Simvastatin attenuates the development of pulmonary and cutaneous fibrosis in a murine model of systemic sclerosis

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Abstract

Objective. The antifibrotic effect of simvastatin has been demonstrated in human lung fibroblasts. This study aimed to measure the effects of simvastatin in the development of pulmonary and cutaneous fibrosis in a murine model of SSc and to explore the mechanisms of these effects.

Methods. Chronic oxidant stress SSc was induced in BALB/c mice by daily s.c. injections of HOCl for 6 weeks. Mice were randomized in three arms: treatment with HOCl, HOCl plus simvastatin or vehicle alone. Statin treatment was initiated 30 min after HOCl s.c. injection and continued daily for 6 weeks. Skin and lung fibrosis were evaluated by histological methods. Immunohistochemical staining for α-smooth muscle actin in cutaneous and pulmonary tissues was performed to evaluate myofibroblast differentiation. Lung and skin concentrations of VEGF, extracellular signal-related kinase (ERK), rat sarcoma protein (Ras), Ras homologue gene family (Rho) and TGF-β were analysed by western blot.

Results. Injections of HOCl induced cutaneous and lung fibrosis in BALB/c mice. Simvastatin treatment prevented both skin thickness and pulmonary fibrosis. Myofibroblast differentiation was also inhibited by simvastatin in the skin and in the lung. Increased cutaneous and pulmonary expression of VEGF, ERK, Ras and Rho in mice treated with HOCl was significantly lower in mice treated with HOCl plus simvastatin.

Conclusion. Simvastatin reduces the development of pulmonary fibrosis, potentially modulating adverse lung remodelling, as shown by the reduced deposition of collagen in alveolar septae. Simvastatin also reduces skin thickness in this model.

Key words: systemic sclerosis, pulmonary fibrosis, simvastatin, animal model, reactive oxygen species.

Introduction

SSc is a progressive connective tissue disease of unknown aetiology characterized by a complex cascade of pathogenic events that leads to fibrosis of skin and internal organs. In this setting, endothelial dysfunction, inflammation and immune activation are considered the major contributors to the development of tissue damage [1]. To date, no therapy has been shown to reverse or arrest the progression of fibrosis, making scleroderma one of the most complex systemic autoimmune diseases in terms of its therapeutic management [2]. Nonetheless, studies of the multifaceted aetiopathogenesis of SSc are providing a growing number of potential therapies [3]. Among these, statins, 3-hydroxy-3-methylglutaryl
coenzyme A (HMG-CoA) reductase inhibitors primarily used as lipid-lowering agents, have shown pleiotropic effects: they could exert an antifibrotic effect by inhibiting TGF-β1 production [3], but also an immunomodulating role shown in other autoimmune diseases. For example, in trinitrobenzene sulphonic acid (TNBS)-induced colitis mice, atorvastatin significantly downregulates systemic Th17 cytokine levels [4], as well as in an autoimmune mouse model of anti-glomerular basement membrane glomerulonephritis [5]. Early statin therapy may suppress T cell-mediated endothelial cell damage in atherosclerotic plaques and thus prevent cardiovascular events [6]. In a prospective population-based cohort study of patients age 55 years and older (n = 2921), statin use is associated with a more than 50% reduction in overall progression of OA of the knee [7]. New therapeutic implications for statins have also been suggested in complicating pulmonary diseases, as evidenced by Zeki et al. [8]. In a mouse model of allergic asthma, statins reduced peribronchiolar eosinophilic inflammation, airway hyperreactivity, goblet cell hyperplasia and lung IL-4 and IL-13 production [8]. Potential preventive pleiotropic effects have been demonstrated in rats exposed to tobacco smoke, whereas 1 week of simvastatin pre-treatment almost completely prevented smoke-induced denudation of the airway epithelial layer [9]. Statins deplete cells of mevalonic acid, the direct product of the enzyme reaction, and thereby prevent the synthesis of downstream products such as cholesterol, haem A and dolichol [10], influencing post-translational modifications of isoprenylated proteins. One key cell-signalling molecule affected by statins is rat sarcoma protein (Ras), a small guanosine triphosphate (GTP) binding protein. Ras-GTP, the active conformation of Ras, is capable of stimulating downstream signalling molecules, including mitogen-activated protein (MAP) kinase, which has been directly implicated in cell survival [11] and proliferation [12]. Lovastatin can inhibit the activation of Ras as a result of interfering with its prenylation and induce, as demonstrated by Tan et al. [13], fibroblast apoptosis in vitro and in vivo. Therefore the current study aims to evaluate the preventive effect of simvastatin on the development of cutaneous and pulmonary fibrosis in a murine model of SSC, as well as test the hypothesis that such an effect might be mediated through Ras-GTP.

**Materials and methods**

**Animals**

Pathogen-free, 6-week-old female BALB/c mice were purchased from Harlan (Italy) and maintained with food and water ad libitum and were given humane care according to our institutional guidelines. The project was reviewed and approved by the ethics committee. All mice were housed in single cages under controlled light and temperature conditions. Mice (n = 23) were randomized to three arms: HOCl (n = 10), HOCl plus simvastatin (n = 8; hereafter Simva) or vehicle alone (n = 5; Sham) for 6 weeks.

**Reactive oxygen species preparation and treatments**

SSC was induced as characterized in detail in the Cochin chronic oxidant stress model [14]. Briefly, HOCl was produced by adding 166 μl of NaClO solution (2.6% as active chlorine) to 11.1 ml of KH2PO4 solution (100 mM, pH 7.2). A total of 100 μl of solution containing HOCl was injected s.c. into the back of the mice, using a 27-gauge needle, every day for 6 weeks. Mice (n = 8) from the HOCl group (n = 18) were randomly chosen to be treated with Simvastatin (Sigma-Aldrich, Italy) at a dose of 40 mg/kg/day s.c. The dosage of 40 mg/kg/day was chosen as being consistent with previously published papers on simvastatin use in mice [15, 16]. The administration of simvastatin was initiated 30 min after HOCl injection and continued for 6 weeks. All agents were prepared daily. Sham animals received injections of 100 μl of saline solution.

**Experimental procedure**

At the end of the experiment, animals were sacrificed by an overdose of pentothal sodium (80 mg/kg i.p.). Serum samples were collected by cardiac puncture from each mouse and stored at −80°C until use. Lungs were removed from each mouse and collected for histopathology, inflated with 400 μl of 10% formalin/PBS and fixed in formalin for 24 h. After paraffin embedding, 5 μm sections were cut throughout the whole lung. Five sections, with 1 mm intervals, were stained with Masson’s trichrome (MT) and systematically scanned with a light microscope, as previously described [17, 18]. A skin biopsy was performed on the back region, involving the skin of the injected area. Tissue samples were stored at −80°C for determination of protein expression or fixed in 10% neutral buffered formalin for histopathological analysis.

**Determination of Rho, Ras, ERK and VEGF by western blot analysis**

Briefly, lung and skin samples were homogenized in RIPA buffer (25 mM Tris/HCl, pH 7.4; 1.0 mM EDTA; 1.0 mM EGTA) together with 1% of Nonidet P40, 0.5% of phenyl methylsulphonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin (10 μg/ml each), with an Ultra Turrax (IKA, Staufen, Germany) homogenizer. The lysate was subjected to centrifugation at 15 000g for 15 min at 4°C. The supernatant was collected and used for protein determination using the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA). Protein samples (30 μg) were denatured in reducing buffer (62 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred onto to a PVDF membrane (Amersham, UK) using the transfer buffer (39 mM glycine, 48 mM Tris pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were blocked with 5% nonfat dry milk (Bio-Rad) in TBS with 0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS with 0.1% Tween and incubated with a primary Ras homologue gene family (Rho) or Ras (Abcam, Cambridge, UK) or
extracellular signal-regulated kinase (ERK) (Cell Signaling, Danvers, MA, USA), TGF-β (Cell Signaling, Danvers, MA, USA) or VEGF (Abcam) antibody in TBS with 0.1% Tween overnight at 4°C. After being washed three times for 10 min each in TBS with 0.1% Tween, the membranes were incubated with a secondary peroxidase-conjugated goat anti-rabbit antibody (Pierce, UK) for 1 h at room temperature. After washing, the membranes were analysed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham, UK). The protein signal was quantified by scanning densitometry using a bioimage analysis system (Bio-Profil, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with Sham lung or skin tissue measured with the same batch. β-Actin (Cell Signalling, Danvers, MA, USA) was used on stripped blots to confirm equal protein loading.

Histological and immunohistochemical evaluation

At the end of the experimental phase, lungs and skin were removed from the animals and fixed in 10% buffered formalin, processed for paraffin embedding, sectioned at 5 μm thickness and subsequently stained with haematoxylin and eosin (H&E) or MT, for examination under a light microscope. For immunohistochemistry, paraffin-embedded tissues were sectioned (5 μm), rehydrated and antigen retrieval was performed using 0.05 M sodium citrate buffer. Tissues were treated with 1% hydrogen peroxide to block endogenous peroxidase activity, and with horse normal serum (Vector Laboratories, Burlingame, CA, USA) to prevent nonspecific staining. A primary antibody against α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK) was used and kept overnight at 4°C in a humid box. After washing in PBS, a secondary antibody was used (Vector Laboratories) and the location

**Fig. 1** Effect of simvastatin on the development of pulmonary fibrosis.

Representative MT-stained section of lung was examined by light microscopy. (A) Normal histology of a representative lung tissue from a Sham mouse. (B) Representative lung section from an HOCl animal. (C) Representative lung section from a Simva mouse (original magnification 10×). (D) Semiquantitative analysis of lung tissue graded using the Ashcroft score, as described in the Materials and methods section. The degree of pulmonary fibrosis was evaluated in MT-stained sections using the Ashcroft score [15]. The grade of lung fibrosis was scored on a scale of 0–8 using the following criteria: grade 0, normal lung; grade 1–2, minimal fibrous thickening of alveolar or bronchiolar wall; grade 3–4, moderate thickening of walls without obvious damage to lung architecture; grade 5–6, increased fibrosis with definite damage to lung structure; grade 7–8, severe distortion of structure and large fibrous areas. Values are the mean ± S.D.* P < 0.001 vs Sham; # P < 0.001 vs HOCl. HOCl group: n = 10; Simva group: n = 8; Sham group: n = 5.
of the reaction was visualized with diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Milan, Italy). Slides were counterstained with haematoxylin, dehydrated and mounted with coverslips. As a part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment, using masked slides with a Leica microscope (Leica Microsystems, Milan, Italy). In each section, α-SMA-positive cells were counted in three randomly chosen high-power fields.

Assessment of dermal thickness

Dermal thickness, defined as the thickness of skin from the top of the granular layer to the junction between the dermis and s.c. fat was examined in histology samples (MT stain) using the Leica application suite software, as previously described [19, 20]. Ten random measurements were taken per section. The results were expressed in micrometers as mean values of dermal thickness for each group. Two investigators examined all of the sections independently in a blinded fashion.

Assessment of pulmonary fibrosis

The degree of pulmonary fibrosis was evaluated in H&E-stained sections using the Ashcroft score [21]. Briefly, the grade of lung fibrosis was scored on a scale of 0–8 using the following criteria: grade 0, normal lung; grade 1–2, minimal fibrous thickening of alveolar or bronchiolar wall; grade 3–4, moderate thickening of walls without obvious damage to lung architecture; grade 5–6, increased fibrosis with definite damage to lung structure; grade 7–8, severe distortion of structure and large fibrous areas.

Statistical analysis

All quantitative data are expressed as mean ± s.d. for each group. Data were compared using the nonparametric Mann–Whitney test or the Student’s paired t-test. When the analysis included more than two groups, one-way analysis of variance was used. P-values <0.05 were considered significant.
Results

HOCl-induced pulmonary fibrosis is prevented by simvastatin administration

We next investigated whether simvastatin affects HOCl-induced pulmonary fibrosis. At the end of the SSc induction, most of the alveolar walls were thickened, the air spaces were collapsed and collagen deposition in the lungs was markedly present. Semiquantitative assessment using the Ashcroft score demonstrated that the degree of pulmonary fibrosis in the HOCl group (n = 10) was significantly higher than in the Sham group (n = 5). In contrast, simultaneous administration of simvastatin and HOCl markedly prevented the development of pulmonary fibrosis in the Simva group (n = 8) (Fig. 1).

Dermal thickness in HOCl-injected mice is abrogated by simvastatin administration

At the end of the experiment, histological examination of MT-stained skin sections of the HOCl (n = 10), Simva (n = 8) and Sham (n = 5) groups demonstrated that the Simva animals were strikingly protected from HOCl-induced dermal fibrosis. Skin samples of BALB/c mice treated with HOCl showed an increase in the dermal thickness compared with the Sham group. When compared with HOCl, the Simva group showed a significantly reduced dermal thickness (Fig. 3). In addition, simvastatin

Myofibroblast differentiation, as determined by \( \alpha \)-SMA staining in pulmonary tissues, was less evident in the Simva group than in the HOCl mice (Fig. 2).

Dermal thickness was determined using photomicrographs of MT-stained sections by measuring the distance between the epidermal-dermal junction and the dermal-fat junction at 10 randomly selected sites/high-power fields (HPFs), for 10 HPFs per section. Skin fibrosis was induced in mice by s.c. injection of HOCl. The resultant increase in dermal thickness was significantly reduced by s.c. injection of simvastatin. Representative MT-stained sections were examined by light microscopy. (A) Normal histology of representative skin tissue obtained from a Sham mouse. (B) Representative histology of skin tissue of an HOCl mouse. (C) Representative histology of skin tissue of a Simva mouse (original magnification 10×). (D) Dermal thickness in mice from the three treatment groups \( (n = 5 \text{ for Sham, } n = 10 \text{ for HOCl, } n = 8 \text{ for Simva}) \). Values are the mean ± S.D. \( ^* P < 0.001 \text{ vs Sham, } ^# P < 0.001 \text{ vs HOCl. HOCl group: } n = 10; \text{ Simva group: } n = 8; \text{ Sham group: } n = 5 \).
administration prevented myofibroblast differentiation as determined by α-SMA staining (Fig. 4).

The increase in VEGF, pERK, RAS and RHO protein expression in cutaneous and pulmonary tissues of HOCl-treated mice is prevented by simvastatin administration.

Greater amounts of VEGF, ERK, RAS and RHO proteins were found in both the lungs (Fig. 5) and the skin (Fig. 6) of HOCl mice than in the Sham group as demonstrated by western blot analyses. Administration of simvastatin significantly abrogated the expression of these proteins. No significant difference in the expression of TGF-β (data not shown) was observed in mice exposed to HOCl vs Sham mice or between HOCl vs Simva mice.

Discussion

In these studies, simvastatin prevented the development of cutaneous and pulmonary fibrosis in the HOCl mouse model of SSC. Both the western blot analyses here (Figs. 5 and 6) and in the previous literature [4–7] bring some mechanistic insights into the question of how such a pleiotropic statin effect could potentially work in patients with SSC. The western blot analyses indicate that the Ras-ERK pathway and Rho expression (Figs. 5A–C and 6A–C) are modified by the addition of simvastatin, supporting the biological plausibility of the hypothesis that pleotropic effects of statins can modify fibrotic outcome in scleroderma via guanosine triphosphate (GTPase) intracellular signalling.

Previous reports have shown that statins exert an inhibitory effect on acute and chronic hypoxic pulmonary adventitial fibroblast proliferation [22, 23]. Some investigators have associated the use of statins in SSC with reduced endothelial activation [24] and increased levels of circulating endothelial precursor cells [25], which in some clinical trials led to improvements in severe RP [26] and the overall number of ulcers [27]. However, animal SSC model studies have not yet focused on the potential anti-inflammatory and antifibrotic effects of statins.

Fig. 4 Immunostaining for α-SMA (arrows show myofibroblast cells) in cutaneous samples.

Representative tissue sample from (A) a Sham mouse, (B) an HOCl mouse and (C) a Simva mouse (original magnification 40x). The arrows show strong diffuse staining of myofibroblast nuclei (dark brown staining), the number of which is lower in controls (A) than in HOCl (B) and lower in Simva (C) than in HOCl. *P < 0.001 vs Sham; #P < 0.001 vs HOCl. HOCl group: n = 10; Simva group: n = 8; Sham group: n = 5.
Statins abrogate the cholesterol synthesis pathway by inhibiting the enzyme HMG-CoA reductase. However, the cholesterol pathway is also involved in small guanosine triphosphatase (GTPase) signalling. The isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), intermediates of the cholesterol synthesis pathway, are important lipid attachments for the post-translational modification of proteins [28], such as small GTP-binding protein Ras and Ras-like proteins, such as Rho, Rab, Rac, Ral or Rap [29]. The antifibrotic actions may result from disruption of small GTPase signalling arising from inhibition of isoprenylation and farnesylation. This is supported by the observation that the small GTPases of the Rho family are important for basal as well as induced ccm2 (CTGF) expression in human renal mesangial cells [30]. CTGF is inhibited by statins through the inhibition of Rho protein [31]. Simvastatin triggers apoptosis of cardiac fibroblasts and myofibroblasts through the isoprenylation of Rho protein [32], and is a powerful inhibitor of type I collagen gene expression in normal and SSC fibroblasts [33]. Our findings confirm that simvastatin inhibits in vivo the extension of fibrosis through the inhibition of Rho protein, as was previously described in the bleomycin-induced animal model of pulmonary fibrosis [34]. Rho kinase cascade has been shown to be directly involved in the production of collagen by cardiac fibroblasts [35]. An oxidant-antioxidant imbalance has been suggested as another aetiopathogenic mechanism in SSC, especially in the development of pulmonary fibrosis [36]. Exposure of human lung fibroblasts to hyperoxia resulted in RhoA activation and an increase in collagen-I synthesis and cell proliferation, and ROS-dependent RhoA activation is responsible for the increase in collagen-I synthesis in hyperoxic lung fibroblasts and mouse lungs [37]. More interestingly, the inhibition of RhoA target protein, Rho-kinase (ROCK), may interrupt signalling pathways known to contribute to pulmonary fibrosis, as already evidenced in bleomycin-induced experimental pulmonary fibrosis [38].

In addition to the possible statin effects on pulmonary fibroblast proliferation and the process of fibrosis itself within the lung and elsewhere, endothelial cells are also subject to pleiotropic effects of statins through the same mechanisms involving small GTPase signalling. Activation of Rho GTPases is also a key step in the epithelial-to-

![Effect of simvastatin on RAS (A), RHO (B), pERK (C) and VEGF (D) protein expression in skin tissue samples.](https://academic.oup.com/rheumatology/article-abstract/52/8/1377/1791707/1383)
mesenchymal transition (EMT) of renal tubular cells, which contributes to renal interstitial fibrosis [39]. EMT has also been demonstrated to be a potential source of fibroblasts in idiopathic pulmonary fibrosis [40, 41]. Endothelial cells also display a significant amount of plasticity and endothelial-to-mesenchymal transition (EndMT) is an example of such plasticity. Analogous to EMT, during EndMT, endothelial cells lose characteristic markers such as CD31 and gain mesenchymal markers (FSP-1 and α-SMA). EndMT contributes to the progression of cardiac fibrosis [42]. Whether or not endothelial cells contribute to populations of fibroblasts during pulmonary fibrosis remains unclear. Recently it has been demonstrated that in an animal model of pulmonary fibrosis, endothelial cells can undergo mesenchymal transition to serve as sources of fibroblasts during pulmonary fibrosis [43]. That study provides evidence for a central role of the Ras/ERK pathway in the completion of EndMT, since only treatment with TGF-β in combination with activated Ras was able to induce α-SMA expression [44].

One of the predominant growth factors associated with vascular endothelial proliferation, survival and migration is VEGF [45]. Several groups of investigators have reported that VEGF is upregulated in skin of patients affected by SSC [46, 47], consistent with our results (Figs. 5D and 6D).

Fibrosis appears to be a consequence of linked processes, including the proliferation of resident fibroblast cell types, the increased production and deposition of extracellular matrix components and the transition of fibroblasts into cells exhibiting a myofibroblast phenotype. Myofibroblasts are atypical fibroblasts expressing α-SMA and exhibiting an uncontrolled activated phenotype. Besides resident fibroblasts, myofibroblasts have multiple origins, such as vessel wall pericytes, adventitial fibroblasts, bone marrow-derived circulating fibroblasts, epithelial cells and more recently endothelial cells [48]. Our results show that the increase in the myofibroblast population in the HOCl group was abrogated by simvastatin administration (Figs. 2C1504), probably acting on the inhibition of Rho protein expression, which exerts a central role in mechanotransduction in the regulation of matrix stiffness-induced myofibroblast differentiation, as evidenced in matrix stiffness-induced myofibroblast differentiation experiments [49].

Conclusions
Pulmonary fibrosis and pulmonary hypertension are the most morbid and the most easily quantifiable features of human SSC. The histological evidence from these
experiments in the Cochin model suggests that, given the low 1 per million death rate from statin prescriptions and the morbidity and mortality of SSc pulmonary disease, consideration of human trials may eventually be warranted if further animal studies confirm that simvastatin can affect the fibrotic component of SSc in different, independent animal models of SSc and perhaps in other fibrotic states as well.

Rheumatology key messages

- Simvastatin exerts antifibrotic effects in the reactive oxygen species murine model of SSc.
- Simvastatin administration during the experimental induction of SSc modulates end-organ damage.

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