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Inhibition of Systemic Sclerosis Dermal Fibroblast Type I Collagen Production and Gene Expression by Simvastatin

Natalia Louneva, Gonzalo Huaman, Joanna Fertala, and Sergio A. Jiménez

***Objective*. To examine whether statins are capable of modulating collagen gene expression in cultured systemic sclerosis dermal fibroblasts.**

***Methods*. Cultured dermal fibroblasts from 3 patients with diffuse systemic sclerosis of recent onset were treated with 5 μM and 10 μM of simvastatin for 3 or 4 days. Morphologic features, cytotoxicity, and type I collagen production and messenger RNA (mRNA) levels in the fibroblasts were examined. The effects of mevalonate, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP), which are lipids downstream from the hydroxymethylglutaryl–coenzyme A block, were also examined. Transient transfections with COL1A1 promoter-reporter constructs and electrophoretic gel mobility shift assays were utilized to examine COL1A1 transcription and Sp1 and CCAAT-box binding factor (CBF) binding.**

***Results*. Simvastatin did not cause morphologic changes or cytotoxicity in the fibroblasts, even after 4 days of treatment. Type I collagen production and mRNA levels showed a potent and dose-related inhibition following 3 and 4 days of treatment. The inhibition of collagen gene expression by simvastatin was completely reversed by mevalonate and GGPP, but not by FPP. The statin effects occurred at the transcriptional level and involved the proximal COL1A1 promoter region encompassing −174 bp. A significant reduction in Sp1 and CBF binding activity was also found in simvastatin-treated cells.**

***Conclusion*. Simvastatin is a powerful inhibitor of type I collagen gene expression in normal and systemic sclerosis fibroblasts. The pleiotropic protective effects of statins on various endothelial and immune cell functions in conjunction with their potent inhibitory effects on type I collagen gene expression suggest that statins may be effective therapeutic agents in systemic sclerosis.**

Systemic sclerosis is characterized by the excessive deposition of collagen and other connective tissue components in skin and multiple internal organs, prominent and often severe alterations in the microvasculature, and humoral and cellular immune system abnormalities (for review, see refs. 1–3). Although the exact mechanisms involved in the pathogenesis of systemic sclerosis are not known, progressive cutaneous, visceral, and vascular fibrosis is responsible for most of the clinical manifestations and severity of the disease as well as the resultant mortality (4,5). Numerous studies have shown that the pathologic increase in tissue collagen deposition is largely the result of increased expression of collagen genes (for review, see refs. 2 and 3).

Fibroblasts from affected systemic sclerosis skin cultured in vitro produce excessive amounts of various collagens (6,7) and display increased transcription of the corresponding genes (7–10). However, the mechanisms responsible for the increased transcriptional activity of collagen genes in systemic sclerosis fibroblasts are not fully understood. Transforming growth factor β (TGFβ) plays a crucial role in the development of tissue fibrosis (11), and a plausible hypothesis has suggested that this growth factor may be intimately involved in the pathogenesis of systemic sclerosis (12). The intracellular pathways involved are mediated through complex interactions between specific cell surface receptors and several intracellular proteins of the Smad family (13,14). However, recent studies indicate that non-Smad pathways

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Natalia Louneva, PhD, Gonzalo Huaman, MD, Joanna Fertala, Sergio A. Jiménez, MD: Thomas Jefferson University, Philadelphia, Pennsylvania.

Address correspondence and reprint requests to Sergio A. Jiménez, MD, Thomas Jefferson University, Division of Rheumatology, 233 S. 10th Street, Room 509 BLSB, Philadelphia, PA 19107-5541. E-mail: Sergio.jimenez@jefferson.edu.

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may also be involved in some of the effects of TGFβ (15).

Posttranslational protein isoprenylation is of fundamental importance in the regulation of cell survival, proliferation, and differentiation, as well as in cytoskeletal organization. Isoprenylation reactions also participate in the regulation of collagen genes and in some of the effects of TGFβ. Investigators in our group previously demonstrated that specific inhibition of geranylgeranyl transferase I with the compound GGTI298 caused a profound and selective inhibition of expression of type I and type III collagen genes in normal and systemic sclerosis dermal fibroblasts (16). Since statins also affect the synthesis of lipid intermediates involved in isoprenylation reactions (17,18), we postulated that statins may also directly inhibit the expression of type I collagen genes, and examined this hypothesis in normal and systemic sclerosis dermal fibroblasts in vitro. The results obtained indicate that statins are powerful inhibitors of type I collagen gene expression in normal and systemic sclerosis fibroblasts, and this is likely attributable to their effects on geranylgeranyl prenylation of key intracellular mediators. Previous results demonstrating the pleiotropic protective effects of these drugs on various endothelial and immune cell functions (19–21) coupled with the potent inhibitory effects of statins on type I collagen gene expression suggest that these drugs may be effective therapeutic agents in systemic sclerosis and other diseases that are characterized by tissue fibrosis and vascular dysfunction.

PATIENTS AND METHODS

Fibroblast cultures. Dermal fibroblast cell lines were established from patients with systemic sclerosis who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of the disease (22). To avoid variability introduced by heterogeneity of the clinical characteristics and differences in the stage of evolution of systemic sclerosis, only cells from untreated patients with diffuse systemic sclerosis of recent onset and rapid progression were studied, as described previously (7,16). The cell lines were obtained from full-thickness skin biopsy tissue surgically excised for diagnostic purposes from the leading edge of clinically apparent systemic sclerosis lesions. Normal fibroblast cell lines were obtained from age- and sex-matched individuals undergoing surgical procedures. All studies were approved by the institutional review board of Thomas Jefferson University.

For all studies, only early-passage fibroblasts (<12) were used, to avoid changes in their original phenotype during subculture. Cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1% vitamins, 2 m*M* glutamine, and antibiotics, and were incubated at 37°C in a 5% CO_2 humidified atmosphere. The cultures were supplemented with 40 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan) for 24 hours prior to the addition of the test compounds to optimize their level of collagen production, as described previously (23). At the conclusion of all experiments, the cells were counted and their viability was assessed by trypan blue exclusion and, in certain experiments, the MTT TOX-1 assay kit (Sigma, St. Louis, MO) was used, as described previously (24).

Treatment of cultured fibroblasts. Simvastatin was purchased from Calbiochem (La Jolla, CA) in its active form. All other chemicals were of reagent grade. Three strains of systemic sclerosis fibroblasts and 3 strains of normal fibroblasts were studied. Normal and systemic sclerosis cells were plated in 6-well plates and cultured until confluent. Following preincubation with 40 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate, the cells were incubated with 5 μM and 10 μM of simvastatin in 10% FBS medium for 48 hours. The medium was then changed with fresh medium containing the same components and the cultures incubated for an additional 24 or 48 hours. The type I collagen present in the culture media was quantified by Western blotting using a specific anti-human type I collagen polyclonal antibody (Rockland, Gilbertsville, PA). Geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), and mevalonic acid were from Sigma and were used at concentrations that have been shown in other studies to be sufficient for complete reversal of the inhibition of activity of the corresponding enzymes.

Northern hybridization. Fibroblasts were grown to confluency and, following incubation either under control conditions or with simvastatin, total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). 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, Piscataway, NJ) and the filters hybridized to ^{32}P-radiolabeled human complementary DNA (cDNA) for COLlAl, as described previously (16,24). Equivalent amounts of RNA were loaded and RNA loading and transfer were evaluated by probing with 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 storage phosphor technology (Image-Quant version 5.1 software; Amersham Biosciences).

Transient transfections of normal and systemic sclerosis fibroblasts with COL1A1 promoter–chloramphenicol acetyltransferase (CAT) constructs. Transient transfections of normal and systemic sclerosis fibroblasts were performed with the lipid-based FuGene 6 Kit (Roche, Indianapolis, IN) as described previously (25). Normal and systemic sclerosis fibroblasts were transfected at 80% confluence in 60-mm dishes with 2.5 μg of each plasmid. COL1A1 transcription was assessed by using deletion constructs of the COL1A1 promoter fused to the CAT reporter gene. All of the constructs examined ended at nucleotide +42 to ensure transcription in a proper reading frame, and their 5′ ends were at −675 bp, −174 bp, and −84 bp. The detailed procedures for preparation of these constructs have been described previously (26). Four hours later, fresh medium with or without 5 μM simvastatin was added, and the cells were harvested 72 hours after

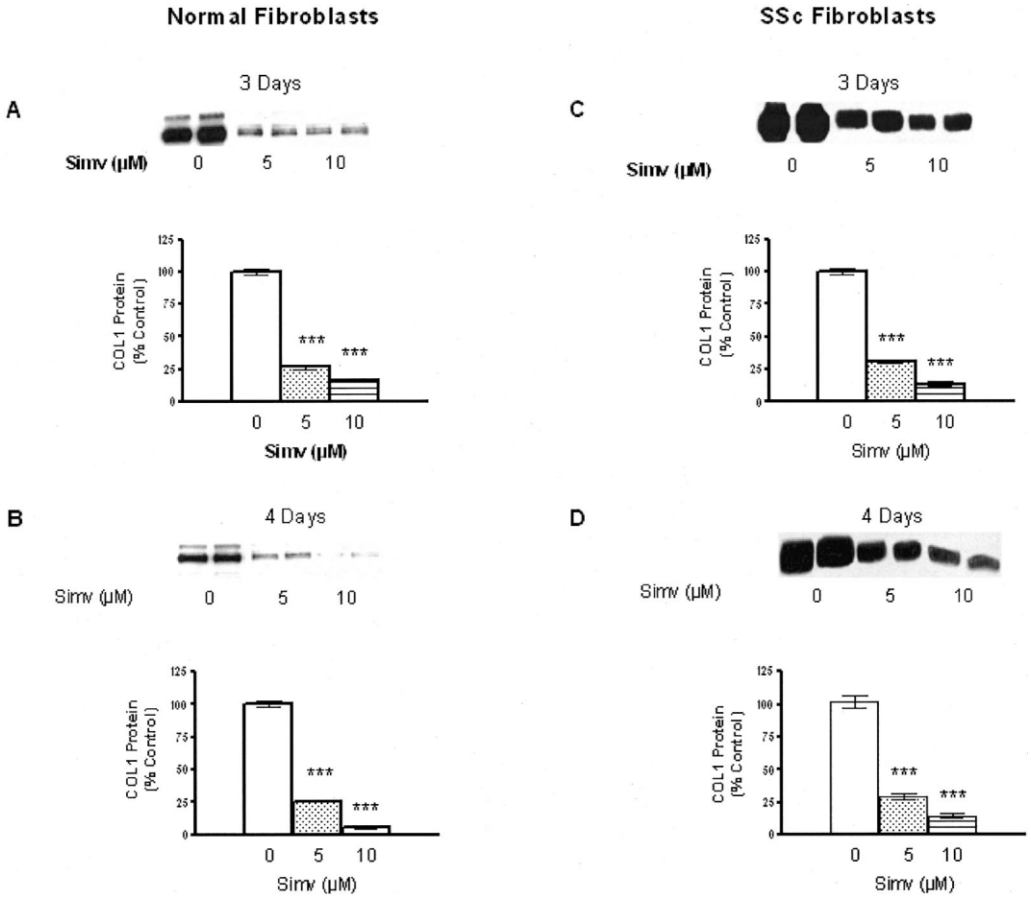

Figure 1. Effect of various concentrations of simvastatin (Simv) on normal and systemic sclerosis (SSc) dermal fibroblast type I collagen (COL1) production. **A** and **B,** Western blot and densitometric analysis of culture media from normal fibroblasts, after 3 days **(A)** and 4 days **(B)** of treatment with simvastatin. **C** and **D,** Western blot and densitometric analysis of culture media from SSc fibroblasts after 3 days **(C)** and 4 days **(D)** of treatment with simvastatin. Results are representative of 3 independent experiments, each performed in duplicate. Bars show the mean ± SEM percentage relative to the values in untreated (control) fibroblasts. *** = $P < 0.0001$ versus untreated control.

transfection; CAT activity was then determined in cell extracts. The efficiency of transfection was normalized by cotransfecting 0.2 μg of vector containing *Escherichia coli* β-galactosidase cDNA (pCMV β-galactosidase; Clontech, Palo Alto, CA) followed by assays of β-galactosidase enzymatic activity.

Electrophoretic gel mobility shift assay (EMSA). Confluent cells in two 175-mm flasks were treated with 5 μM of simvastatin for 4 days in the same manner as that described above, and nuclear extracts were prepared using a kit from Sigma. EMSA was performed as described previously (24,25). A −125-bp DNA fragment from the COL1A1 promoter, encompassing nucleotides −174 to −50, was used as a probe for the EMSA experiments. The fragment was obtained by polymerase chain reaction amplification with primers at −174 bp (5′-GGTGGACTCCCTTCCCTCCTC-3′) and at −50 bp (5′-AGGACCCCTGCCCCTCGGAGA-3′) of the human COLlA1 promoter. Radioactive probes were prepared by phosphorylation of the 5′ ends with polynucleotide kinase (Promega, Madison, WI) and α^{32}-ATP (Amersham Biosciences). Ten micrograms of nuclear extracts, 4 μg of poly(dI-dC), and 5×10^4 counts per minute of radiolabeled probe were incubated for 20 minutes in a buffer containing 40 mM KCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, and 5% glycerol.

To test the specificity of binding, competition experiments using 100-fold molar excess of unlabeled Sp1 and CCAAT-box binding factor (CBF) consensus oligonucleotides and their mutated forms were performed. These oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). EMSA was performed in 5% nondenaturing polyacrylamide gels as described previously (24,25). Sp1 and CBF binding activities were quantified by densitometry (ImageQuant, version 5.1; Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Results are expressed as the mean ± SEM. Student's *t*-test was used for comparing 2 groups

of data, utilizing GraphPad software (San Diego, CA). *P* values less than 0.05 were considered significant.

RESULTS

Effects of simvastatin on the viability and morphologic features of normal and systemic sclerosis fibroblasts. To examine the morphologic and/or cytotoxic effects of simvastatin, confluent cultures of normal and systemic sclerosis fibroblasts were treated with various simvastatin concentrations (1.25 μM, 2.5 μM, 5 μM, and 10 μM) for 24, 48, 72, and 96 hours. Morphologic changes were evaluated by dark field microscopy, while cytotoxicity was measured by an MTT reduction assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The highest concentration of simvastatin examined in these experiments (10 μM) did not cause morphologic changes; furthermore, there was no detectable cytotoxicity even after 4 days of treatment with the highest concentration (results not shown). Therefore, all subsequent experiments were performed with 5 μM and/or 10 μM of the drug for 3 or 4 days.

Effect of simvastatin on type I collagen production. The effects of simvastatin on the expression of type I collagen were examined in 3 different systemic sclerosis cell lines and in 3 normal cell lines treated for 3 or 4 days with either 5 μM or 10 μM simvastatin (Figure 1). Simvastatin reduced type I collagen production in a

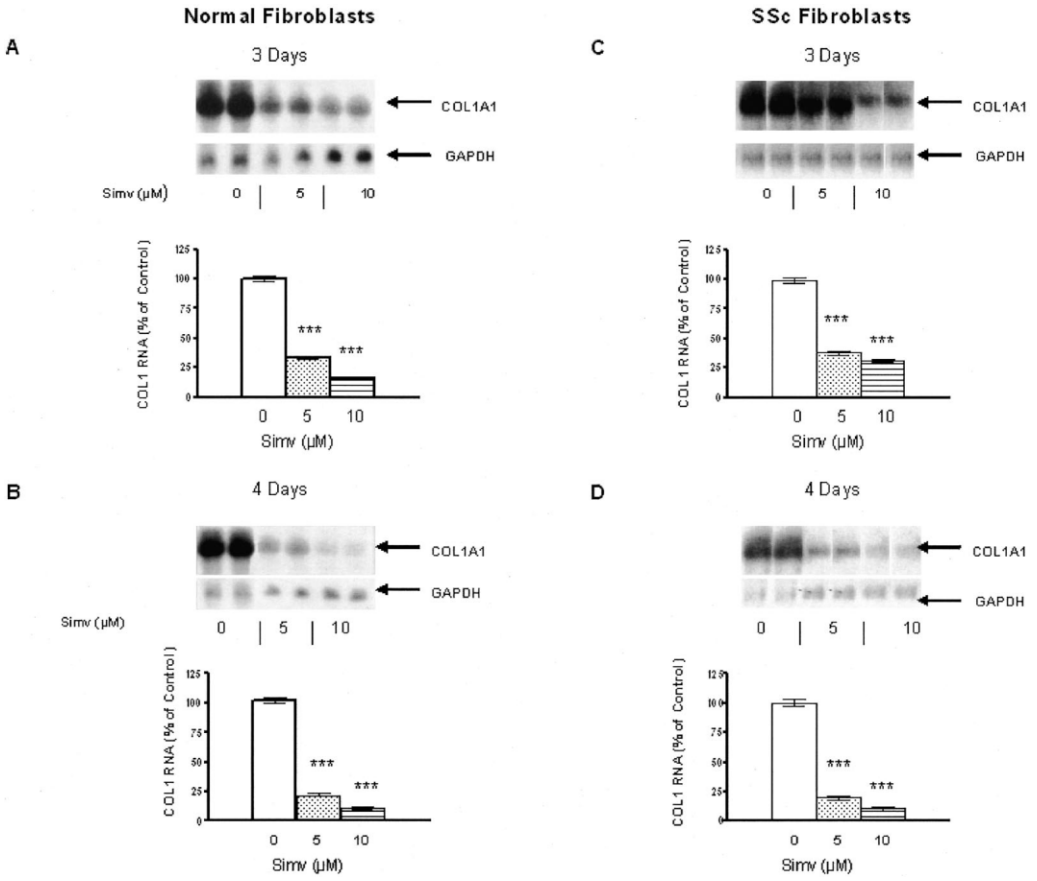

Figure 2. Northern hybridizations of total RNA from normal and SSc fibroblasts with cDNA for COL1A1, after treatment with simvastatin. **A** and **B,** Northern hybridization and densitometric analysis of normal fibroblasts after 3 days **(A)** and 4 days **(B)** of treatment. **C** and **D,** Northern hybridization and densitometric analysis of SSc fibroblasts after 3 days **(C)** and 4 days **(D)** of treatment. Densitometry values were corrected for differences in loading and transfer by normalization with the values obtained for GAPDH hybridizations. Northern blot results are representative of 3 independent experiments, each performed in duplicate. Bars show the mean ± SEM percentage relative to untreated (control) fibroblasts. *** = $P < 0.0001$ versus untreated control. See Figure 1 for definitions.

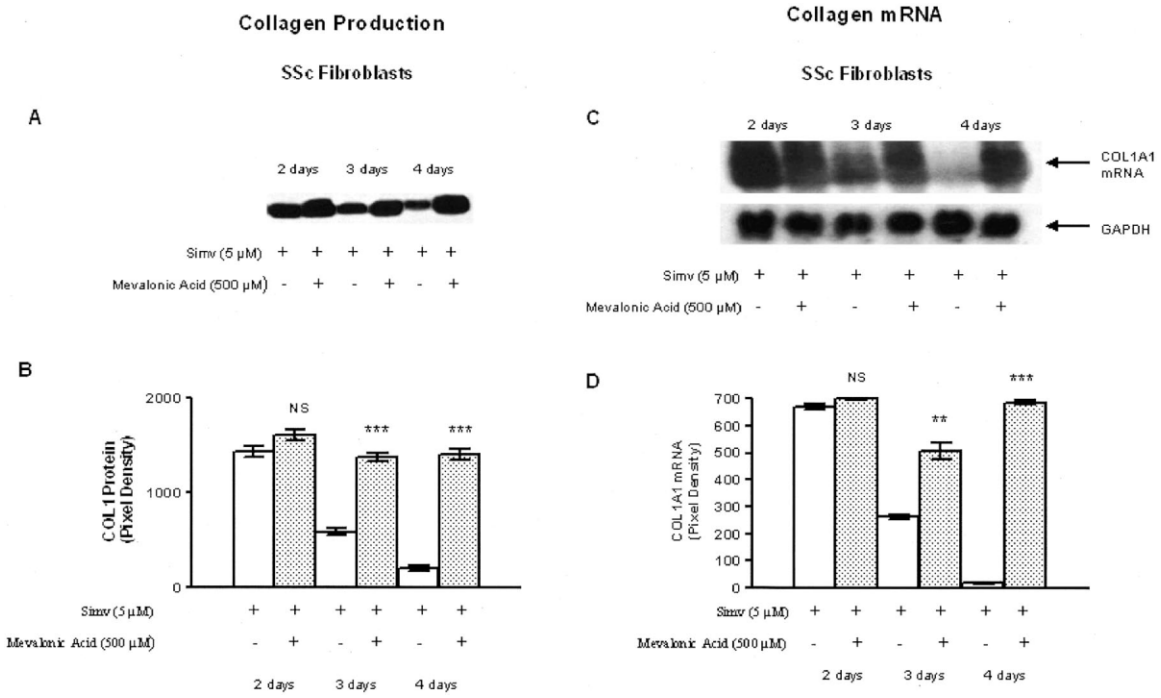

Figure 3. Effect of mevalonic acid on type I collagen production and steady-state COL1A1 mRNA levels in simvastatin-treated SSc fibroblasts. Western blot analysis **(A)** and densitometric analysis of the Western blots **(B)** were carried out on media of SSc fibroblasts after treatment with 5 μM simvastatin with or without 500 μM mevalonic acid for 2, 3, and 4 days. Northern hybridization **(C)** and densitometric analysis of the Northern blots **(D)** were carried out for COL1A1 mRNA in SSc fibroblasts after treatment with 5 μM simvastatin with or without 500 μM mevalonic acid. Values were corrected by normalization with the values obtained for GAPDH hybridizations. Densitometry results are representative of 2 independent experiments, each performed in duplicate. Bars show the mean ± SEM pixel density. ** = $P < 0.001$; *** = $P < 0.0001$, versus cultures without mevalonic acid. NS = not significant (see Figure 1 for other definitions).

dose-dependent manner in both cell types. In normal fibroblasts, simvastatin at a concentration of 5 μM reduced collagen production by 74% and 75% at days 3 and 4, respectively, and at a concentration of 10 μM, it reduced collagen production by 82% and 93% at days 3 and 4, respectively (Figures 1A and B). In systemic sclerosis fibroblasts, simvastatin treatment for 3 days at a concentration of 5 μM reduced collagen production by 77%, and at a concentration of 10 μM, by 84%. After 4 days, 5 μM simvastatin caused a 70% reduction and 10 μM caused an 86% reduction in type I collagen in SSc fibroblasts (Figures 1C and D). Therefore, the sensitivity to simvastatin was very similar between normal and SSc cells.

Down-regulation of COL1A1 steady-state mRNA levels by simvastatin. To investigate whether the effects of simvastatin on type I collagen production were reflected at the COL1A1 mRNA level, confluent cultures of fibroblasts were treated with 5 μM or 10 μM of the drug for 3 and 4 days. The results are shown in Figure 2. In normal fibroblasts, following 3 days of simvastatin treatment at concentrations of 5 μM and 10 μM, COL1A1 transcripts were reduced by 75% and 87%, respectively (Figure 2A). Following 4 days of treatment with 5 μM or 10 μM of the drug, COL1A1 mRNA levels were reduced by 80% and more than 92%, respectively (Figure 2B). In all experiments, GAPDH mRNA levels were not affected. These data were strongly correlated with the protein measurements. In systemic sclerosis fibroblasts, 5 μM and 10 μM of simvastatin reduced COL1A1 mRNA levels after 3 and 4 days to a similar extent as that in normal fibroblasts (Figures 2C and D).

Effect of mevalonic acid on type I collagen expression and COL1A1 mRNA levels in simvastatin-treated systemic sclerosis fibroblasts. Inhibition of hydroxymethylglutaryl–coenzyme A (HMG-CoA) reductase by simvastatin is known to reduce intracellular levels of mevalonate. To investigate the effect of exog-

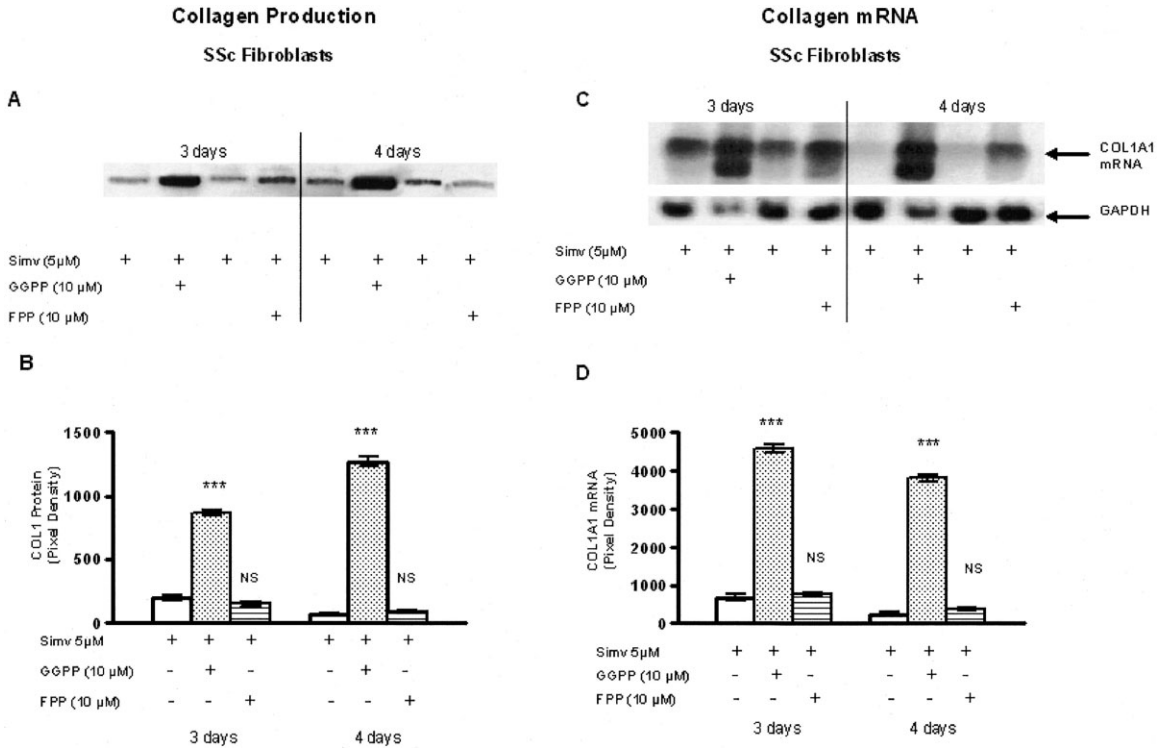

Figure 4. Effect of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) on type I collagen production and COL1A1 mRNA steady-state levels in SSc fibroblasts treated with simvastatin. Western blot **(A)** and densitometric analysis of the Western blots (from 3 separate experiments) **(B)** were carried out on media of 5 μM simvastatin–treated SSc fibroblasts cultured with either 10 μM FPP or 10 μM GGPP for either 3 or 4 days. Northern hybridization **(C)** and densitometric analysis of the Northern blots (from 3 separate experiments) **(D)** for COL1A1 mRNA, after normalization to GAPDH, were carried out on 5 μM simvastatin–treated SSc fibroblasts with or without FPP (10 μM) or GGPP (10 μM) after 3 and 4 days of treatment. Results from Western and Northern blotting are representative of 3 separate experiments. Bars show the mean ± SEM pixel density. *** = $P < 0.0001$ versus cultures without GGPP or FPP. NS = not significant (see Figure 1 for other definitions).

enous addition of mevalonate to fibroblasts treated with simvastatin, systemic sclerosis fibroblasts were treated with 5 μM simvastatin with or without the addition of 500 μM mevalonic acid. Collagen production was measured at 2, 3, and 4 days of incubation. The results (Figures 3A and B) show that coincubation of systemic sclerosis fibroblasts with mevalonate completely abrogated the inhibitory effects of simvastatin on collagen production, thus indicating that these effects were directly caused by HMG-CoA reductase inhibition of the synthesis of mevalonate-derived lipid molecules, and could be fully reversed by addition of the metabolite immediately below the enzymatic block. This effect was also observed when COL1A1 mRNA levels were measured at 2, 3, and 4 days in 5 μM simvastatin–treated systemic sclerosis fibroblasts to which 500 μM mevalonic acid was added (Figures 3C and D). Similar effects were observed in normal fibroblasts (results not shown).

Effects of FPP and GGPP on type I collagen production and COL1A1 mRNA levels in simvastatin-treated systemic sclerosis fibroblasts. The role of the HMG-CoA downstream lipids FPP and GGPP on type I collagen production and mRNA levels was also evaluated in systemic sclerosis fibroblasts. For this purpose, the cells were treated with 5 μM simvastatin for 3 and 4 days with or without the addition of either 10 μM FPP or 10 μM GGPP. The results with regard to type I collagen production are shown in Figures 4A and B. As was observed with mevalonic acid, the addition of GGPP completely reversed the inhibitory effect of simvastatin on type I collagen production at both 3 and 4 days of treatment. In contrast, the addition of FPP failed to reverse the inhibitory effects of the statin on collagen production. Similar results were obtained when COL1A1 mRNA levels were measured at 3 and 4 days in 5 μM simvastatin–treated systemic sclerosis fibroblast

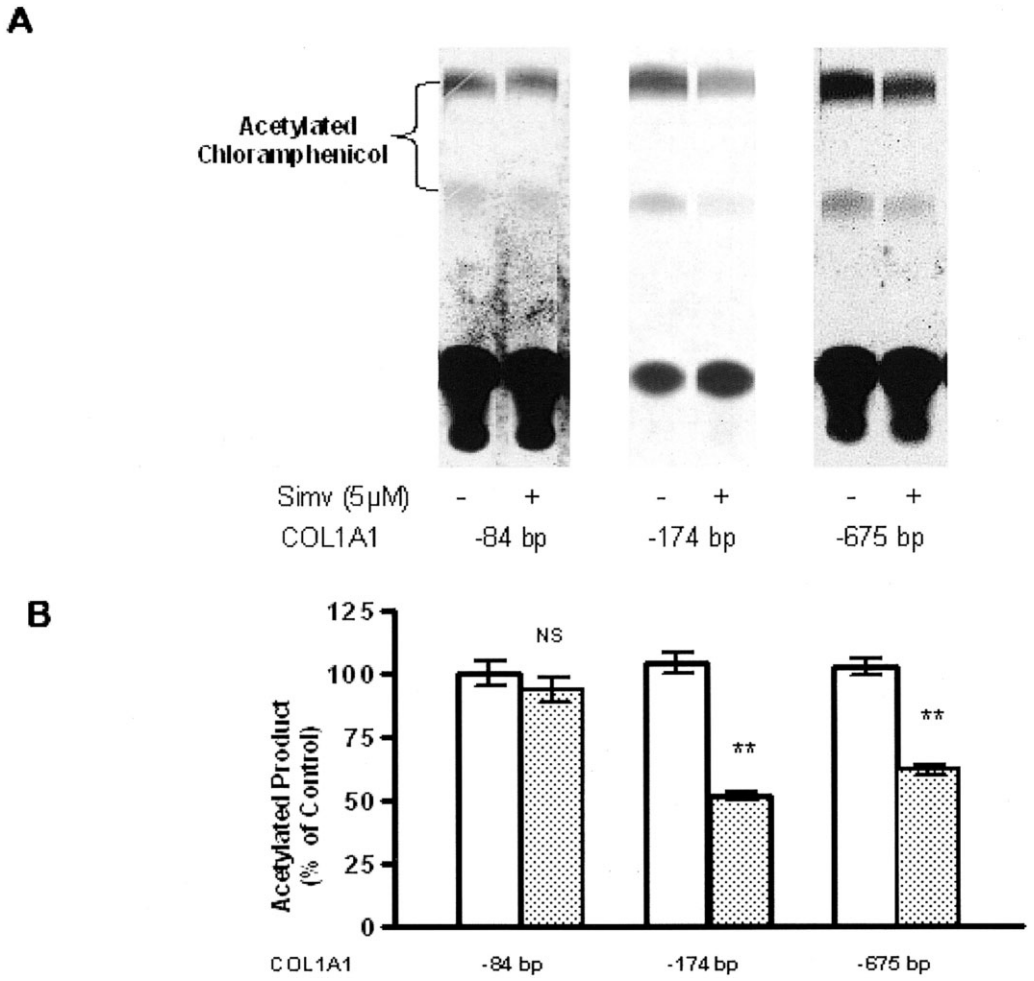

Figure 5. Simvastatin treatment of SSc fibroblasts transiently transfected with COL1A1 promoter constructs. **A,** SSc fibroblasts were transfected with 2.5 μg of 1 of 3 different COL1A1 promoter constructs plus 0.2 μg of pCMV β-galactosidase DNA, and then either treated with 5 μM simvastatin or left untreated for 3 days. Results were normalized for transfection efficiency, and acetylated chloramphenicol activity was analyzed (as described in Patients and Methods). Results are representative of 3 independent experiments. Note that owing to the much higher transcriptional activity of the −174-bp construct, the image shown is from an underexposed gel (2 hours) as compared with the images obtained with the −675-bp and −84-bp constructs, which were exposed for 24 hours. **B,** Densitometric analysis of the transfection described in **A,** with results representative of 3 separate experiments. Bars show the mean ± SEM percentage of activity (shaded bars) relative to the values in each corresponding untreated control (open bars). ** = $P < 0.001$ versus untreated control. NS = not significant (see Figure 1 for other definitions).

cultures to which 10 μM of GGPP or 10 μM of FPP were added (Figures 4C and D). Moreover, testing of normal fibroblasts in the same manner produced similar results (not shown).

Effect of simvastatin on COL1A1 promoter activity in transient transfections. Transient transfection experiments were performed to determine whether the effects of simvastatin on COL1A1 involved the transcriptional down-regulation of COL1A1. Three COL1A1 promoter constructs (−675 bp, −174 bp, and −84 bp) were transfected into 2 systemic sclerosis cell lines and 1 normal fibroblast cell line, and the cells were then treated with 5 μM simvastatin for 3 days. The results in systemic sclerosis cell cultures demonstrated that the promoter activities of the −675-bp and −174-bp COL1Al constructs were down-regulated by the statin, whereas the −84-bp construct was essentially not affected (Figures 5A and B). The results were similar

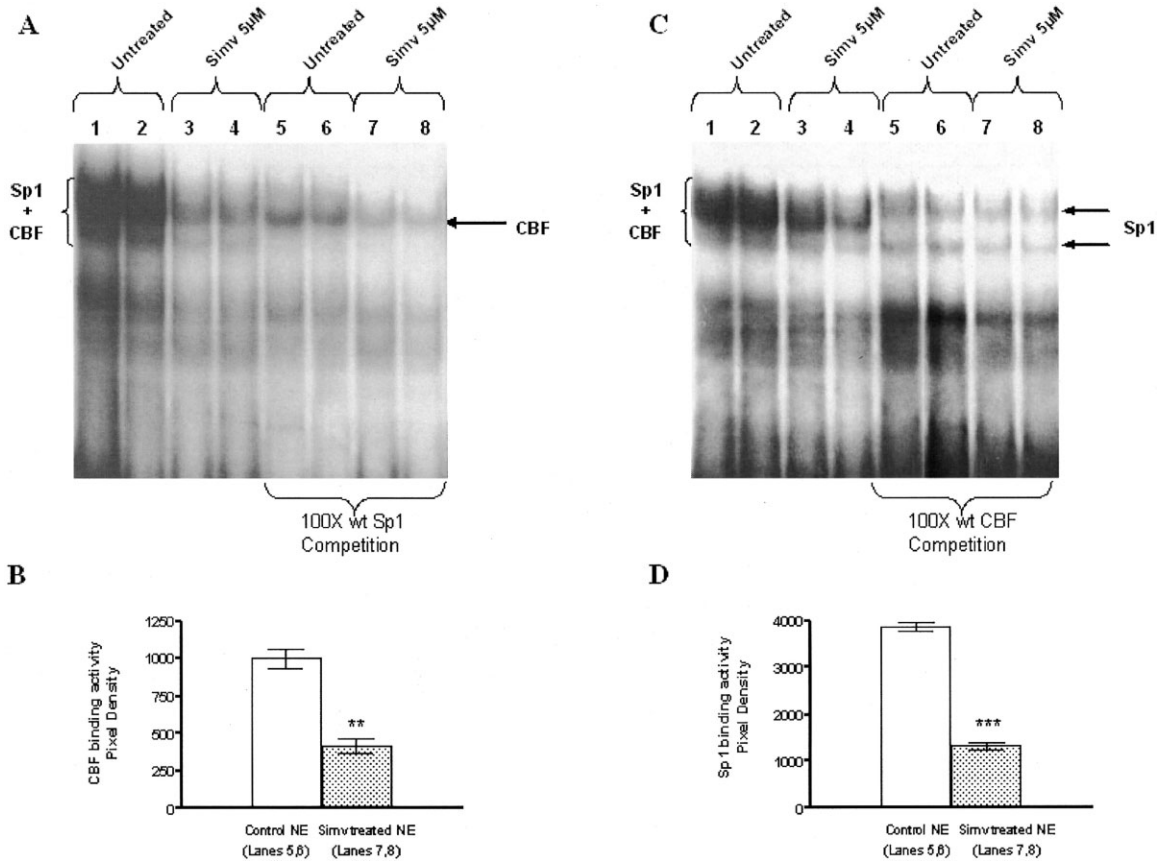

Figure 6. Electrophoretic gel mobility shift assay (EMSA) of nuclear extracts (NE) from systemic sclerosis skin fibroblasts interacting with a −125-bp DNA fragment from the COL1A1 promoter following treatment with 5 μM simvastatin (Simv) for 4 days. **A,** EMSA using competition with a 100-fold molar excess of wild-type (WT) Sp1 oligonucleotides. **B,** Densitometric analysis of results shown in lanes 5 and 6 and lanes 7 and 8 of **A.** Bars show the mean ± SEM pixel density from 3 separate experiments. **C,** EMSA using competition with a 100-fold molar excess of WT CCAAT-box binding factor (CBF) oligonucleotides. **D,** Densitometric analysis of results shown in lanes 5 and 6 and lanes 7 and 8 of **C.** Bars show the mean ± SEM pixel density from 3 separate experiments, each performed in duplicate. ** = $P < 0.001$; *** = $P < 0.0001$, versus untreated control.

between normal and systemic sclerosis fibroblasts. The most profound effect was observed with the −174-bp construct. An average of 3 separate experiments with systemic sclerosis cells revealed that simvastatin caused an ~45% reduction in its promoter activity compared with that in untreated cells, as calculated by densitometric analysis of the combined results from the 3 cell lines.

Comparison of CBF and Sp1 binding activity in nuclear extracts from control and simvastatin-treated fibroblasts. In previous studies, we have shown that Sp1 and CBF bind to a −125-bp fragment of the COL1A1 promoter, and we identified the formation of 4 DNA–protein complexes in nuclear extracts with this COL1A1 fragment. These complexes were identified as Cl, C2, C4 (corresponding to Sp1/Sp3 binding sites), and C3 (corresponding to a CBF binding site) (25–27). To compare the binding activity of CBF and Sp1 in nuclear extracts from control or 5 μM simvastatin–treated cells, we incubated nuclear extracts of treated or untreated systemic sclerosis fibroblasts with the −125-bp COL1A1 probe and performed EMSA with consensus oligonucleotides for Sp1 (Figures 6A and B) or CBF (Figures 6C and D) as competitors. To confirm the specificity of the DNA–protein complexes, mutated oligonucleotides for Sp1 and CBF were used (results not shown).

Competition experiments with Sp1 consensus oligonucleotides enabled us to abolish Sp1 binding to the −125-bp COL1A1 fragment, and thus allowed us to evaluate the binding of only the CBF transcription factor

(Figures 6A and B). It can be seen that CBF binding activity was reduced by ~65% in nuclear extracts from simvastatin-treated fibroblasts. In the experiments in which CBF consensus competition was performed, the binding of CBF to the −125-bp COL1A1 DNA probe was abolished, allowing us to compare the effects of simvastatin on Sp1 binding activity (Figures 6C and D). The results obtained showed that simvastatin caused a level of inhibition of Sp1 binding to the −125-bp COL1A1 probe similar to the level of inhibition induced against CBF binding.

DISCUSSION

It has recently become apparent that posttranslational protein isoprenylation is of fundamental importance in the regulation of expression of numerous genes involved in cell survival, proliferation, and differentiation, as well as in cytoskeletal organization. These processes are regulated by the Rho and RAS superfamilies of proteins, which are small membrane-bound GTPases that require the attachment of a prenyl group (either farnesyl or geranylgeranyl) for their activity. It has also been demonstrated that isoprenylation may participate in the regulation of expression of interstitial collagen genes, as well as in some of the downstream effects of TGFβ. Indeed, specific inhibition of geranylgeranylation caused a significant reduction of collagen gene expression in normal fibroblasts as well as in fibroblasts from patients with systemic sclerosis (16). Other studies have shown that inhibition of HMG-CoA reductase with statins not only causes a potent inhibition of cholesterol biosynthesis, but also inhibits synthesis of lipid molecules required for isoprenylation (17,18).

We therefore examined whether statins are capable of modulating type I collagen gene expression in cultured normal and systemic sclerosis dermal fibroblasts. The results demonstrated that treatment of systemic sclerosis cells with simvastatin (5–10 μM) caused a potent inhibition of COLlAl expression in these cells. The inhibition was not due to a generalized toxic effect, since it was completely abrogated when statin-treated cells were supplemented with the HMG-CoA downstream products mevalonic acid and GGPP. Of note was the observation that FPP, the other important downstream product of HMG-CoA, failed to reverse the inhibitory effects of the statin on COLlAl expression, indicating that geranylgeranylation, but not farnesyl prenylation, was involved. We further demonstrated that the statin acts ultimately at the transcriptional level, and that its effect on COL1A1 expression is mediated through a −174-bp gene segment in the proximal COLlAl promoter. Thus, it appears that the effects of this statin are mediated by COL1A1 promoter elements previously recognized as participants in the up-regulation of collagen gene expression in systemic sclerosis (9,10). This possibility was explored further by the assessment of the binding activity of Sp1 and CBF, which are 2 transcriptional factors that play a crucial role in the regulation of COLlAl transcription, both under normal conditions and under the influence of TGFβ (25,27–33).

It is well established that reduction and normalization of elevated cholesterol levels by statins promote primary and secondary prevention of ischemic, atherosclerotic-related events in patients with hypercholesterolemia (34–36). This effect was initially believed to be solely related to the cholesterol-lowering effects of the statins. However, there is increasing evidence that these agents may exert profound endothelial and vascular protective effects beyond their lipid-lowering action (19–21). These later clinical and experimental observations have led to a wealth of research studies examining the pleiotropic effects of statins, including modification of vascular disease and certain immune responses (for review, see ref. 21). Indeed, statins have been shown to down-regulate the expression of TGFβ1 by renal mesangial cells in models of renal nephropathy in which fibrosis is mediated by TGFβ1 and connective tissue growth factor (CTGF), and to reduce CTGF mRNA levels in normal human and rodent mesangial cell lines, in human renal fibroblast cell lines, and in human lung fibroblasts from patients with idiopathic pulmonary fibrosis (37–39).

To understand these pleiotropic effects, it is necessary to understand the metabolic pathways on which these agents exert their cholesterol-lowering effect. Statins inhibit the HMG-CoA reductase, which is the enzyme that catalyzes the formation of mevalonate from HMG-CoA, the earliest step in the production of cholesterol. Mevalonate is also the precursor of isoprenoid groups that are required to modify posttranslationally the Rho and Ras superfamilies of proteins by attaching a hydrophilic prenyl group, either farnesyl or geranylgeranyl. The Rho and Ras proteins are small membrane-bound GTPases, which are active in the GTP-bound state and inactive in the GDP-bound state. In their active state, these proteins regulate important cellular functions including cell survival, proliferation, and differentiation, as well as cytoskeletal organization, by relaying extracellular ligand–stimulated signals to cytoplasmic signaling cascades (40,41). It is the inhibition of prenylation of the Rho- and Ras-related proteins

by statins that is postulated to be responsible for some of the pleiotropic effects of this class of agents (42,43).

We are very aware that the concentrations of simvastatin required in vitro in the systemic sclerosis fibroblast culture system to observe the effects on collagen gene expression are between 50 and 100 times greater than those achieved in plasma following oral administration of the drug at doses currently utilized for cholesterol-lowering effects. However, the simvastatin concentrations used in our study are very similar to those used in numerous other studies examining the pleiotropic effects of the drug in vitro. For example, the concentration of simvastatin considered effective for modulating cardiomyocyte proliferation was in the range of 10–20 μM (44) and the concentration required to influence apoptosis of malignant lung epithelial cell lines ranged from 8.4 μM to 45.4 μM depending on the cell line (45). Similarly, numerous other studies with mesenchymal cells utilized concentrations in the range of 1–10 μM for modulation of various cellular functions, including the expression of growth factors and the production of various extracellular matrix molecules (42).

It is likely that the exquisite sensitivity of the cholesterol biosynthetic pathway to the inhibitory effects of statins in vivo is related to the facility of the compounds for cell penetration, since, for example, liver cell membranes are extremely permeable to statins, whereas membranes from other cell types are more resistant. Another factor that may be responsible for the requirement of higher statin concentrations in the in vitro studies relates to the need to empty or substantially reduce the intracellular stores of cholesterol-derived lipid precursors to achieve the observed effects. It is likely that in vivo depletion of these lipid precursor pools can be accomplished following extended periods of treatment at lower concentrations of the drug; however, for in vitro studies, it is necessary that depletion of these storage pools be accomplished in a much shorter period of time (in hours), therefore requiring much higher concentrations of the drug.

It is worthwhile to mention that some in vivo human studies that used much higher statin doses than those used to lower cholesterol levels reached statin serum concentrations in the μM range. For example, a recent study to examine the effects of very high lovastatin doses on cerebral gliomas in human subjects showed that serum concentrations as high as 3.92 μM could be achieved (46). Thus, it may be feasible to reach, in vivo, the statin concentrations required to accomplish the effects that we have described herein by increasing the administered dose and by using statins that may have an improved or preferential ability to penetrate mesenchymal cell membranes.

The potent inhibitory effects of simvastatin on collagen demonstrated in the present study provide strong support to the testable hypothesis that statins may be of benefit in the treatment of systemic sclerosis, a disease in which the prominent endothelial cell dysfunction and vascular abnormalities have been suggested to play a crucial role in its pathogenesis and evolution. We therefore propose that statins, which are readily available and have been demonstrated to be remarkably safe, are a promising alternative for the treatment of systemic sclerosis, a disease for which few proven disease-modifying agents are available.

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REFERENCES

1. Seibold JR. Scleroderma. In: Harris ED, editor. Kelley's textbook of rheumatology. Philadelphia: Elsevier Saunders; 2005. p. 1279–308.
2. Jimenez SA, Hitraya E, Varga J. Pathogenesis of scleroderma: collagen. Rheum Dis Clin North Am 1996;22:647–74.
3. Jimenez SA, Derk CT. Following the molecular pathways toward an understanding of the pathogenesis of systemic sclerosis. Ann Intern Med 2003;140:37–50.
4. D'Angelo WA, Fries JF, Masi AT, Shulman LE. Pathologic observations in systemic sclerosis (scleroderma): a study of fifty-eight autopsy cases and fifty-eight matched controls. Am J Med 1969;46:428–40.
5. Medsger TA Jr. Systemic sclerosis: classification, prognosis. In: Clements PJ, Furst DE, editors. Systemic sclerosis. Philadelphia: Lippincott Williams and Wilkins; 2004. p. 17–28.
6. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest 1974;54:880–9.
7. Jimenez SA, Feldman G, Bashey RI, Bienkowski R, Rosenbloom J. Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis. Biochem J 1986; 237:837–43.
8. Kikuchi K, Smith EA, LeRoy EC, Trojanowska M. Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts. Biochem Biophys Res Commun 1992;187:45–50.
9. Hitraya EG, Jimenez SA. Transcriptional activation of the α(I) procollagen gene in systemic sclerosis dermal fibroblasts: role of intronic sequences. Arthritis Rheum 1996;39:1347–54.
10. Hitraya EG, Varga J, Artlett CM, Jimenez SA. Identification of elements in the promoter region of the α1(I) procollagen gene involved in its up-regulated expression in systemic sclerosis. Arthritis Rheum 1998;41:2048–58.
11. Branton MH, Kopp JB. TGF-β and fibrosis. Microbes Infect 1999;1:1349–65.
12. Smith EA, LeRoy EC. A possible role for transforming growth factor-β in systemic sclerosis. J lnvest Dermatol 1990;95:125S–7S.
13. Massague J, Wotton D. Transcriptional control by the TGFβ/Smad signaling system. EMBO J 2000;19:1745–54.

14. Attisano L, Wrana JL. Smads as transcriptional co-modulators. Curr Opin Cell Biol 2000;12:235–43.
15. Moustakas A, Heldin CH. Non-Smad TGFβ signals. J Cell Sci 2005;118:3573–84.
16. Rosenbloom J, Saitta B, Gaidarova S, Sandorfi N, Rosenbloom JC, Abrams WR, et al. Inhibition of type I collagen gene expression in normal and systemic sclerosis fibroblasts by a specific inhibitor of geranylgeranyl transferase I. Arthritis Rheum 2000;43: 1624–32.
17. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425–30.
18. Holstein SA, Wohlford-Lenane CL, Hohl RJ. Consequences of mevalonate depletion. J Biol Chem 2002;277:10678–82.
19. The ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. Major outcomes in moderately hypercholesterolemic, hypertensive patients randomized to pravastatin vs. usual care. JAMA 2002;288:2998–3007.
20. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, et al, and the Cholesterol and Recurrent Events Trial Investigators. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. N Engl J Med 1996;335:1001–9.
21. Derk CT, Jimenez SA. Statins and the vasculopathy of systemic sclerosis: potential therapeutic agents? Autoimmun Rev 2006;5: 25–32.
22. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581–90.
23. Jimenez SA, Freundlich B, Rosenbloom J. Selective inhibition of human diploid fibroblast collagen synthesis by interferons. J Clin Invest 1984;74:1112–6.
24. Gaidarova S, Jimenez SA. Inhibition of basal and transforming growth factor-β-induced stimulation of COL 1A1 transcription by the DNA intercalators, Mitoxantrone and WP631, in cultured human dermal fibroblasts. J Biol Chem 2002;277:38737–45.
25. Saitta B, Gaidarova S, Cicchillitti L, Jimenez SA. CCAAT binding transcription factor binds and regulates human COL1A1 promoter activity in human dermal fibroblasts: demonstration of increased binding in systemic sclerosis fibroblasts. Arthritis Rheum 2000;43: 2219–29.
26. Jimenez SA, Varga J, Olsen A, Li L, Diaz A, Herhal J, et al. Functional analysis of human αl(I) procollagen gene promoter: differential activity in collagen-producing and non-producing cells and response to transforming growth factor-β1. J Biol Chem 1994;269:12684–91.
27. Artlett CM, Chen SJ, Varga J, Jimenez SA. Modulation of basal expression of the human αl(I) procollagen gene (COL 1A1) by tandem NF-1/Sp1 promoter elements in normal human dermal fibroblasts. Matrix Biol 1998;17:425–34.
28. Ghosh AK. Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. Exp Biol Med 2002;227: 301–14.
29. Chen SJ, Artlett CM, Jimenez SA, Varga J. Modulation of human αl(I) procollagen gene activity by interaction with Sp1 and Sp3 transcription factors in vitro. Gene 1998;215:101–10.
30. Nehls MC, Grapilon ML, Brenner DA. NF-I/Sp1 switch elements regulated collagen α1(I) gene expression. DNA Cell Biol 1992;11: 443–52.
31. Inagaki Y, Truter S, Ramirez F. Transforming growth factor-β stimulates α2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem 1994;269: 14828–34.
32. Rossi P, Karsenty G, Roberts AB, Roche NS, Sporn MB, de Crombrugghe B. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-β. Cell 1988;52:405–14.
33. Coustry F, Maity SN, Sinha S, de Crombrugghe B. The transcriptional activity of the CCAAT-binding factor CBF is mediated by two distinct activation domains, one in the CBF-B subunit and the other in the CBF-C subunit. J Biol Chem 1996;271:14485–91.
34. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, et al, for the AFCAPS/TexCAPS Research Group. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. JAMA 1998;279:1615–22.
35. Scandinavian Simvastatin Survival Study Group. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study(4S). Lancet 1994;344:1383–9.
36. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20536 high-risk individuals: a randomized controlled trial. Lancet 2002; 360:1623–30.
37. Kim SI, Han DC, Lee HB. Lovastatin inhibits transforming growth factor-β1 expression in diabetic rat glomeruli and cultured rat mesangial cells. J Am Soc Nephrol 2000;11:L80–7.
38. Li C, Yang CW, Park JH, Lim SW, Sun BK, Jung JY, et al. Pravastatin treatment attenuates interstitial inflammation and fibrosis in a rat model of chronic cyclosporine-induced nephropathy. Am J Physiol Renal Physiol 2004;286:F46–57.
39. Goppelt-Struebe M, Hahn A, Iwanciw W, Rehm M, Banas B. Regulation of connective tissue growth factor (ccn2; ctgf) gene expression in human mesangial cells: modulation by HMG CoA reductase inhibitors (statins). Mol Pathol 2001;54:176–9.
40. Holstein SA, Wohlford-Lenane CL, Hohl RJ. Isoprenoids influence expression of Ras and Ras-related proteins. Biochemistry 2002;41:13698–704.
41. Vojtek AB, Der CJ. Increasing complexity of the Ras signaling pathway [review]. J Biol Chem 1998;273:19925–8.
42. Eberlein M, Heusinger-Ribeiro J, Goppelt-Struebe M. Rho-dependent inhibition of the induction of connective tissue growth factor (CTGF) by HMG CoA reductase inhibitors (statins). Br J Pharmacol 2001;133:1172–80.
43. Danesh FR, Sadeghi MM, Amro N, Philips C, Zeng L, Lin S, et al. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors prevent high glucose-induced proliferation of mesangial cells via modulation of Rho GTPase/p21 signaling pathway: implications for diabetic nephropathy. Proc Natl Acad Sci U S A 2002;99:8301–5.
44. Soma MR, Corsini A, Paoletti R. Cholesterol and mevalonic acid modulation in cell metabolism and multiplication. Toxicol Lett 1992;64:1–15.
45. Khanzada UK, Pardo OE, Meier C, Downward J, Seckl MJ, Arcaro A. Potent inhibition of small-cell lung cancer cell growth by simvastatin reveals selective functions of Ras isoforms in growth factor signaling. Oncogene 2005:1–11. E-pub ahead of print.
46. Thibault A, Samid D, Tompkins C, Figg WD, Cooper MR, Hohl RJ, et al. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 1996;2:483–91.