Decreased Expression of Caveolin 1 in Patients With Systemic Sclerosis

Crucial Role in the Pathogenesis of Tissue Fibrosis

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Objective. Recent studies have implicated caveolin 1 in the regulation of transforming growth factor β (TGFβ) downstream signaling. Given the crucial role of TGFβ in the pathogenesis of systemic sclerosis (SSc), we sought to determine whether caveolin 1 is also involved in the pathogenesis of tissue fibrosis in SSc. We analyzed the expression of CAV1 in affected SSc tissues, studied the effects of lack of expression of CAV1 in vitro and in vivo, and analyzed the effects of restoration of caveolin 1 function on the fibrotic phenotype of SSc fibroblasts in vitro.

Methods. CAV1 expression in tissues was analyzed by immunofluorescence and confocal microscopy. The extent of tissue fibrosis in Cav1-knockout mice was assessed by histologic/histochemical analyses and quantified by hydroxyproline assays. Cav1-null and SSc fibroblast phenotypes and protein production were analyzed by real-time polymerase chain reaction, immunofluorescence, Western blot, and multiplexed enzyme-linked immunosorbent assay techniques. The effects of restoration of caveolin 1 function in SSc fibroblasts in vitro were also examined using a cell-permeable recombinant CAV1 peptide.

Results. CAV1 was markedly decreased in the affected lungs and skin of SSc patients. Cav1-knockout mice developed pulmonary and skin fibrosis. Down-regulation of caveolin 1 was maintained in cultured SSc fibroblasts, and restoration of caveolin 1 function in vitro normalized their phenotype and abrogated TGFβ stimulation through inhibition of Smad3 activation.

Conclusion. Caveolin 1 appears to participate in the pathogenesis of tissue fibrosis in SSc. Restoration of caveolin 1 function by treatment with a cell-permeable peptide corresponding to the CAV1 scaffolding domain may be a novel therapeutic approach in SSc.

Systemic sclerosis (SSc) is characterized by excessive deposition of collagen and other connective tissue macromolecules in skin and multiple internal organs, prominent and often severe alterations in the microvasculature, and humoral and cellular immunologic abnormalities (1). The excessive collagen deposition in SSc is due to overproduction of this protein by fibroblasts (2–4). Indeed, it is the persistent activation of the genes encoding various collagens in SSc fibroblasts that distinguishes controlled repair, such as that occurring during normal wound healing, from the uncontrolled fibrosis that is the hallmark of SSc (5).

Numerous alterations in the expression of cytokines and growth factors with potent effects on fibroblast collagen synthesis, various endothelial cell functions, and T cell responses have been demonstrated in SSc (6–8). Transforming growth factor β (TGFβ) plays a crucial role in tissue fibrosis (9–12) and has been implicated in the pathogenesis of SSc (1,12–17). An important effect of TGFβ is the stimulation of the expression of genes encoding various collagens and other matrix proteins and the processing and tissue
deposition of interstitial collagens (9–11). Although SSc fibroblasts display increased TGFβ signaling (18,19), the mechanisms responsible are not completely known. A recent study confirmed the importance of the increased TGFβ pathway activation in the pathogenesis of SSc (20). In that study, the conditional, fibroblast-specific gene expression of a constitutively active TGFβ receptor type I (TGFβRI) recapitulated the fibrotic process occurring in the skin, the vasculature, and possibly the lung of SSc patients.

Caveolin 1 is the most important member of a family of membrane proteins that are the major coating proteins of caveolae. Caveolae are 50–100-nm flask-shaped invaginations that represent a morphologically identifiable subset of lipid rafts (21). The spatial organization of cell receptors in lipid rafts can modulate the subsequent transmission of the specific signal (22,23). Indeed, TGFβ1 receptors are internalized both by caveolin 1-associated lipid rafts and by early endosome antigen 1 non–lipid raft pathways. Non–lipid raft–associated internalization increases TGFβ signaling, whereas, caveolin-associated internalization increases TGFβ receptor degradation, thus, effectively decreasing or abolishing TGFβ signaling (24,25). The increased TGFβRII degradation is mediated by an interaction between the receptor and the scaffolding domain of CAV1; the decreased availability of the activated TGFβRI diminishes the phosphorylation of Smad2/3 and disrupts its interaction with Smad4 and its subsequent nuclear translocation (26).

The possible participation of caveolin 1 in the pathogenesis of fibrotic diseases was first suggested by Tourkina et al (27), who demonstrated that knock-down of CAV1 in vitro resulted in a 5-fold increase in COL1A2 gene expression by normal human lung fibroblasts, whereas increased CAV1 expression caused a reduction in collagen production. A recent study (28) described a marked reduction of CAV1 expression caused in lung tissues and in lung fibroblasts from patients with idiopathic pulmonary fibrosis as compared with cells from normal lungs. Those investigators also demonstrated that induction of CAV1 expression markedly ameliorated bleomycin-induced pulmonary fibrosis and suppressed TGFβ1-induced stimulation of extracellular matrix production in cultured fibroblasts.

Based on these observations, we raised the question of whether caveolin 1 is involved in the pathogenesis of tissue fibrosis in SSc. Here, we present evidence supporting the hypothesis that caveolin 1 plays a role in the pathogenesis of tissue fibrosis in SSc. Furthermore, our results suggest that an increase in caveolin 1 function through the use of a cell membrane–permeable CAV1 scaffolding domain peptide may be a novel therapeutic approach to limiting the progression of tissue fibrosis in SSc.

**PATIENTS AND METHODS**

**Patients.** Full-thickness skin biopsy samples were surgically obtained from 3 patients with SSc of recent onset (<18 months from the appearance of clinically detectable skin induration) and 3 normal control subjects, with approval of the Institutional Review Board. The SSc patients satisfied the criteria for the classification of SSc (29) and had the diffuse cutaneous clinical subset of the disease, as defined by LeRoy et al (30). Skin biopsy samples were obtained from the leading edge of the lesion on the forearm. The tissues remaining after diagnostic histopathology were used for fibroblast isolation and for immunohistologic assessment. Paraffin-embedded sections from surgical open-lung biopsy samples from 2 patients with SSc-associated pulmonary fibrosis and from 1 autopsy sample from a subject with SSc-associated pulmonary hypertension were obtained. The histopathologic examination of skin showed the typical changes of SSc. The 2 lung biopsy samples displayed the typical features of nonspecific interstitial pneumonitis. The lung sample from the autopsy case showed typical histopathologic findings of pulmonary arterial hypertension (PAH).

**Animals and histopathology studies.** Cav1-deficient mice were generated as previously described (31) on the C57BL/6 genetic background. Six same-sex mice per group were killed at 3 months of age. After hair removal with a commercial depilatory, full-thickness skin samples were surgically excised from the interscapular region, stretched and pinned in histopathology cassettes, and fixed in phosphate buffered formalin for 24 hours. The lungs were removed, and the left lung was processed by perfusion of the left pulmonary artery with paraformaldehyde to attain fixation of the parenchyma, avoiding alveolar collapse or atelectasis, as described previously (32). The entire right lung was hydrolyzed and used for hydroxyproline assays. Paraffin-embedded skin and lung tissues were stained with hematoxylin and eosin and Masson’s trichrome according to standard procedures.

**Hydroxyproline assays.** The collagen content in the lungs and skin of the same animals that were killed for the histopathology studies was determined by hydroxyproline assay. Skin samples from an area adjacent to that used for histopathologic assessment were obtained with a 4-mm tissue punch, and the skin samples and right lung were weighed and hydrolyzed overnight at 110°C in 6M HCl for determination of their hydroxyproline content, as described elsewhere (33).

**Confocal microscopy studies.** Caveolin 1 protein was analyzed by immunofluorescence using an anti–caveolin 1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit IgG (Sigma, St Louis, MO) was used as isotype control. For Smad localization studies, cells were fixed and permeabilized as described previously (34). Anti-Smad3 antibodies (Upstate, Lake Placid, NY) were used at a 1:200 dilution. Secondary antibodies used in the studies were affinity-purified sheep Fab’ anti-rabbit IgG conjugated with
manufacturer’s protocol, including a genomic DNA digestion (RNeasy kit; Qiagen, Valencia, CA) according to the EDTA, washed in PBS, and then processed for RNA extraction.

Fibroblasts were used at passages 4–10. Both human and mouse fibroblasts from Cav1-knockout or wild-type (WT) mice were obtained by subculturing full-thickness skin biopsy samples from age- and sex-matched animals. Both human and mouse fibroblasts were used at passages 4–10.

Photoshop software (Adobe Systems, San Jose, CA), without separate signal from noise. Panels were assembled using a Zeiss LSM 510 META confocal laser scanning microscope system and software (Zeiss, Wetzlar, Germany). The 2 channels were recorded simultaneously if no cross talk could be detected. The breakthrough of the DAPI signal into the red channels were recorded simultaneously if no cross talk could be detected. The breakthrough of the DAPI signal into the red and the green channels was recorded separately and subtracted from the DAPI blue channel. Each image was scanned 8 times to separate signal from noise. Panels were assembled using Photoshop software (Adobe Systems, San Jose, CA), without any RGB modification.

**Cell culture.** Fibroblasts from normal and SSc dermis were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics, and glutamine (complete medium) until confluent. Fibroblasts from Cav1-knockout or wild-type (WT) mice were obtained by subculturing full-thickness skin biopsy samples from age- and sex-matched animals. Both human and mouse fibroblasts were used at passages 4–10.

**Western blot analysis.** Intracellular proteins in whole cell lysates were obtained by lysing the confluent cells in 100-mm dishes as described elsewhere (36). Fifty micrograms of protein was used per sample. Primary antibodies used in the different experiments were as follows: caveolin 1 polyclonal rabbit antibody (Santa Cruz Biotechnology), Woburn, MA) were performed to measure the levels of interleukin-6 (IL-6) and matrix metalloproteinase 3 (MMP-3) in supernatants from mouse fibroblasts as well as the levels of MMP-3 and plasminogen activator inhibitor (PAI) in tissue culture supernatants from human fibroblasts, as described previously (36). Briefly, samples were diluted 1:5, 1:50, or 1:1,000 before a 1-hour incubation on the array plates, which had been prespotted with capture antibodies specific for each protein. Plates were decanted and washed 3 times before adding a mixture of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates were washed 3 times and incubated for 30 minutes with streptavidin–horseradish peroxidase. Plates were then washed before adding SuperSignal Femto chemiluminescent substrate. The plates were immediately imaged using the SearchLight imaging system, and data were analyzed using ArrayVision software (Pierce).

**RNA isolation and quantitative real-time polymerase chain reaction (PCR).** Fibroblasts were trypsin–EDTA, washed in PBS, and then processed for RNA extraction (RNeasy kit; Qiagen, Valencia, CA) according to the manufacturer’s protocol, including a genomic DNA digestion step. Total RNA (2 μg) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green Master Mix chemistry according to a standard amplification protocol on a Bio-Rad MyiQ real-time PCR system (Bio-Rad, Hercules, CA). Reactions were conducted as described elsewhere (36). To confirm the amplification specificity, the PCR products were subjected to melting temperature dissociation curve analysis. No amplification and no template controls were examined in parallel. The differences in the number of messenger RNA (mRNA) copies in each PCR reaction were corrected for endogenous control transcript levels; the control experiment levels were arbitrarily set at 100, and all other values were calculated as multiples thereof.

**Primer sequences.** All primers were designed using Primer Express (Applied Biosystems, Foster City, CA) and validated on the National Center for Biotechnology Information (NCBI) database for specificity (Table 1).

**Enzyme-linked immunosorbent assay (ELISA).** SearchLight Multiplexed Proteome Arrays (Pierce Biotechnology, Woburn, MA) were performed to measure the levels of interleukin-6 (IL-6) and matrix metalloproteinase 3 (MMP-3) in supernatants from mouse fibroblasts as well as the levels of MMP-3 and plasminogen activator inhibitor (PAI) in tissue culture supernatants from human fibroblasts, as described previously (36). Briefly, samples were diluted 1:5, 1:50, or 1:1,000 before a 1-hour incubation on the array plates, which had been prespotted with capture antibodies specific for each protein. Plates were decanted and washed 3 times before adding a mixture of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates were washed 3 times and incubated for 30 minutes with streptavidin–horseradish peroxidase. Plates were then washed before adding SuperSignal Femto chemiluminescent substrate. The plates were immediately imaged using the SearchLight imaging system, and data were analyzed using ArrayVision software (Pierce).

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Table 1. Oligonucleotides used in the SYBR Green quantitative real-time polymerase chain reaction analysis
Treatment of cells with cell-permeable peptides. It has been demonstrated that caveolin 1 regulates TGFβ/Smad signaling through an interaction between the TGFβ1 receptor and a peptide corresponding to the scaffolding domain of CAV1 (DGIWKASFTFTVTKYWFY) (26). Confluent cells between passages 4 and 9 were treated for 24 hours with either Penetratin peptide, a 16–amino acid cell-permeable peptide corresponding to the homeodomain of the Drosophila transcription factor Antennapedia (RQPKIWFPNRRKPWKK) or with a peptide consisting of the fusion of the Antennapedia peptide and the peptide corresponding to the scaffolding domain of CAV1 (32). This fusion peptide, referred to as Cav1p, was used at 2.5 μM and 5 μM final concentrations. Fibroblasts were incubated for 24 hours in duplicate for each experiment, with or without 10 ng/ml of TGFβ. Following a 24-hour incubation with or without TGFβ, one experimental plate was harvested and processed for RNA extraction for real-time PCR and the other was processed to obtain cell lysates for Western blots. Tissue culture supernatants were collected to analyze collagen production by Western blotting.

Statistical analysis. The resulting data were analyzed by 2-tailed t-test to determine statistical significance. *P values less than 0.05 were considered significant.

RESULTS

Decreased levels of caveolin 1 in affected SSc lung. Affected tissues from patients with SSc-related pulmonary fibrosis and PAH displayed reduced caveolin
immunofluorescence compared with normal lungs (Figure 1). The reduction in fluorescence staining for caveolin 1 was observed in the thickened alveolar septa, typical of SSc alveolitis (Figure 1B). This reduction was even more apparent in the neointima of pulmonary arteries in lung tissues from SSc patients with PAH (Figure 1C) and in the interstitium of a lung section from a region where the normal lung architecture has been replaced by fibrotic tissue (Figure 1D). In contrast, intense caveolin 1 immunofluorescence was observed in histopathologically unaffected areas of SSc lung tissues from the same patients (Figure 1E).

Quantitative analysis of the integrated density of fluorescence in 3 different fields per type of lung involvement (Figures 1A–E) was performed using ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsbweb.nih.gov/ij/). The ratio between caveolin 1 immunofluorescence staining (green fluorescence) and the number of cells in the sample as assessed by DAPI staining of their nuclei was 1.95 in normal lung and 2.1 in histopathologically unaffected SSc lung samples, compared with 0.79 in the alveolitis sections, 0.60 in the PAH sections, and 0.66 in the late fibrotic lesion sections. These data are shown in Figure 1F. Hence, the 3 types of SSc lung involvement analyzed showed, respectively, a 59%, 69%, and 66%
decrease in relative caveolin 1 immunofluorescence levels compared with either normal lung tissue or histopathologically unaffected areas of SSc lung tissue.

**Decreased levels of caveolin 1 in skin and in dermal fibroblasts from SSc patients.** The expression of caveolin 1 observed under confocal microscopy was markedly reduced in affected skin from SSc patients (Figures 2A–D). We then compared by quantitative analysis of the integrated density of fluorescence the levels of caveolin 1 in skin biopsy samples from 3 normal subjects and 3 SSc patients. The results indicated that the ratio of caveolin 1 to DAPI immunofluorescence in
the affected skin of SSc patients, on average, <50% of that observed in normal skin (Figure 2E). To confirm these results we studied in vitro–cultured fibroblasts derived from SSc lesional skin because these cells recapitulate the most important molecular alterations responsible for the fibrotic phenotype observed in SSc skin in vivo. In these studies, we compared the amount of caveolin 1 protein between SSc and normal dermal fibroblasts by immunofluorescence analysis (Figure 2F) and Western blotting (Figure 2G). Both studies showed a dramatic decrease in caveolin 1 in SSc dermal fibroblasts compared with normal dermal fibroblasts.

**TGFβ down-regulation of CAV1 expression in vitro.** To investigate whether the potent profibrotic growth factor TGFβ is involved in the observed reduction of caveolin 1 in affected tissues from SSc patients, we examined the effects of TGFβ on CAV1 expression in cultured normal human dermal fibroblasts. As shown in Figures 2H and I, we observed a profound decrease in CAV1 mRNA and protein levels following 24 hours of incubation with 10 ng/ml of recombinant human TGFβ.

**Pulmonary and dermal fibrosis and increased collagen content in Cav1-knockout mice.** To investigate whether the observed reduction of caveolin 1 in affected SSc lung and skin biopsy samples in vivo and in SSc cultured fibroblasts in vitro could play a role in the pathogenesis of tissue fibrosis, we analyzed by histopathology the skin and lungs of mice lacking Cav1 expression. We observed that lung tissues from Cav1-null mice had increased staining in the interstitium, indicating an increase of the collagen content in the lungs. The histopathologic analysis of paraffin-embedded sections of skin biopsy tissue from the same mice showed an increase in the thickness of the dermis that was accompanied by an increase in collagen staining (Figure 3A). Hydroxyproline assays of lung and skin samples from 6 Cav1-knockout mice (12 weeks old) and 6 matched WT littermates showed that the collagen content in tissue...
from the Cav1-null mice was 2.5-fold greater than that in tissue from the WT mice (mean 0.86 µg/mg and 0.35 µg/mg, respectively; P < 0.001) and that the skin of Cav1-null mice contained 2.4-fold more collagen than the skin of WT mice (mean 6.8 µg/mg and 2.8 µg/mg, respectively; P < 0.002) (Figure 3B).

**Profibrotic phenotype of Cav1-null mouse fibroblasts in vitro.** To determine whether skin fibroblasts obtained from Cav1-null mice recapitulated in vitro the molecular events associated with a profibrotic phenotype, we examined by real-time PCR the expression of collagen, α-smooth muscle actin (α-SMA), and Mmp genes in Cav1-null mouse fibroblasts. We observed a 2-fold increase in Col1a1 transcript levels, a 3.5-fold more collagen than the skin of WT mice (mean 0.86 µg/mg and 0.35 µg/mg, respectively; P < 0.001) and that the skin of WT mice (mean 6.8 µg/mg and 2.8 µg/mg, respectively; P < 0.002) (Figure 3B).

**Cav1 cell-permeable peptide down-regulation of collagen production in SSc fibroblasts and suppression of the up-regulation of TGFβ-inducible genes in vitro.** We next examined whether Cav1p, a cell-permeable Cav1 scaffolding domain peptide, previously shown to be able to inhibit TGFβ pathway activation and to mimic Cav1 function in a rat model of PAH (26,32), could exert an antifibrotic effect on SSc fibroblasts. We analyzed the effects of Cav1p on SSc fibroblasts under control conditions and following stimulation with TGFβ. We found that treatment of SSc dermal fibroblasts with Cav1p caused a reduction in their collagen expression at both the basal level and following TGFβ stimulation (Figure 4A). Furthermore, gene expression analysis of CTGF, a potent profibrotic TGFβ-inducible gene, showed that treatment with Cav1p reduced its basal level of expression and abrogated the stimulation normally induced by TGFβ (Figure 4A).

These results were confirmed at the protein level for both proteins. Western blot analyses of type I collagen indicated that incubation with 2.5 µM or 5 µM Cav1p caused a profound reduction in basal collagen production (Figure 4B). Furthermore, we also observed that 5 µM Cav1p abrogated TGFβ-induced type I collagen stimulation (Figure 4B). These effects were not caused by cellular toxicity induced by the Cav1p, since cell viability assays did not show any differences between...
treated and untreated cells at the 18-hour and 24-hour time points (Figure 4C). Western blot analyses of CTGF in the cell lysates from SSc fibroblasts incubated with TGFβ for 24 or 48 hours confirmed at the protein level
the inhibitory effects of 24 hours of Cav1p treatment that was observed at the mRNA level (Figure 4D).

PCR analysis and immunofluorescence studies of SSc fibroblasts indicated that the basal expression of α-SMA transcripts and α-SMA–containing stress fibers were reduced by treatment with 10 μM Cav1p and that treatment with the peptide inhibited the TGFβ-induced increased levels of α-SMA mRNA and stress fibers (Figures 5A and B). The Cav1 peptide also reduced the effects of TGFβ on the production of PAI-1 and increased the production of MMP-3 regardless of TGFβ stimulation, as demonstrated by ELISA of tissue culture supernatants (Figure 5C).

**Cav1 cell-permeable peptide inhibition of TGFβ pathway activation through suppression of Smad3 phosphorylation.** To gain insight into the mechanism of the observed inhibitory effects of Cav1p on TGFβ pathway activation, we analyzed Smad3 phosphorylation in SSc fibroblasts following TGFβ stimulation. We observed that Cav1p inhibited the TGFβ-induced phosphorylation of Smad3 as early as 5 minutes following TGFβ stimulation. The inhibition was more pronounced at 15, 30, and 60 minutes following TGFβ (Figure 6A). To examine whether Cav1p also reduced the translocation of activated Smad3/4 complexes from the cytoplasm to the nucleus, we analyzed by immunofluorescence the cellular localization of Smad following incubation of SSc dermal fibroblasts with TGFβ. We observed that the expected nuclear localization of Smad3 at 60 minutes following TGFβ stimulation was almost completely abrogated in the presence of 5 μM Cav1p (Figure 6B).

**DISCUSSION**

Since the discovery of the potent profibrotic and immunomodulatory activities of TGFβ, this growth factor has been recognized as one of the most important molecules in the pathogenesis of SSc and other fibroproliferative diseases (1,9–20). The classic pathway of TGFβ signal transduction involves the ligand-bound TGFβRII, which recruits the receptor TGFβRI and then transphosphorylates specific serine and threonine residues in a short (30–amino acid) regulatory sequence known as the GS region (37,38). Signaling from the phosphorylated TGFβRI receptor to the nucleus then occurs through the Smad family of proteins (39). Smad2 or Smad3, 2 of the 5 receptor-activated Smads, bind to the activated TGFβ receptor complex and become phosphorylated, allowing the formation of a complex with the common-mediator Smad, Smad4, a cytoplasmic protein which translocates the Smad complex through nuclear membrane pores into the nucleus. Once in the nucleus, Smad3/Smad4 complexes act as transcription factors, binding with the help of intranuclear transcriptional partners to specific DNA binding sites in the promoter regions of target genes and activating their expression.

To add to the complexity of the TGFβ activation and signaling cascades, it has been recognized that there are numerous other non–Smad-mediated events involved in TGFβ functions and effects (40,41). Furthermore, it has also recently been demonstrated that other non–TGFβ-dependent pathways may be necessary for the expression of the full SSc fibroblast phenotype. For example, Chen et al (42) found that the overexpression of CTGF and α-SMA in SSc fibroblasts was independent of TGFβ signaling, since it could not be abolished by treatment of the cells with a specific inhibitor of activin receptor–like kinase 5, the receptor involved in the canonical TGFβ activation cascade.

Another important mechanism of the regulation of TGFβ activation involves the intracellular degradation of the activated TGFβ receptor complex. The important role of caveolin 1 in this regulation has recently been recognized (23–26), and it has been suggested that alterations in this process may participate in the initiation and progression of tissue fibrosis (27,28). Thus, the purpose of the present studies was to examine the role of caveolin 1 in the pathogenesis of tissue fibrosis in SSc. We demonstrate here that the levels of caveolin 1 in affected lung and skin from SSc patients were substantially lower in comparison with the levels in normal tissues. Interestingly, our findings that CAV1 expression was not reduced in histopathologically unaffected areas of SSc lungs indicate that reduction of CA1V1 expression may be a specific component of the actual pathologic process, rather than the result of a generalized manifestation of SSc. Additionally, our observations that caveolin 1 levels are also low in lesional skin from SSc patients indicates that regardless of the anatomic site, a reduction in caveolin 1 likely represents a marker of the pathologic process in SSc.

To explore the functional effects of decreased caveolin 1 expression in vivo, we used Cav1-knockout mice (31) and performed a detailed histopathologic analysis of their skin and lung tissues. In these studies, we found that the lack of Cav1 expression was accompanied by skin and lung fibrosis, supporting the notion that the observed decrease in the expression of CA1V1 in SSc tissues may participate in the fibrotic process rather than being only an epiphenomenon. Furthermore, we found that Cav1-null fibroblasts displayed a profibrotic phenotype characterized by an increased expression of
type I collagen, α-SMA, and IL-6 and decreased production of MMP-3. In support of our observations are the results of a recent study (28), which showed that induced overexpression of CAV1 in vitro by means of an adenoviral vector suppressed the expression of various genes encoding extracellular matrix molecules as well as TGFβ-induced α-SMA up-regulation in the MRC-5 fibroblast line.

To further examine the role of decreased CAV1 expression in the development of tissue fibrosis, we explored the effects of increasing CAV1 function in SSc fibroblasts on the expression of genes encoding various key proteins participating in the process of pathologic fibrogenesis. The numerous and potentially serious difficulties encountered with the clinical application of virally mediated gene therapy approaches (43–47) has stimulated the search for alternative methods of delivery of therapeutic proteins into cells. One of the most promising approaches is the utilization of novel cell-permeable carriers capable of shuttling small peptides and proteins inside cells (48). One such carrier that has been used extensively is the small peptide Penetratin. Hence, we treated SSc fibroblasts in vitro with a cell-permeable Cav1 scaffolding domain peptide (Cav1p), previously shown to be capable of increasing the intracellular bioavailability of caveolin 1 (26,32), and examined whether the reconstitution of intracellular CAV1 function resulted in a modulatory effect on the profibrotic phenotype of SSc fibroblasts. We found that the administration of Cav1p in vitro to SSc fibroblasts caused a significant reduction in the basal level of COL1A1 production, as well as a reduction in CTGF protein levels and α-SMA–containing stress fibers. Of further interest was the observation that the TGFβ-induced up-regulation of COL1A1, CTGF, and α-SMA was strongly inhibited by treatment of SSc cells with Cav1p. Subsequently, we demonstrated that the inhibitory effects of Cav1p on TGFβ pathway activation were associated with the inhibition of Smad3 phosphorylation and of its nuclear translocation.

However, it should be noted that in the absence of TGFβ stimulation, SSc fibroblasts did not show appreciable nuclear accumulation of Smad3. Although these results are in contrast with previously published data on Smad3 nuclear localization in SSc fibroblasts (19), they support the participation of non–Smad3-mediated pathways in the activation of the SSc fibroblast phenotype as suggested by Chen et al (42) and suggest that in the absence of exogenous TGFβ stimulation, Cav1p could function in a TGFβ/Smad-independent pathway.

In summary, the studies described herein strongly support the hypothesis that decreased expression of CAV1 in SSc tissues plays a role in the development and progression of tissue fibrosis by mediating a sustained activation of the TGFβ pathway. Furthermore, we demonstrated that an in vitro intervention capable of increasing the function of CAV1 by using a cell-permeable Cav1 peptide reverted the profibrotic phenotype of SSc fibroblasts in vitro and that this effect was mediated by the suppression of the cascade of events induced by TGFβ receptor ligation, as shown by the abrogation of Smad3 phosphorylation and nuclear translocation.

These results suggest that restoration of CAV1 function in vivo by use of cell-permeable Cav1 peptides may be effective in preventing progression and limiting the extension of tissue fibrosis in SSc. Future studies should be performed to fully assess the long-term safety and effectiveness of Cav1p administration in vivo as a novel therapeutic approach for SSc.

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AUTHOR CONTRIBUTIONS

Dr. Jiménez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Del Galdo, de Almeida, Jiménez.

Acquisition of data. Del Galdo, Sotgia, de Almeida, Jasmin, Musiek.

Analysis and interpretation of data. Del Galdo, de Almeida, Lisanti, Jiménez.

Manuscript preparation. Del Galdo, Jiménez.

Statistical analysis. Del Galdo.

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