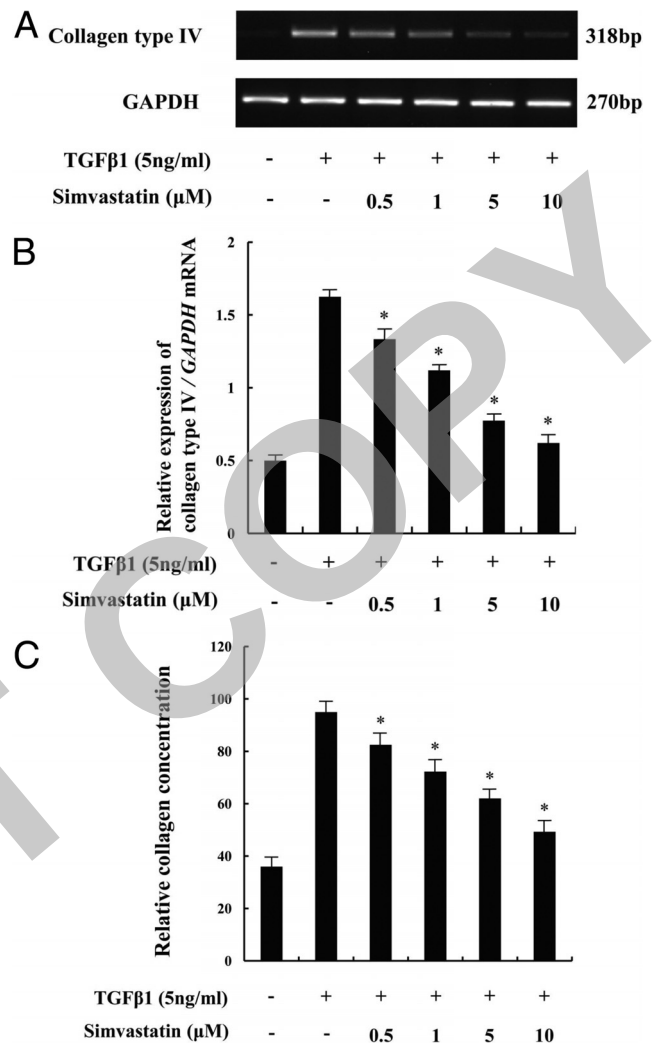


Figure 2. Simvastatin inhibited collagen production in transforming growth factor (TGF) β 1-induced nasal polyp-derived fibroblasts (NPDFs). The expression of collagen type IV mRNA was determined by (A) reverse transcription-polymerase chain reaction (RT-PCR) and (B) densitometric analysis. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown as an internal control. (C) Total soluble collagen production was determined by a SirCol collagen dye-binding assay. Values are expressed as the means $\pm$ SD of six separate experiments (* p < 0.05 versus TGF- β 1 alone).

aminoethyl]-N-[4-pyridyl]cyclohexanecarboxamide dihydrochlorides) is widely used as a specific inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases.⁹ To determine the effect of Y-27632 in TGF- β 1-induced α -SMA expression, we pretreated cells with Y-27632 (30 μ M) for 2 hours and stimulated them with TGF- β 1 for 24 hours. The expression of α -SMA mRNA and protein was significantly decreased by pretreatment with Y-27632 (Fig. 3). These results mean the effect of simvastatin on modulation of TGF- β 1 signaling in NPFDs is related with Rho/ROCK pathway inhibition.

Effect of Simvastatin in Combination with Mevalonate on α -SMA Expression in TGF- β 1-Induced NPFDs

Mevalonate is an earliest intermediate of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-reductase). To confirm statin shows

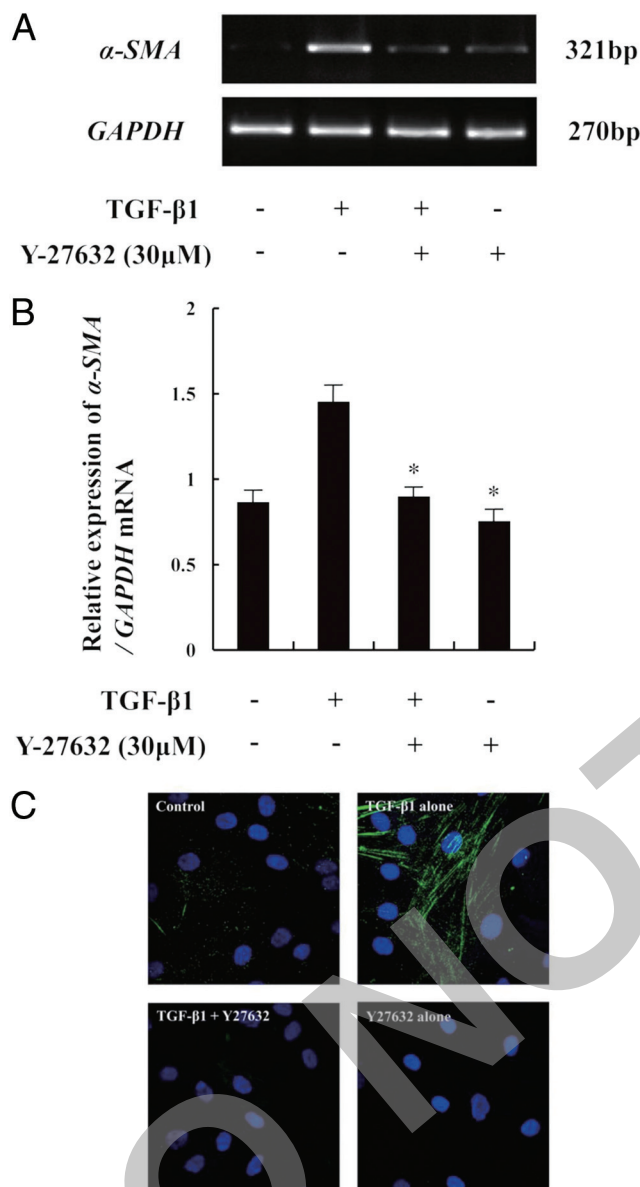


Figure 3. Rho-associated coiled kinase inhibitor, Y27632, inhibited phenotype change (α -smooth muscle actin [α -SMA]) of transforming growth factor (TGF) β 1-induced nasal polyp-derived fibroblasts (NPDFs) and mimicked the effect of simvastatin. The expression of α -smooth muscle actin (α -SMA) mRNA was determined by (A) reverse transcription-polymerase chain reaction (RT-PCR) and (B) densitometric analysis. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown as an internal control. Values are expressed as the means $\pm$ SD of six separate experiments (* p < 0.05 versus TGF- β 1 alone). (C) The expression of α -SMA protein was determined in immunofluorescence microphotography. Nuclei were stained with 4',6-diamidino-2-phenylindole (original magnification, $\times$ 200). Representative of six independent experiments.

the effect by its HMG-CoA reductase activity, we assessed if mevalonate, earliest intermediate by HMG-CoA reductase, which is a target of statin, reverses the effect of simvastatin on the expression of α -SMA in TGF- β 1-induced NPDFs. In the absence of simvastatin, TGF- β 1 increased the expression of α -SMA mRNA expression after 24 hours of stimulation. Simvastatin strongly diminished the TGF- β 1-induced increase in the levels of α -SMA mRNA. Pretreatment with mevalonate reversed the effect of simvastatin (Fig. 4). This result means that

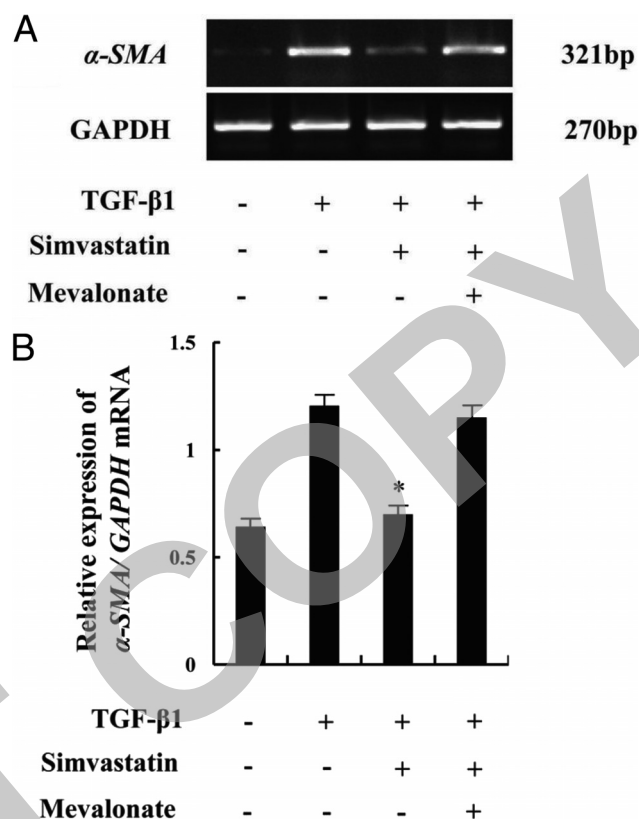


Figure 4. The mevalonate, earliest intermediate by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is target of statin, reversed the effect of simvastatin. The expression of α -smooth muscle actin (α -SMA) mRNA was determined by (A) reverse transcription-polymerase chain reaction (RT-PCR) and (B) densitometric analysis. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown as an internal control. Values are expressed as the means $\pm$ SD of six separate experiments (* p < 0.05 versus transforming growth factor [TGF] β 1 alone).

simvastatin may exert its effect as a HMG-CoA reductase not by other unexpected effects.

Effect of Simvastatin on Phosphorylation of Smad 2/3 in TGF- β 1-Induced NPDFs

To identify the effect of simvastatin on intracellular TGF- β 1 signaling in NPDFs, we evaluated phosphorylated Smad (pSmad) 2/3 as a downstream marker for TGF- β signaling by Western blot analysis. We pretreated cells with simvastatin for 2 hours before stimulating the cells with TGF- β 1 for 24 hours. The expression of pSmad 2/3 protein was increased by stimulation with TGF- β 1 and was notably decreased by pretreatment with simvastatin. Pretreatment with Y-27632 also decreased the expression of pSmad 2/3 protein (Fig. 5).

DISCUSSION

Although the etiology and pathophysiological mechanisms leading to nasal polyp formation remain unclear, experimental and histological studies showed accumulation of ECM is an important process in the characteristic structural modification of nasal polyps.^{1,2,10} Myofibroblasts, which are found in nasal polyps, appear to be involved in the aforementioned process. Myofibroblasts are key cells for the connective tissue remodeling that takes place during wound healing and development of fibrosis. After tissue injury, fibroblasts assume the activated phenotype (myofibroblasts) and synthesize ECM components by cytokines locally released from inflammatory and resident

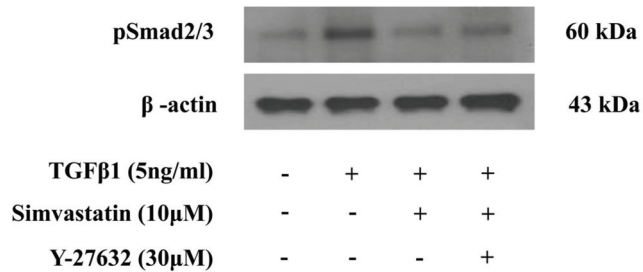


Figure 5. Simvastatin inhibited the phosphorylation of Smad 2/3, a well-known downstream marker for transforming growth factor (TGF) β signaling, in TGF- β 1-induced nasal polyp-derived fibroblasts (NPDFs). The expression of phosphorylated Smad 2/3 was determined by Western blot analysis. The expression of β -actin is shown as an internal control. Values are expressed as the means $\pm$ SD of six separate experiments ($n = 6$; $*p < 0.05$ versus TGF- β 1 alone).

cells.¹¹ Fibroblast apoptosis terminate above tissue repair process in proper time; however, this could be inhibited by persistence of cytokines, such as TGF- β .¹² Overproduction of TGF- β can induce excessive deposition of scar tissue and fibrosis. In inflammatory nasal polyps, TGF- β 1 is the main isoform detected and is present in macrophages and eosinophils.^{13,14} In our previous study, we showed that TGF- β 1 significantly increased the expression of α -SMA, as well as the production of collagen in NPDFs.¹⁵

Statins are the commonly prescribed drugs for the treatment of hypercholesterolemia because of the efficacy of statins in reducing low-density lipoprotein and the tolerability and safety of statins.⁵ However, statins also exert other cellular effects, including immunomodulation, anti-inflammatory effects, and neurogenesis.^{16,17} In NPDFs, we showed the inhibitory effect of simvastatin in TGF- β 1-induced myofibroblast differentiation (α -SMA protein) and collagen production.

The pleiotropic effects of statins are partially explained by inhibition of isoprenylation, the addition of hydrophobic molecules to protein. Isoprenylation enables proteins to bind to the cell membrane and exert signaling activities. Statins down-regulate intermediates of cholesterol synthesis, such as geranylgeranylpyrophosphate, which is important for isoprenylation.⁸ Small guanosine triphosphatase of the Rho and Ras family are included in proteins that require isoprenylation for posttranscriptional modification.¹⁸ Inhibition of the Rho/ROCK pathway makes statins exhibit antifibrotic properties by modulation of TGF- β 1 signaling.⁸ Y-27632, widely used as a specific inhibitor of the ROCK pathway, mimicked the effect of simvastatin in TGF- β 1-induced myofibroblast differentiation and collagen production in NPDFs in this study.

Several studies have suggested a close interaction between the Rho/ROCK pathway and Smad of TGF- β 1 signaling. Dominant-negative RhoA blocked nuclear translocation of Smad2 and Smad3 because of the inhibition of phosphorylation of both Smads.¹⁹ A recent study showed that simvastatin inhibits TGF- β 1-mediated fibroblast activation by inhibition of Smad3 phosphorylation in intestinal fibroblasts.²⁰ We evaluated pSmad 2/3 as a downstream marker for TGF- β signaling by Western blot analysis in NPDFs and confirmed the inhibitory effect of simvastatin on phosphorylation of Smad 2/3 in TGF- β 1-induced NPDFs.

This study is the first study to show the inhibitory effect of statins on myofibroblast differentiation and collagen production in TGF- β 1-induced NPDFs. In the present study, we showed that simvastatin inhibits TGF- β 1-induced myofibroblast differentiation and collagen production in NPDFs and indicated that Rho/Rock and TGF- β /Smad

signaling may be involved as the underlying mechanisms. Frequent recurrence of nasal polyps after endoscopic sinus surgery requires additional therapeutic modalities. The results of our study suggest simvastatin as a possible candidate for the inhibition of nasal polyp formation and further testing in patients will be required to verify this finding.

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