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Simvastatin Abrogates TGF-β Induced Stimulation of Normal Human Dermal Fibroblast Collagen Gene Expression in Culture

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Abstract

A potent stimulation of fibroblast collagen production is one of the crucial pleotropic effects of transforming growth factor β (TGF-β) and has been considered to play a crucial role in the pathogenesis of fibrotic diseases including Systemic Sclerosis and Pulmonary Fibrosis. This complex process involves numerous intracellular reactions mediated by canonical Smad-dependent or non-canonical pathways that transduce the extracellular stimuli into the nucleus. Here, we demonstrated that Simvastatin, a widely used statin, induces a potent inhibition of TGF-β1 profibrotic effects in cultured normal human dermal fibroblasts, and studied the molecular mechanisms involved in these effects. We also examined Simvastatin modulation of TGF-β1 induced fibroblast to myofibroblast transition. Normal human dermal fibroblasts were cultured with various concentrations of Simvastatin in the presence or absence of TGF-β1 (10ng/ml) for 24, 48, and 72 h. The effects of Simvastatin on TGF-β1 stimulation of COL1A1 expression and type 1 collagen production were examined. Assessment of Smad2/3 and Erk1/2 phosphorylation, chromatin immunoprecipitation assays for Sp1 transcription factor binding to the COL1A1 proximal promoter, siRNA-mediated RhoA knockdown, and F-actin immunofluorescence microscopy was performed to examine the molecular mechanisms involved.

The results showed that Simvastatin caused a potent and dose-dependent abrogation of TGF-β1 stimulation of COL1A1 expression and type I collagen production in cultured normal human dermal fibroblasts. The molecular mechanisms of these effects were complex and involved reduction of Sp1 binding to the COL1A1 proximal promoter, decreased Erk1/2 phosphorylation and inhibition of Rho-A- mediated prenylation effects. Additional studies indicated that Simvastatin also prevented the TGF-β- induced phenotypic conversion of fibroblasts into activated myofibroblasts. Collectively, these results suggest that statins may be effective therapeutic agents for TGF-β1-mediated fibrotic diseases including Systemic Sclerosis and Pulmonary Fibrosis.

INTRODUCTION

The fibrotic diseases comprise a highly heterogeneous group of pathologic conditions caused by multiple etiologic factors and involving numerous and complex molecular alterations that result in the excessive and disorganized accumulation of fibrotic tissue in various organs of the body causing abnormalities in their function and eventually leading to organ failure [1-4]. The fibrotic diseases are quite frequent and it has been estimated that, collectively, they may contribute to 40 to 45% of the overall mortality in the USA [1]. Despite their serious clinical consequences and high mortality, there is currently no effective therapy for these disorders and they remain among the most frequent causes of morbidity and mortality worldwide. Furthermore, the precise molecular alterations involved in their development and progression have not been fully elucidated although numerous highly relevant pathogenetic pathways have been identified [5,6].

Transforming Growth Factor β (TGF-β), the most potent profibrotic polypeptide identified to date [7-12], plays a crucial role in the pathogenesis of various human fibrotic diseases including systemic fibrotic diseases such as Systemic Sclerosis (SSc), and organ-specific fibrotic diseases including Idiopathic Pulmonary Fibrosis (IPF) and cardiac, kidney, and liver fibrosis [13-19]. One of the most important molecular effects of TGF-β is the stimulation of expression of genes encoding various extracellular matrix (ECM) proteins including fibrillar collagens (COL1A1, COL1A2 and COL3A1) and fibronectin, resulting in the increased production and exaggerated tissue accumulation of the corresponding proteins [20-24]. These effects are initiated by TGF-β binding to the type II TGF-β specific cell surface receptor, followed by recruitment and phosphorylation of the type I TGF-β

receptor, and transduction of the binding signal through the canonical signaling cascade of Smad proteins into the nucleus. This cascade of reactions results in modulation of expression of TGF-β responsive genes including those encoding fibrillar collagens, fibronectin, and other ECM macromolecules [25-29]. The TGF-β effects may also be mediated by other non-Smad pathways depending on the initial stimulus and the specific target cells [30-32]. Owing to the potent profibrotic TGF-β effects, it has been suggested that inhibition of TGF-β profibrotic effects may represent an effective therapeutic approach for TGF-β- mediated fibrotic diseases. In a previous study we demonstrated that the statin Simvastatin was a powerful inhibitor of type I collagen gene expression in cultured SSC dermal fibroblasts [33]. However, the effects of Simvastatin on TGF-β stimulated collagen gene expression were not examined. Here, we investigated the effects of Simvastatin on the TGF-β1-stimulated expression of COL1A1 and on the production of type I collagen in cultured normal human dermal fibroblasts. The results showed that Simvastatin caused a potent reduction of TGF-β1-stimulated expression of COL1A1 and type I collagen production. Studies of the molecular mechanisms involved in these effects showed that the abrogation of TGF-β stimulation of collagen gene expression was mediated by a decreased binding of the transcription factor Sp1 to the COL1A1 proximal promoter and was associated with a profound inhibition of ERK 1/2 phosphorylation and Rho-A prenylation.

MATERIALS AND METHODS

Cell Culture normal human dermal fibroblasts were obtained from discarded tissues from normal individuals undergoing dermatological surgical procedures. All studies were approved by the Institutional Review Board of Thomas Jefferson University. For all studies, only early passage fibroblasts (<10) were employed. Normal dermal fibroblasts were cultured in 100 mm dishes until confluency in DMEM medium supplemented with 10% fetal bovine serum (Mediatech Inc.) as previously described [21,22]. Once confluency was achieved, cells were serum-depleted by culture in DMEM supplemented with 10% Nu serum (BD Biosciences) for 24 h before the addition of test compounds. For immunofluorescence studies, cells were cultured in 4-well glass slides in the same culture media, then in DMEM media supplemented with 1% bovine serum albumin for 24 h, and finally in DMEM media supplemented with 0.5% BSA with the addition of the test compounds. For studies of collagen gene expression and production, cells were maintained in DMEM containing 10% fetal bovine serum (Invitrogen), 1% vitamins, 2 mM glutamine, and antibiotics and were incubated at 37°C in a 5% CO2 humidified atmosphere. The cultures were supplemented with 40 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Waco Biochemical) 24 h prior to the addition of the test compounds to optimize the level of collagen production.

Treatment of cultured fibroblasts. Simvastatin (in active form) was from CalBiochem. All other chemicals were of reagent grade. Normal human dermal fibroblasts were plated in 6-well plates and cultured until confluent as described above. The confluent cells were preincubated with 5 μM of Simvastatin in serum-free medium containing ascorbic acid and 10% Nu serum (BD Biosciences) for 24h. The medium was then changed with fresh 10% Nu serum medium containing 10ng/ml TGF-β1 (From Sigma-Aldrich) and/or other reagents and the cultures incubated for additional 24, 48, or 72 h. Western blots. For Western blot analysis of collagen type I production under basal conditions and under TGF-β1 +/- Simvastatin treatment, the media of the cell cultures were removed and equal aliquots were boiled in an SDS buffer containing 1% β-mercaptoethanol for 5 min, then loaded into 8% or 10% Tris- glycine polyacrylamide gels (Invitrogen) and the proteins separated by electrophoresis as described previously [33]. The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad), and incubated in a blocking buffer containing 5% nonfat dry milk in Tris-buffered saline with Tween (TBST) for 1 h. The membrane was incubated with a polyclonal anti-collagen type 1 antibody (Rockland) in a buffer containing 3% nonfat dry milk with TBST at room temperature overnight, washed with TBST and then incubated for 1.5 h with HRP-conjugated anti-rabbit IgG (Amersham Biosciences). After several washes with TBST the immunoreactive bands corresponding to collagen type 1 were visualized by ECL (Amersham Biosciences). Western blots for phospho-Smad2/3, total Smad, phospho-Erk1/2 and total Erk were performed with mouse monoclonal phospho-Erk1/2 and total Erk antibodies (Santa Cruz Biotechnology), mouse monoclonal phospho-Smad2/3 antibodies (Chemicon-Millipore), and mouse monoclonal Smad2/3 antibodies (BD Biosciences-Pharmingen). For control of protein loading andtransfer, the blots were probed with a mouse monoclonal β-actin antibody (Sigma).

Northern hybridizations. Fibroblasts were grown to confluence and following incubation either under control conditions or with TGF-β1 +/- Simvastatin total RNA was isolated employing an RNeasy Mini- Kit (Qiagen). Aliquots of total RNA (5-10 μg/well) were electrophoresed on 1.0% formaldehyde agarose gels. The RNA was then transferred to Hybond™ N+ membranes (Amersham Biosciences) and the filters 32hybridized to P-radiolabeled human cDNA for COL1A1, as described previously [33]. Equivalentamounts of RNA were loaded and RNA loading and transfer were evaluated by probing with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. Equivalent loading and transfer were also verified by quantitative image analysis of ethidium bromide staining of ribosomal RNA in the same blots. The filters were analyzed using phosphor technology (Image-Quant V5.1 software; Amersham Biosciences). Chromatin Immunoprecipitation Assays (ChIP). For ChIP assays, normal dermal fibroblasts were plated in 150 mm dishes and cultured until confluency, then treated with TGF-β1 (10 ng/ml) +/- Simvastatin (5 μM) for 72 h. The ChIP assay [29] was performed using the ChIP- IT kit (Active Motif), as described previously [34]. Briefly, cells from the various cultures were treated with a solution of 1% formaldehyde in PBS for 10 min at room temperature to crosslink the DNA, washed twice with ice cold PBS and harvested. The cells were then resuspended in lysis buffer, and the nuclei were released following Dounce

homogenization and sonicated on ice to shear the DNA. For immunoprecipitation, a chromatin aliquot was pre-cleared with protein G agarose beads for 1-2 h at 4oC and then incubated with Sp1 monoclonal antibody overnight with gentle rotation. The antibody-chromatin mixtures were then incubated with Protein G beads for 1.5 h at 4°C, washed extensively and the DNA eluted. Crosslinks were reversed and the DNA was purified and subjected to RT-PCR using specific primers for the COL1A1 proximal promoter encompassing the Sp1 recognition site as previously described [35]. The sequences of the primers employed were: forward5'-GGTGGACTCCCTTCCCTCCTC-3'; reverse5'- AGGAACCCTGCCCCTCGGAG-3'.

RhoA siRNA knockdown: SiRNA-mediated knockdown of RhoA was performed using transfection of a double strand siRNA oligonucleotide as previously described [36]. The sequences for siRNA transfection used were: anti-RhoA siRNA; sense 5'- GACAUGCUUGCUCAUAGUCTT-3'; antisense 3'- TTCUGUACGAACGAGUAUCAG-5'. Briefly, normal human dermal fibroblasts were plated onto 60 mm dishes in 10% FBS containing medium. When cultures were 80% confluent, RhoA siRNA was transfected into the cells with Hypertexts transfection reagent (Quiagen) in 10% FBS-containing medium. Then, 5nM of siRNA was added to 100 µl of serum-free media followed by addition of 20 µl of Hyperfect transfection reagent. The solution was mixed gently and incubated at room temperature for 15 min and 120 µl of the mixture was added to each 60 mm dish. After 24 h, cells were transferred to 10% Nu serum containing ascorbic acid for 6 h and then a second siRNA dose added. The cells were treated with 10 ng/ml TGF-β1 for 48 h, then harvested and total RNA and total cell lysate prepared. Real Time PCR. Total RNA was extracted employing the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with DNase treatment. One microgram of each total RNA sample was reverse transcribed using a Superscripts preamplification system (Invitrogen). COL1A1 mRNA expression was assessed by real-time PCR in triplicate as described previously [36]. The differences in the number of mRNA copies in each PCR were corrected for human β-actin endogenous control transcript levels.

Immunofluorescence staining of F actin in human dermal fibroblasts following TFG-β1 treatment with or without Simvastatin. Immunofluorescence microscopy was performed with Alexa fluor 568 phalloidin against F-actin in cells treated for 48 h with TGF-β1 + Simvastatin. For this purpose, cells were plated on glass coverslip wells, grown until confluency, treated with Simvastatin as described above, and then treated with TGF-β1 (10 ng/ml) for 48 h in a 10% Nu serum containing medium. Aftertreatment, cells were washed with PBS pH 7.4 twice, then fixed in 3.7% paraformaldehyde solution in PBS for 10 min, and washed again twice. Following the washes, a cold acetone solution was added to the wells for 3 to 5 min after which the Alexa fluor was added and the wells visualized in a Nikon epifluorescence.

Statistical analysis: Data are shown as means + SD. An unpaired Student's t test was employed for comparing two groups of data with the GraphPad Software. Values of $p<0.05$ were considered significant.

RESULTS

Simvastatin abrogates TGF-β1 induced stimulation of COL1A1 expression and type I collagen production in cultured normal human dermal fibroblasts. As expected, TGF-β1 treatment of the cells resulted in a marked increase in the amount of type I collagen levels reaching between 150 and 200% of the values obtained in the control untreated cells (Figure 1A). Treatment with Simvastatin caused a potent inhibitory effect of TGF-β1 type I collagen stimulation. These observations were confirmed by Northern hybridization analysis showing that the stimulatory effect of TGF-β1 on α1(I) collagen mRNA levels was abrogated by Simvastatin (Figure 1B). These studies showed the expected increase of greater than 200% in type I collagen production

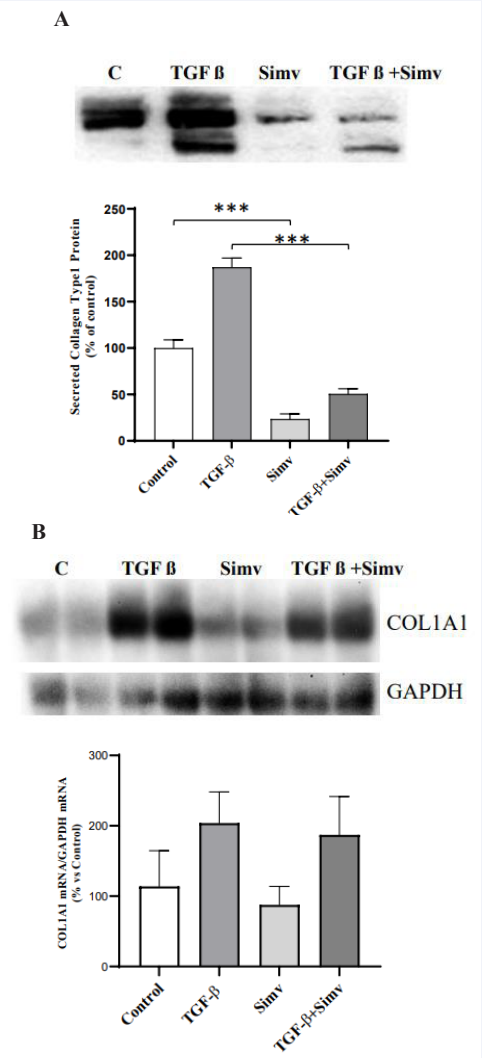

Figure 1 Effect of Simvastatin on collagen I production and COL1A1 mRNA steady-state levels in normal fibroblasts treated with TGF-β. (A) Western analysis of type I collagen levels secreted by normal fibroblasts treated with TGF-β (10ng/ml), Simvastatin /Simv (5µM), or both for 72 h. and densitometric Western blots analysis of three cell lines. (B) Northern analysis of COL1A1 mRNA in normal fibroblasts treated with TGF-β (10ng/ml), Simvastatin /Simv (5µM), or both for 72 h. and densitometric analysis of Northern hybridization of three cell lines. GAPDH mRNA levels were used for normalization.

and COL1A1 transcript levels induced by TGF-β1 treatment compared to untreated control fibroblasts. In contrast, when cells were treated with TGF-β1 plus Simvastatin, there was a marked reduction in the level of TGF-β1-stimulated collagen production and a confirmatory reduction in COL1A1 transcript levels.

Simvastatin inhibits the binding of transcription factor Sp1 to the proximal COL1A1 promoter in control and TGF-β1 treated fibroblasts. To examine the molecular mechanisms responsible for the potent inhibition of the TGF-β1-induced stimulation of type I collagen gene expression and type I collagen production caused by Simvastatin we examined the binding of transcription factor Sp1 to the COL1A1 proximal promoter. We chose Sp1 owing to previous studies demonstrating that Sp1 binding to the COL1A1 promoter is a crucial determinant involved in the upregulation of COL1A1 gene expression by TGF-β1 [33,35] and because Sp1 is also involved on the transcriptional regulation of numerous gene promoters including the COL1A2 [37,38]. Sp1 binding to the proximal COL1A1 promoter region was examined in control and TGF-β1 stimulated fibroblasts with or without Simvastatin treatment employing ChIP assays with specific antibodies against Sp1. The DNA amplification was with specific primers for the proximal -125 bp region from the COL1A1 promoter, a previously known binding site for Sp1 [39]. The results shown in Figure 2 demonstrate that the binding of Sp1 to the COL1A1 proximal promoter DNA was markedly increased in the TGF-β1-treated fibroblasts compared to control fibroblasts and that the increase in Sp1 binding caused by TGF-β was essentially completely abrogated when fibroblasts were treated with Simvastatin. Simvastatin effects on phosphorylated Smad2/3 and Erk1/2 levels. The effects of Simvastatin on certain phosphorylated mediators of TGF-β Smad and non-Smad pathways were examined in a time course experiment. Western blot analyses of the cell lysates using antibodies to phosphorylated Smad2/3 and Erk1/2 were performed (Figure 3). As expected, phospho-Smad2/3 levels markedly increased following TGF-β1 stimulation and these levels were substantially reduced by treatment with Simvastatin. Similar results were obtained with phospho-Erk1/2 levels which also were markedly increased in cells treated with TFG-β1 and then profoundly decreased in the cells treated with TGF-β1 + Simvastatin. These results indicated that the abrogation of collagen type I production observed following Simvastatin treatment was not exclusively due to a change in the levels of phosphorylated Smad2/3 but that a non-Smad pathway involving Erk1/2 signaling also played an important role in these Simvastatin effects.

Effect of geranylgeranyl pyrophosphate (GGPP) on collagen type I production in normal dermal fibroblasts treated with TGF-β1 +/- Simvastatin. Previous studies indicated that inhibition of geranyl geranyl prenylation played a crucial role in the Simvastatin effects on the regulation of COL1A1 gene expression [33]. To confirm whether protein geranylgeranyl prenylation was involved in the inhibitory effects of Simvastin on TGF-β1-induced stimulation of COL1A1 gene expression and type I collagen production, cultured dermal fibroblasts were treated with 5 μM Simvastatin for 24 h, and then TGF-β1 (10 ng/ml) was

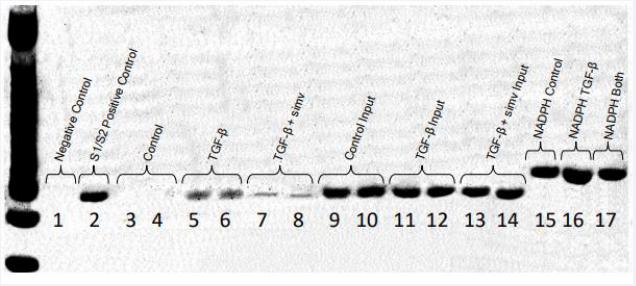

Figure 2 Chromatin Immunoprecipitation (ChIP) analysis of a -125 bp DNA fragment from the COL1A1 promoter with a specific monoclonal antibody against Sp1. A chromatin immunoprecipitation (ChIP) assay was performed as described in Materials and Methods to examine changes in the interaction of the transcription factor Sp1 with the COL1A1promoter. Lane 1: Negative control; lane 2: S1/S2 positive control; lanes 3,4: Control fibroblasts; lanes 5,6: TGF-β treated fibroblasts; lanes 7,8: fibroblasts treated with both TGF-β and Simvastatin; lanes 9,10: input DNA from control cells; lanes 11,12: input DNA from TGF-β treated fibroblasts; lanes13,14: input DNA from fibroblats treated with both TGF-β and Simvastatin; lanes 15,16,17: NADH PCR amplification from DNA obtained from control, TGF- β, and TGF- β plus Simvastatin treated fibroblasts.

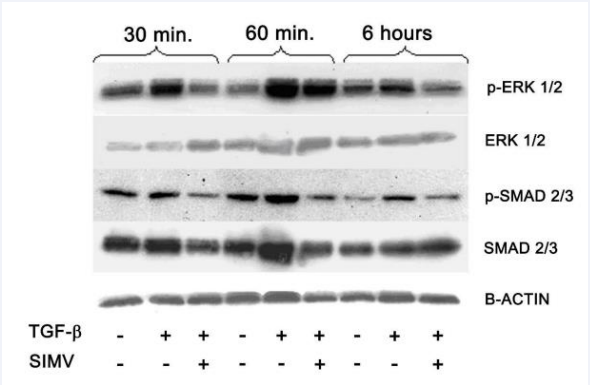

Figure 3 Western blot analysis of phosphorylated Smad2/3 and Erk1/2 in normal fibroblasts after treatment with TGF-β or TGF-β plus Simvastatin. Western analysis of the TGF-β pathway mediator phospho-SMAD2/3 and total Smad 2/3 and the RhoA pathway mediator phospho- Erk1/2 and total Erk in normal fibroblasts treated with TGF-β (10 ng/ml) +/- Simvastatin (5 μM) for 30, 60 min and 6 h

added for 48 h in the presence or absence of the geranyl geranyl transferase inhibitor, GGPP (10 μM). The results shown in Figure 4 demonstrate that GGPP completely reversed the inhibitory effect of Simvastatin on type I collagen production following TGF-β1 stimulation. To confirm the observations that inhibition of RhoA activity was involved in the Simvastatin abrogation of the effects of TGF-β1, we performed transfections of a double-stranded siRNA oligonucleotide to knockdown RhoA mRNA, and then treated normal dermal fibroblasts with TGF-β1 (Figure 5). The efficiency of the RhoA siRNA on the knockdown of this gene was assessed by quantitative real time PCR with specific primers. The results showed a greater than 70% decrease in the levels of RhoA mRNA (Figure 5A). We observed an almost complete abrogation of TGF-β1 stimulation of COL1A1 gene expression in fibroblasts previously transfected with the double stranded RhoA siRNA (Figure 5B and C). This effect was confirmed with

quantitative real time PCR of RNA obtained from treated and untreated fibroblasts which showed a similar pattern of response with a significant stimulation of COL1A1 mRNA levels in TGF-β1 treated cells (250%) compared to untreated controls, and an essentially complete abrogation of the COL1A1 transcript levels stimulation in fibroblasts previously treated with the RhoA siRNA (Figure 5D).

Effects of Simvastatin and RhoA siRNA on Fibroblast to Myofibroblast Transition assessed by immunofluorescence. We next examined whether Simvastatin also modified the effects of TGF-β1 on the phenotypic conversion of fibroblasts into myofibroblasts. For this purpose, confluent normal fibroblasts were treated with 10ng/ml of TGF-β1 in the presence or absence of 5μM Simvastatin and F-actin filaments indicative of the fibroblast to myofibroblast conversion were visualized by staining with Alexa fluor (Figure 6). The images showed a marked increase in F-actin filaments indicative of the fibroblast to myofibroblast transition following TGF-β1 stimulation compared to untreated control cells and the abolishment of this effect in cells treated with Simvastatin (Figure 6A). RhoA siRNA abrogation of TGF-β1 stimulation also inhibited the TGF-β1 induced phenotypic conversion as shown above for Simvastatin. The images obtained showed the expected increase in F-actin filaments following TGF-β1 stimulation compared to untreated control and the abrogation of this effect in cells treated with TGF-β1 plus RhoA siRNA (Figure 6B).

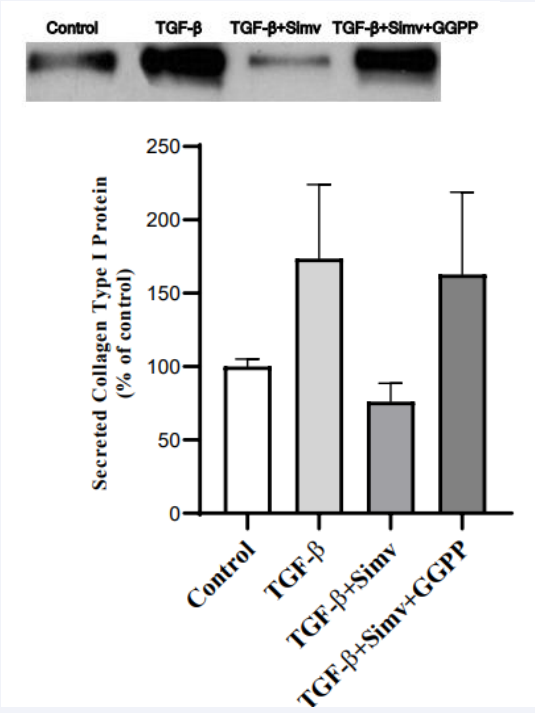

Figure 4 Effect of geranylgeranyl pyrophosphate (GGPP) on collagen type I production in normal dermal fibroblasts after treatment with TGF-β +/- Simvastatin. Western analysis with specific antibodies against collagen type I of media from normal dermal fibroblasts treated with GGPP (10 μM) following 48 h of treatment with TGF-β +/- Simvastatin and densitometric Western blots analysis of three cell lines.

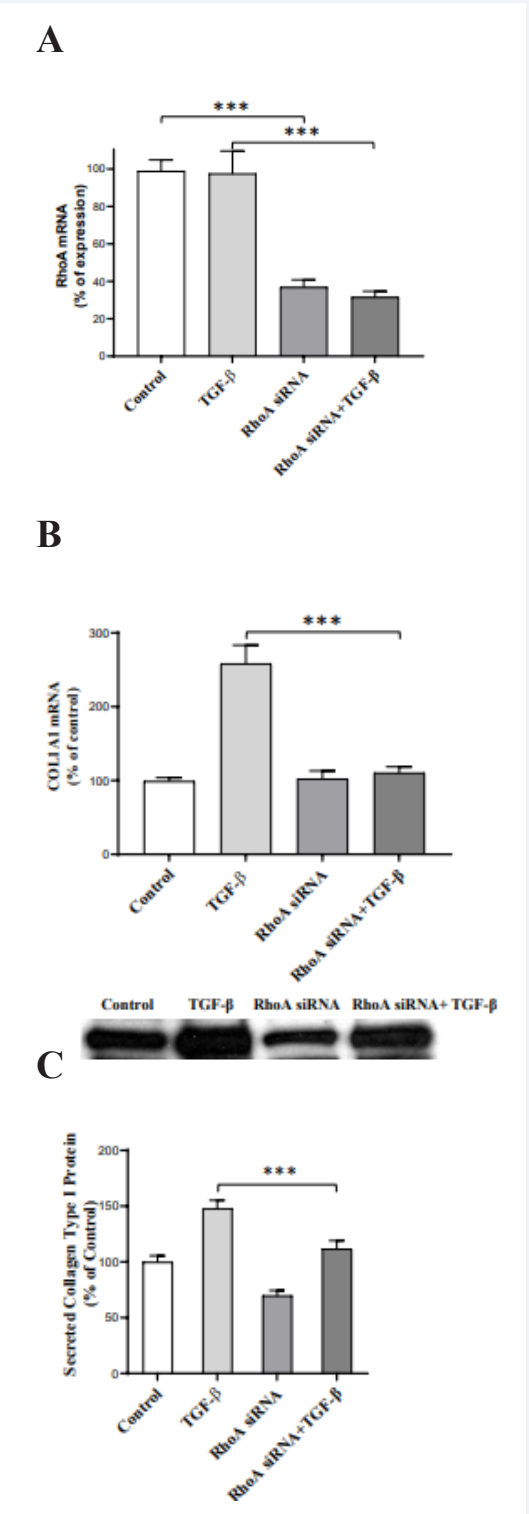

Figure 5 Effect of RhoA siRNA transfection on collagen I production in normal fibroblasts treated with 21 TGF-β. Quantitative real-time PCR of cDNA prepared from total RNA obtained from normal dermal fibroblasts transfected with a double stranded RhoA siRNA +/- TGF-β treatment. (A) RhoA mRNA relative expression following RhoA siRNA +/- TGF-β treatment. (B) COL1A1 mRNA relative expression following RhoA siRNA +/- TGF-β treatment. (C) Western analysis with specific antibodies to type I collagen present in the media from normal fibroblasts treated with TGF-β (10 ng/ml) or with a double-stranded RhoA siRNA (5 nM) after 2 days of treatment and densitometric Western blots analysis of three cell lines.

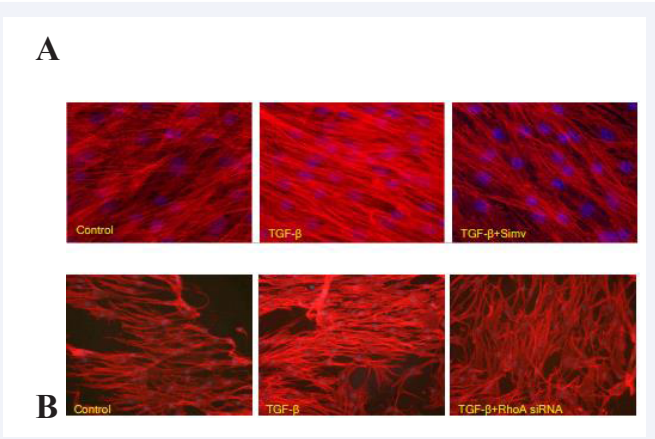

Figure 6 Effects of Simvastatin and RhoA siRNA on immunofluorescence staining of F actin in TGF- β - treated normal human dermal fibroblasts. (A) Immunofluorescence microscopic image of normal human dermal fibroblasts treated for 48 h with TGF-β (10ng/ml) +/- Simvastatin (5μM). (B) Immunofluorescence microscopic image of normal human dermal fibroblasts treated with TGF-β + RhoA siRNA.

DISCUSSION

The mechanisms involved in the regulation of expression of the genes encoding the interstitial collagens in the fibrotic diseases including SSc, IPF, and cardiac, renal and liver fibrosis have not been completely elucidated. Numerous studies have shown that members of the TGF-β family of proteins play a crucial role in the initiation and progression of pathologic fibrogenesis [7-12], although the mechanisms involved have not been fully elucidated and have been the subject of intensive investigations. Of particular importance are the intracellular transduction pathways that transmit the activated TGF-β receptor-initiated cell surface signals to the transcriptional response elements of specific TGF-β-responsive genes. Furthermore, it is of great interest to determine how these signals provide the selectivity required to influence only certain genes under given conditions and cellular contexts. In this regard, it has been shown that canonical Smad-mediated pathways are intimately involved in the potent stimulation of interstitial collagen gene expression and ECM protein production by TGF-β1 [11,38], however, non-Smad pathways (non-canonical) also participate in the regulation of expression of genes encoding these proteins by TGF- β1 [40]. Given the high mortality rates caused by the fibrotic diseases that have been estimated to be responsible for a much as 45% of the mortality in the Western developed countries [1] there is great interest in the identification of effective antifibrotic therapeutic interventions. In the present study, we demonstrated a potent inhibition of TGF-β1- induced stimulation of COL1A1 gene expression induced by in vitro treatment with the statin, Simvastatin and examined the molecular mechanisms responsible for these effects. Our results showed that Simvastatin abrogated the potent stimulation of COL1A1 gene expression induced by TGF-β1 in normal fibroblasts in a dose-and length of exposure-dependent manner. As shown in Figure 1, we found that TGF-β1 treatment caused the expected increase in type I collagen production and COL1A1 mRNA levels and that this effect was abrogated in cells treated simultaneously with Simvastatin. We also demonstrated employing a ChIP assay that the Simvastatin effects occurred at the COL1A1 transcriptional level and were mediated by the reduction of Sp1 transcription factor binding to the proximal COL1A1 promoter region in contrast to increased Sp1 binding in cells treated with TGF-β1 (Figure 2). We chose Sp1 owing to previous studies demonstrating that Sp1 binding to the COL1A1 promoter is a crucial determinant involved in the upregulation of COL1A1 gene expression by TGF-β1 [39]. Thus, this is the first report showing that statin treatment caused a potent reduction of TGF-β1-stimulated binding of the Sp1 transcription factor to the proximal COL1A1 promoter. We also showed that the Simvastatin- induced abrogation of TGF-β1-stimulated COL1A1 gene expression and collagen type 1 production involved both the canonical and non-canonical Smad pathways. Our results showed that phospho-Smad2/3 (canonical) and phospho-Erk1/2 (non-canonical) levels were increased in the TGF-β1-treated cells and that these levels were significantly reduced in the cells treated with Simvastatin (Figure 3). The results obtained with phospho-Erk1/2 also confirmed studies that indicated that Erk1/2 is involved in collagen type 1 production regulation in a Smad-independent pathway. It is well known that Erk1/2 is activated by TGF-β, therefore, the decrease in Erk1/2 phosphorylation is likely to play an important role in the reduction of TGF-β1-induced increase in collagen type 1 production and COL1A1 gene expression observed here.

Numerous studies have shown that important effects of Simvastatin and related compounds are mediated by a potent inhibition geranyl-geranyl RhoA prenylation [41-43] and that RhoA activation and localization to the cell membrane require geranylgeranylation. We, therefore, examined whether the inhibitory effect of Simvastatin on TGF-β induced stimulation of COL1A1 expression was caused by inhibition of RhoA geranylgeranylation. The results showed an essentially complete restoration of TGF-β1 stimulated collagen production when geranyl geranyl pyrophosphate (GGPP) was added to cells treated with TGF-β and Simvastatin (Figure 4). This finding indicates that post- transcriptional protein geranylation is fundamental for COL1A1 gene upregulation induced by TGF-β1. To conclusively demonstrate the involvement of RhoA in the Simvastatin-induced downregulation of the TGF-β1 induced stimulation of collagen type 1 production we transfected a RhoA siRNA into either cells treated with TGF- β1 or cultured under control conditions. The effects obtained in these experiments were similar to those obtained with Simvastatin treatment as the stimulation of collagen type 1 production caused by TGF-β1 was essentially completely abolished in cells transfected with RhoA siRNA (Figure 5). To provide additional support to the notion that the abrogation of TGF-β1 induced COL1A1 gene expression stimulation by Simvastatin is mediated by inhibition of RhoA activation, we showed that TGF-β1 effects on the cytoskeleton were also affected by Simvastatin and by RhoA siRNA. These results are in agreement with other studies demonstrating that Simvastatin inhibits the expression of some profibrotic markers including CTGF, α-smooth muscle actin (αSMA), and fibronectin through the inhibition of RhoA activation [44-47].

SUMMARY

In summary, we demonstrate that Simvastatin treatment abrogates the stimulation of type I collagen production and COL1A1 gene expression induced by TGF-β1 and that these effects appear to be mediated by inhibition of binding of the transcription activator Sp1 to the proximal COL1A1 promoter and by a reduction in the levels of phosphorylated Erk1/2. It is important to emphasize that the inhibitory effects of Simvastatin on the potent profibrotic activities of TGF-β are extremely selective causing abrogation of the increased transcriptional activation of COL1A1 and reduction of the production of type I collagen, the most relevant protein in the context of pathologic tissue fibrosis. The selective abrogation of TGF-β-induced profibrotic molecular events is of substantial practical relevance for the development of novel anti-TGF-β therapeutic approaches since the numerous other beneficial TGF-β activities that are involved in homeostatic processes would not be affected, thus limiting the potential side effects that may result from a more extensive blockage of the TGF-β pleotropic and multifunctional effects. These results suggest that statins may represent potentially effective therapeutic agents for TGF-β mediated tissue fibrotic disorders.

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