Halofuginone, an Inhibitor of Type-I Collagen Synthesis and Skin Sclerosis, Blocks Transforming-Growth-Factor-β-Mediated Smad3 Activation in Fibroblasts

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Halofuginone is a drug that has been shown to have an antifibrotic property *in vitro* and *in vivo*. Whereas halofuginone shows promise as a therapeutic agent for a variety of diseases including scleroderma, liver cirrhosis, cystic fibrosis, and certain types of cancer, the mechanism of action remains unknown. Using the tight skin mouse (TSK) model for scleroderma, we evaluated the ability of halofuginone to inhibit spontaneous development of dermal fibrosis. We found that administration of a low dose of halofuginone both in adult and newborn animals for 60 d prevented the development of cutaneous hyperplasia (dermal fibrosis). *In vitro* halofuginone was found to reduce the amount of collagen synthesized by fibroblasts. This effect was due to a reduction in the promoter activity of the type-I collagen genes as treatment of fibroblast cultures with 10^{-8} M halofuginone reduced the level of α2(I) collagen message detectible by northern blot and greatly reduced the activity of a reporter construct under control of the –3200 to +54 bp α2(I) collagen promoter. In addition, analysis of transforming growth factor β signaling pathways in fibroblasts revealed that halofuginone inhibited transforming-growth-factor-β-induced upregulation of collagen protein and activity of the α2(I) collagen promoter. Further we found that halofuginone blocked the phosphorylation and subsequent activation of Smad3 after transforming growth factor β stimulation. Apparently the inhibitory property was specific to Smad3 as there was no inhibitory effect on the activation of Smad2 after stimulation with transforming growth factor β. Our results demonstrate that halofuginone is a specific inhibitor of type-I collagen synthesis and may elicit its effect via interference with the transforming growth factor β signaling pathway. ***Key words: fibrosis/scleroderma/signal transduction/TGF-β/tight skin. J Invest Dermatol 118:461–470, 2002***

Scleroderma is an idiopathic disorder characterized by over-synthesis and deposition of collagen leading to fibrosis of the skin and other internal organs (Steen, 1998). The etiology of the disease is unknown, but both genetic and environmental factors have been implicated (Fanning *et al*, 1998; Steen, 1998; Tan *et al*, 1998). Recently it was reported that halofuginone causes a drastic reduction in the skin tensile strength of broiler chickens and inhibits the synthesis of collagen type I in avian, murine, and human fibroblasts (Granot *et al*, 1991, 1993; Halevy *et al*, 1996). Subsequently it was found that halofuginone also inhibited the expression of the matrix metalloproteinase 2 (MMP-2) gene and angiogenesis associated with bladder carcinoma metastasis (Elkin *et al*, 1999). These findings indicated that halofuginone may have important clinical applications in diseases such as scleroderma, a disorder in which fibrosis plays a significant role in morbidity and mortality (Liu and Connolly, 1998; Steen, 1998). In spite of the tremendous potential benefit of this drug, however, little research has been invested in understanding its mechanism of action.

Several lines of evidence link transforming growth factor β (TGF-β) stimulation with the regulation of extracellular matrix protein production. First TGF-β stimulation has been shown to upregulate the production of collagen types I, III, and VII (Uitto *et al*, 1979; Kulozik *et al*, 1990; Inagaki *et al*, 1994; Yoshiro *et al*, 1997; Vindevoghel *et al*, 1998), and fibronectin (Hocevar *et al*, 1999), and to regulate the expression of several MMPs including MMP-1, MMP-2, MMP-3, and MMP-13 (Kubota *et al*, 1991; Uria *et al*, 1998). Second, increased TGF-β production has been observed in several human disorders associated with connective tissue fibrosis. For instance, immunohistochemical analysis of biopsies from the lesions of scleroderma patients demonstrated the increased presence of cells producing TGF-β (Gay *et al*, 1992; Sfikakis *et al*, 1993; Higley *et al*, 1994). In addition, Arkwright *et al* (2000) found a strong association between the overproduction of TGF-β and an accelerated decline in lung function in cystic fibrosis patients. Finally, several groups have shown a direct correlation between the inhibition of TGF-β and the abrogation of fibrosis in the skin and lung. In two notable examples, bleomycin-induced lung fibrosis was prevented in mice by the introduction of the inhibitory Smad7 gene via *in vivo* DNA transfection (Nakao *et al*, 1999), and the administration of anti-TGF-β antibodies prevented cutaneous

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Abbreviations: EMSA, electrophoretic mobility shift assay; MMP, matrix metalloproteinase; TSK, tight skin mouse.

hyperplasia in a model for chronic graft *versus* host disease (McCormick *et al*, 1999). Likewise, our laboratory has found that disruption of the TGF-β gene prevented skin fibrosis in tight skin (TSK) mice (McGaha *et al*, 2001).

In this study we examined the effect of halofuginone on skin fibrosis and TGF-β signaling pathways leading to the upregulation of collagen gene expression. We found that halofuginone prevented dermal fibrosis in TSK mice. Additionally, we found that treatment of fibroblasts with 10^{-8} M halofuginone totally inhibited collagen protein and α2(I) collagen mRNA production. Furthermore, analysis of TGF-β signaling pathways demonstrated that halofuginone inhibited DNA binding and nuclear localization of Smad3 after TGF-β stimulation and that this effect is due to the inhibition of Smad3 phosphorylation. To our knowledge, this is the first compound shown to directly inhibit TGF-β-induced Smad signaling, and in this respect halofuginone may become important not only as a therapeutic agent for some connective tissue diseases but as a basic research tool in TGF-β biology.

MATERIALS AND METHODS

Cell lines and cell culture conditions Primary fibroblast lines were generated as previously described (McGaha *et al*, 2001). Briefly, 9-d-old embryos were minced and incubated for 30 min at room temperature in Hanks' balanced salt solution containing 0.25% bovine pancreatic trypsin (Mediatech, Herndon, VA). The cells were collected and incubated for three passages in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Mediatech) containing 20% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). The TSK fibroblasts were genotyped to confirm the presence of the partially duplicated fibrillin-1 gene (Kasturi *et al*, 1997). From the third passage on all primary fibroblast lines were maintained in DMEM supplemented with 100 U per ml penicillin/100 μg per ml streptomycin and 10% fetal bovine serum (Mediatech). Primary fibroblasts were used in experiments from the third to the tenth passage only.

Mice and *in vivo* halofuginone treatment Newborn or 4-wk-old male TSK and C57BL/6 mice bred at the Mount Sinai Animal Facility were used in all experiments. For the *in vivo* treatment 1 μg of halofuginone (a gift from Hoechst Marion Roussel, Puteaux, France) in 500 μl of sterile phosphate-buffered saline (PBS) or 500 μl of PBS alone was administered via intraperitoneal injection to TSK and C57BL/6 mice once every other day for 60 d. At the end of the treatment period the animals were sacrificed and skin and lung samples were removed for analysis of the effect of halofuginone on TSK syndrome.

Histologic examination Skin samples (4.0 cm^2) were removed from the dorsal side immediately below the neck in a manner that minimized stress. The samples were fixed for 16 h in buffered saline containing formalin, cut into 2–3 mm wide longitudinal strips, dehydrated, embedded in paraffin, and stained with hematoxylin–eosin according to routine histologic methods. The thickness of the skin was determined by measuring at least 12 randomly selected sections from the top of the granular layer to the junction between the dermis and subcutaneous fat on hematoxylin–eosin-stained sections as previously described (Phelps *et al*, 1993).

Cell proliferation and ^{35}S incorporation To determine the effects of halofuginone on cell growth 10^3, 5×10^3, or 10^4 fibroblasts were plated in 96-well plates. After a 4 h incubation halofuginone was added and the cells were allowed to grow at 37°C for 24, 48, or 72 h. The number of cells present was then determined using the Cell Titer Proliferation Assay (Promega, Madison, WI) according to the manufacturer's directions.

For the determination of the effect of halofuginone on protein synthesis 10^5 fibroblasts were plated in 60 mm^2 plates. After a 24 h incubation the medium was replaced with serum-free DMEM lacking the amino acid methionine ± halofuginone. Twenty-four hours after the addition of halofuginone 50 μCi of ^{35}S-labeled methionine (Amersham, Piscataway, NJ) was added for a 4 h incubation. The cells were then lyzed with RIPA buffer as described elsewhere in this section. The proteins were precipitated with trichloroacetic acid (Peterkofsky and Diegelmann, 1971), washed twice with 70% EtOH, and resuspended in 50 μl RIPA. Protein concentration was determined using the D_c Protein Assay (Biorad) and the level of ^{35}S-Met incorporation was determined with a scintillation counter.

Measurement of collagen synthesis Fibroblasts were grown to confluence in 75 cm^2 flasks, detached via trypsinization, and plated at a density of 10^5 cells per well in 24-well plates. After 24 h the fibroblasts were washed with PBS and the medium was replaced with proline-free DMEM supplemented with 3 μCi per well ^3H-proline (Amersham), 50 μg per ml ascorbic acid, 50 μg per ml β-aminopropionitrile, and halofuginone ± 10 ng per ml TGF-β. After a 24 h incubation, the medium was collected for determination of collagen synthesis and the cell numbers were counted. To measure collagen synthesis, 100 μl aliquots of medium from labeled fibroblasts were incubated at 37°C for 18 h in either the presence or absence of highly purified bacterial collagenase (Sigma, St. Louis, MO) at a concentration of 200 U per aliquot. The samples were then extensively dialyzed in microdialysis chambers (Pierce, Rockford, IL) against PBS at 4°C to remove unincorporated ^3H-proline and digested collagen. The total counts per sample were then determined utilizing a liquid scintillation counter. Biosynthetic labeling of collagen was estimated by subtracting the counts per minute of the collagenase-digested aliquot from the counts per minute of the aliquot without collagenase added.

Electrophoretic mobility shift assay (EMSA) EMSA was performed according to protocols previously described (Funk *et al*, 1992). Briefly, confluent 100 mm^2 plates of fibroblasts were incubated for 16 h with 10^{-8} M halofuginone in 3 ml of serum-free DMEM. The fibroblasts were then stimulated for 30 min with 10 ng per ml of recombinant human (rh) TGF-β1 (R&D Systems, Minneapolis, MN). At the end of this period nuclear extracts were generated as previously described by Funk *et al* (1992). Ten micrograms of nuclear extract were incubated for 10 min at room temperature in binding buffer containing 20 mM HEPES, 83.3 mM NaCl, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 10% glycerol, 1.9 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.01% Triton X-100, and 25 μg per ml poly(dI-dC) (Sigma Aldrich) in either the presence or absence of 40 ng of the unlabeled competitor oligonucleotide OCT-1 or Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA). One nanogram of radiolabeled oligonucleotide (200,000 cpm) containing the Smad3 consensus binding site CAGACA (Santa Cruz) was added and the binding reaction was incubated for 20 min at room temperature. The samples were then loaded on a 4% nondenaturing gel and electrophoresed for 3 h at 200 V. The shifted bands were visualized by autoradiographic exposure for 6 h at −70°C.

Smad3 nuclear localization Confluent TSK fibroblasts in 100 mm^2 plates were serum starved for 16 h in the presence of 10^{-8} M halofuginone. The fibroblasts were then stimulated with 10 ng per ml of rhTGF-β1 for 5 min to 1 h at 37°C. Nuclear extracts were obtained as previously described (Funk *et al*, 1992). Fifty micrograms of nuclear extract were electrophoresed on a denaturing 12% polyacrylamide gel for 2 h at 100 V. The proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was then blocked with a solution of TBST [TBS containing 0.05% Tween-20 (Sigma)] containing 3% nonfat milk overnight at 4°C. The PVDF membrane was probed with 1 μg per ml of polyclonal rabbit anti-Smad3 (Santa Cruz) for 1 h at room temperature. The membrane was then washed 3×10 min with TBST and probed with a 1:5000 dilution of polyclonal goat antirabbit IgG horseradish peroxidase conjugate (Chemicon, Temecula, CA) for 30 min at room temperature. The membrane was washed as above and the stained bands were visualized with Lumi-Light plus chemiluminescent substrate (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Western blot analysis To analyze the phosphorylation of the TGF-βRI or the PDGFR-AA confluent fibroblast culture was serum starved for 16 h and stimulated with 10 ng per ml rhPDGF-AA or TGF-β1 (R&D Systems) for 10 min or 30 min. The fibroblasts were then lyzed with RIPA buffer [TBS, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 100 μg per ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1:100 dilution of protease and phosphatase inhibitor cocktails (Sigma)] and 200 μg of cellular lysate was incubated overnight with 1 μg per ml of polyclonal rabbit α-PDGFR-AA or α-TGF-βRI (Santa Cruz). The ab–ag complexes were precipitated with 50 μl of a protein A:agarose and the proteins were separated on a 12% SDS polyacrylamide gel. Western blot analysis of the TYR-phosphorylation of the immunoprecipitated PDGFR-AA was then carried out as described above using a mouse monoclonal α-phospho-TYR ab (Cell Signaling Technology, Beverly, MA), and SER-phosphorylation of the immunoprecipitated TGF-βRI was carried out with a goat polyclonal α-phospho-SER ab (Zymed Laboratories, San Francisco, CA) according to the manufacturer's directions.

For analysis of TGF-β receptor expression and Smad2 phosphorylation confluent fibroblast cultures were treated with halofuginone for 16 h in serum-free conditions. The fibroblasts were stimulated with 10 ng per ml of TGF-β (R&D Systems) and the cells were lyzed in RIPA buffer. A hundred micrograms of protein were then electrophoresed on a 12% SDS polyacrylamide gel and analyzed via western blot as described above with 1 μg per ml of polyclonal rabbit α-phospho-Smad2 or αSmad2 (Upstate Biotechnology), α-TGF-βRII, α-TGF-βRI, or α-β-actin (Santa Cruz).

^{32}P incorporation assay As TSK fibroblasts do not express enough endogenous Smad3 to visualize ^{32}P incorporation in this assay (data not shown), we transfected the fibroblasts with a Smad3:FLAG expression construct (a gift from Maria Trojanowska). Confluent 60 mm^2 plates of TSK fibroblasts were transfected with 20 μg of the Smad3:FLAG vector using Fugene6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's directions. It was previously determined that this gave the highest levels of Smad3:FLAG expression (data not shown). The transfected fibroblasts were incubated for 48 h to allow expression of the recombinant protein. The fibroblasts were then serum starved for 16 h in the presence of 10^{-8} M halofuginone. At the end of this period the fibroblasts were incubated for 4 h at 37°C with 1 ml of phosphate-free DMEM (Mediatech) followed by an incubation with 1 mCi per ml of ^{32}P-labeled orthophosphate (Amersham) for 2 h. The cells were washed twice with PBS and placed in 1 ml of serum-free DMEM. After a 30 min incubation with 10 ng per ml rhTGF-β1 (R&D Systems) the cells were lyzed with 0.4 ml of RIPA buffer. One microgram of monoclonal anti-FLAG IgG (Sigma) was added to 250 μg of lysate in 500 μl of RIPA buffer and the solution was incubated for 16 h at 4°C. Then 50 μl of a 1:1 protein G:agarose slurry (Roche) was added to precipitate the protein–antibody complex. The immunoprecipitated samples were electrophoresed on a 12% polyacrylamide gel and transferred to PVDF. The labeled Smad3 was visualized by exposure to X-ray film for 6 h at –70°C. The membrane was then blocked with TBST + 3% nonfat milk. To stain for the presence of the FLAG peptide the membrane was incubated with a 1:2500 dilution of monoclonal anti-FLAG horseradish peroxidase conjugate (Sigma) for 1 h at room temperature. The membrane was washed and the bands were visualized as described above.

Reporter assays For the collagen promoter primary fibroblasts were grown to 90% confluence in 35 mm^2 plates in DMEM supplemented with 100 U per ml penicillin/100 μg per ml streptomycin and 10% fetal bovine serum (Mediatech). The cells were transfected with 2 μg of a luciferase reporter construct under control of the –300 to +54 collagen α2(I) collagen promoter (a gift from Dr. Beniot DeCrombrugghee, University of Texas Southwest Medical Center, Houston, TX) or the –3200 to +54 collagen α(2)I collagen promoter (a gift from Dr. Christine Power, Serono Pharmaceutical Research Center, Geneva, Switzerland) and 0.5 μg of pEGFP-C1 (Clontech, Palo Alto, CA) using Fugene6 transfection reagent (Roche) according to the manufacturer's directions. Six hours after transfection the medium was replaced with fresh serum-free DMEM alone or containing various concentrations of halofuginone alone or in the presence of 10 ng per ml TGF-β. Forty-eight hours after the addition the samples were assayed for the levels of luciferase activity using the Bright Glow luciferase assay (Promega) according to the manufacturer's directions.

In one set of experiments fibroblasts were grown to 90% confluence in 35 mm^2 plates. The cells were then transfected with 2 μg of a CAT reporter construct under control of three tandem repeats of the A + B box of the α2(I) collagen promoter (a gift from Dr. F. Ramirez, Mount Sinai School of Medicine, New York, NY) and 0.5 μg of pEGFP-C1 (Clontech) using the same reagent described above. Six hours after the transfection the cells were serum starved for 12 h in DMEM alone or containing
10^{-8} M halofuginone. The fibroblasts were stimulated with 10 ng per ml rhTGF-β1 for 48 h and the CAT protein level was determined via enzyme-linked immunosorbent assay according to the manufacturer's directions (Roche).

Northern blot analysis of α2(I) collagen expression Confluent 100 mm^2 plates of TSK fibroblasts were serum starved for 16 h in DMEM. The cells were then placed in fresh DMEM containing 10^{-8} M halofuginone. Cytoplasmic RNA was extracted at various time points using the RNeasy RNA isolation kit (Quiagen, Santa Clarita, CA) according to the manufacturer's directions. Five micrograms of RNA was denatured in 62% formamide, 2 × MESA buffer (0.4 M MOPS, 0.1 M NaOAc, 10 mM EDTA), and 25% formaldehyde by heating at 65°C for 5 min. The RNA was electrophoresed on a 1% agarose gel and transferred to a nitrocellulose filter for 16 h in 2 × sodium citrate/chloride buffer (300 mM NaCl, 40 mM sodium citrate). The RNA was crosslinked to the membrane in an ultraviolet oven (Stratagene, La Jolla, CA) and probed for the presence of α2(I) collagen and β-actin mRNA as previously described (Phelps *et al*, 1993).

In one set of experiments the cells were pretreated with 100 ng per ml cyclohexamide (Sigma) for 2 h prior to the addition of halofuginone to block protein synthesis. This concentration was experimentally found to inhibit protein synthesis without the induction of significant levels of apoptosis (data not shown).

Statistical analysis Statistical significance analysis was performed using the unpaired Student's *t* test. Differences between groups were considered to be statistically significant when the p-value was 0.05.

RESULTS

Halofuginone inhibits cutaneous hyperplasia in TSK syndrome To assess the *in vivo* efficacy of halofuginone on fibrosis we used the TSK mouse model for scleroderma. The TSK mouse is an attractive model to study human fibrotic disease as the animals spontaneously develop fibrosis (Green *et al*, 1976) unlike other fibrosis models such as murine cGVHD and bleomycin-induced fibrosis.

We administered 1 μg of halofuginone every other day for 60 d to newborn and 1-mo-old TSK mice via intraperitoneal injection. This protocol was adopted as it was previously shown to prevent skin sclerosis in the murine cGVHD model (Levy-Schaffer *et al*, 1996). At the end of the treatment period skin samples were taken for measurements of dermal thickness. Histologic analysis demonstrated that halofuginone significantly affects skin fibrosis in TSK mice (**Fig 1**, **Table I**). The C57BL/6 mice had a dermal thickness of 132 ± 42 μm whereas TSK mice injected with PBS had a dermis that was almost twice as thick (228 ± 72 μm) (**Fig 1**, *panels 1*, *2*, **Table I**). The administration of halofuginone significantly reduced the thickness of the dermis in both adults ($p = 0.022$) and neonates ($p = 0.01$) compared to TSK mice injected with saline (155 ± 32 μm and 113 ± 15 μm, respectively) (**Fig 1**, *panels 3*, *4*, **Table I**). Previously we have shown that decreased skin thickness was paralleled by a decreased number of dermal cells expressing the type-I collagen transcript (McGaha *et al*, 2001).

The effect of halofuginone on collagen synthesis by fibroblasts It has been reported that halofuginone can inhibit *in vitro* collagen type I production at concentrations as low as 10^{-10} M (Granot *et al*, 1993). The effect of halofuginone on collagen synthesis was assessed on primary culture fibroblasts obtained from 9-d-old embryos. The level of collagen protein present in the culture medium was assayed using a modified version of the protocol by Peterkofsky and Diegelmann (1971). It was observed that the C57BL/6 fibroblasts seemed resistant to the effects of halofuginone-mediated inhibition with no significant decrease in collagen production observed until the fibroblasts were incubated with 10^{-8} M halofuginone (**Fig 2**). These results are in agreement with previous studies, which found that fibroblasts from nonfibrotic skin were more resistant to the effects of halofuginone than the fibroblasts from animals with skin fibrosis (Levy-Schaffer *et al*, 1996). It is noteworthy that halofuginone concentrations used in the experiment did not affect fibroblast viability (data not shown).

Decreased synthesis of collagen by halofuginone can be due to several factors: (i) the inhibition of proliferation of fibroblasts; (ii) the inhibition of global protein synthesis; or (iii) a selective inhibitory effect on the synthesis of type-I collagen. To study the effect of halofuginone on proliferation, fibroblasts were incubated with various concentrations of halofuginone over a 72 h time course. The data depicted in **Fig 3**(***A***) clearly show that, at concentrations up to 10^{-8} M, halofuginone had no effect on fibroblast proliferation. Similarly, as can be seen in **Fig 3**(***B***), no inhibition of overall protein synthesis was observed upon exposure to 10^{-8}–10^{-10} M halofuginone. Therefore, the halofuginone concentrations used in the *in vitro* studies were apparently nontoxic

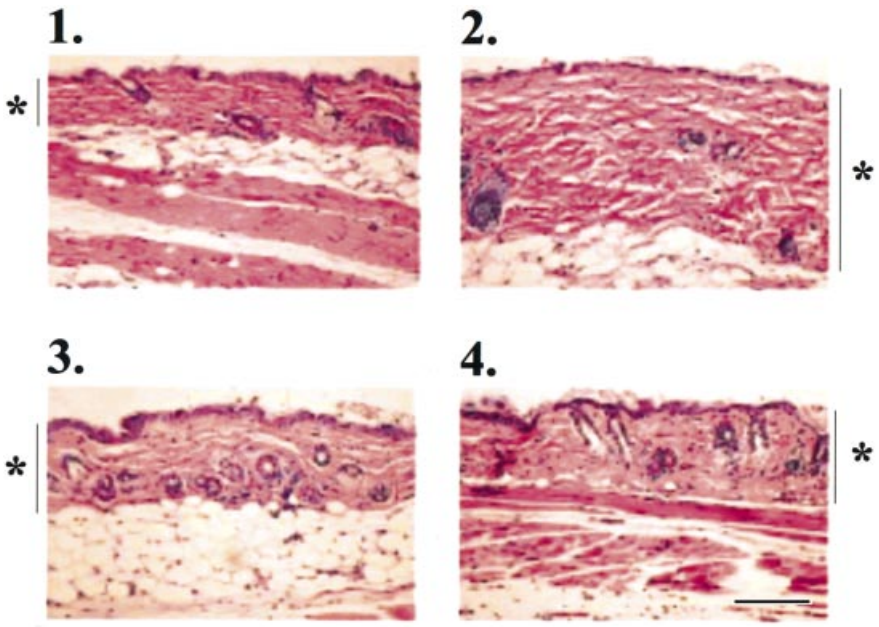

Figure 1. Effect of halofuginone on dermal fibrosis. Representative histologic skin sections from C57BL/6 mice (*panel 1*), TSK mice (*panel 2*), adult TSK mice given halofuginone intraperitoneally (*panel 3*), and neonatal TSK mice given halofuginone intraperitoneally (*panel 4*). Brackets highlight the dermal area of the sections. All sections were paraffin embedded and stained with hematoxylin–eosin. *Scale bar*: 100 μm.

to the cells and blocked collagen protein synthesis in a specific manner.

The effect of halofuginone on the level of the collagen type I message was studied by northern blot analysis of α2(I) collagen and β-actin mRNAs over a 24 h time course after the addition of 10^{-8} M halofuginone. There was no early effect during the assay (0–4 h, colα2(1):β-actin ratio 1.01, 0.92, and 1.01, respectively); however, 8 h after the addition of halofuginone there was a drastic reduction in the amount of α2(I) collagen mRNA and total inhibition by 12 h (colα2(1):β-actin ratio 0.29 and 0.008, respectively) (**Fig 4*A***).

The kinetics of mRNA inhibition by halofuginone suggested that protein synthesis may be necessary to block collagen type I synthesis. To investigate this possibility, TSK fibroblasts were preincubated with 100 ng per ml cyclohexamide for 2 h to inhibit protein translation prior to the addition of 10^{-8} M halofuginone. The fibroblasts were then assayed at 8, 12, and 24 h after the addition of halofuginone for the presence of α2(I) collagen mRNA. We found that cyclohexamide had no effect on the kinetics of inhibition with a partial inhibition of α2(I) collagen mRNA at 8 h and a total inhibition by 24 h (colα2(1):β-actin ratio 0.34 and 0.03, respectively) (**Fig 4*B***). Cyclohexamide alone had no effect on the production of collagen type I mRNA (data not shown) demonstrating that the inhibition was specific to the presence of halofuginone and was due to a translationally independent mechanism.

As we established that halofuginone affected the transcript level of the COL1A2 gene we next studied the effect of halofuginone on collagen promoter activity of fibroblasts transfected with two luciferase reporter constructs under control of the –300 to +54 α2(I) collagen promoter (**Fig 5*A***) or the –3200 to +54 α2(I) collagen promoter (**Fig 5*B***), or a CAT reporter construct under the control of three tandem repeats of the α2(I) collagen A + B box (**Fig 5*C***). The A + B box is a small (–330 to –250 bp) region of the α2(I) collagen promoter that contains several transcription factor binding sites and has been shown to be essential for TGF-β responsiveness of the COL1A2 gene (Zhang *et al*, 2000). In these experiments 6 h after transfection the cells were treated with increasing concentrations of halofuginone and assayed for luciferase activity or CAT protein concentration in the cultures. There was a highly significant decrease in promotor activity upon treatment with 10^{-8} M halofuginone (**Fig 5**). In addition, there was a high degree of similarity between the luciferase activity and CAT protein levels in the treated fibroblast and collagen production with a lack of inhibition until a halofuginone concentration of 10^{-8} M

Table I. The effect of halofuginone on the development of cutaneous hyperplasia

Mouse strain	N	Treatment	Skin thickness	p value[a]
C57BL/6	6	PBS	132 ± 42 μm	–
TSK	13	PBS	228 ± 72 μm	0.02
TSK (adult)	4	HF	155 ± 32 μm	0.38 (0.02)
TSK (neonate)	5	HF	113 ± 14 μm	0.37 (0.01)

[a]p values compared various experimental groups to C57BL/6 (and untreated TSK) mice.

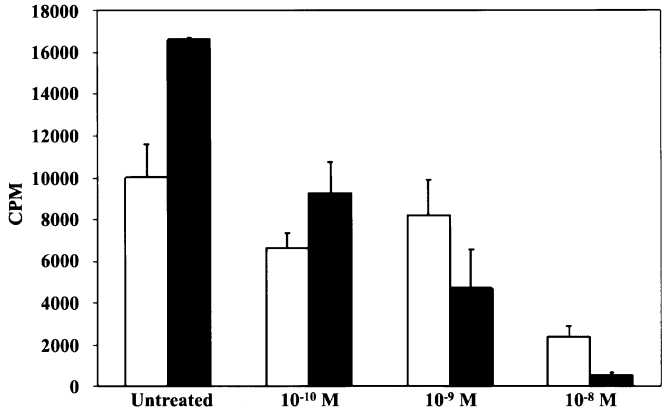

Figure 2. The effect of halofuginone on collagen protein production in normal (C57BL/6) and TSK fibroblasts. Primary fibroblast lines were incubated in serum-free medium in the presence of increasing concentrations of halofuginone for 48 h. The amount of secreted collagen protein was then assayed as described in *Materials* and *Methods*. *Y* axis values represent CMP of collagenase-digestible protein per 10^5 fibroblasts. For this figure open bars represent C57BL/6 fibroblasts and filled bars represent TSK fibroblasts. Bars represent the mean value for triplicate samples ± SD. This experiment was repeated four times with similar results.

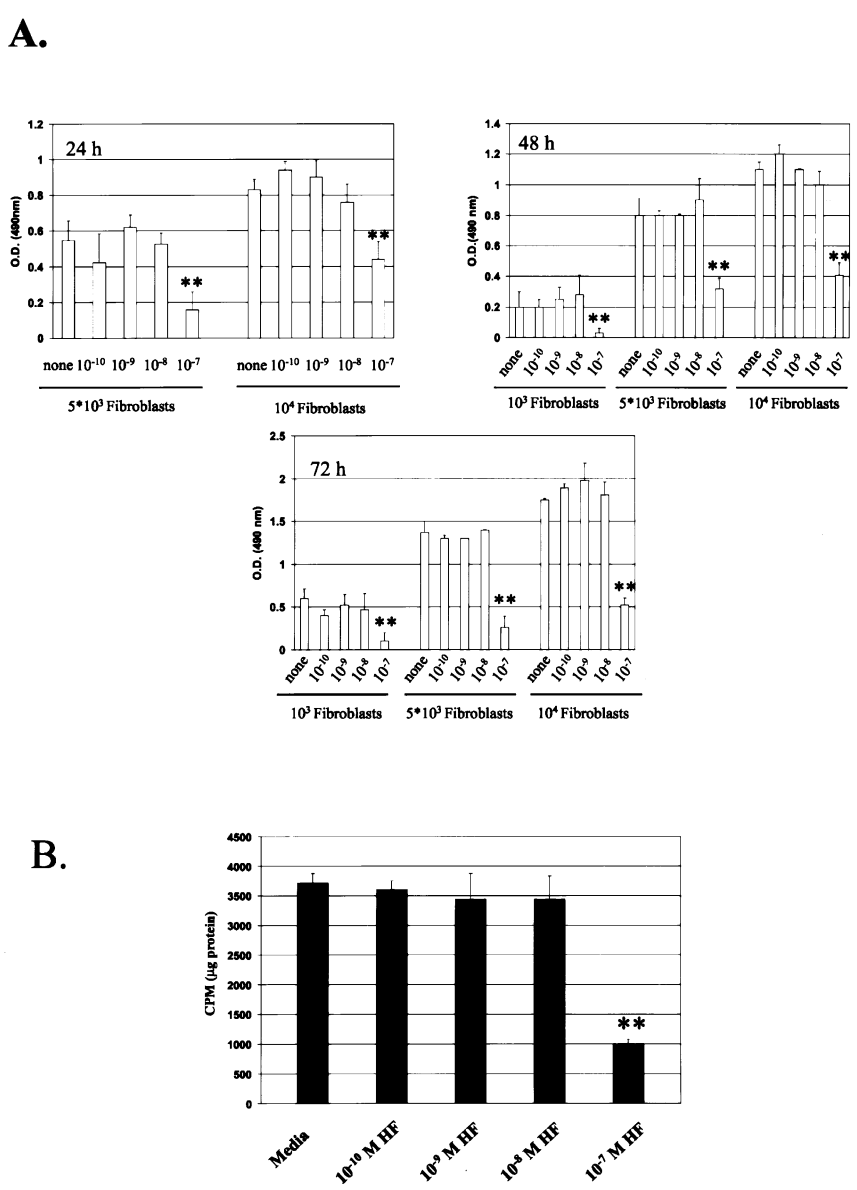

Figure 3. Effect of halofuginone on fibroblast proliferation and protein production. (*A*) The effect of halofuginone on cellular growth. Fibroblasts plated in 96-well plates were incubated for 24, 48, and 72 h with increasing concentrations of halofuginone. At the end of the times indicated in the figure the cells were assayed for proliferation as described in *Materials and Methods*. Bars represent the mean absorbance at 490 nm for triplicate wells ± SD. This experiment was repeated twice with similar results. (*B*) The effect of halofuginone on protein synthesis. Fibroblasts were incubated for 24 h in increasing concentrations of halofuginone and its effect on overall protein synthesis was determined via incorporation of ^{35}S-methionine. The values represent the mean cpm per μg of precipitated protein for triplicate samples ± SD. This experiment was repeated three times with similar results. For both (*A*) and (*B*) two asterisks represent a $p \leq 0.02$ as determined by the unpaired Student's *t* test.

was achieved. Thus, our results show a paralleled inhibition of message levels, promoter activity, and collagen synthesis by fibroblasts upon exposure to a halofuginone concentration of 10^{-8} M. It is therefore likely that halofuginone inhibits cutaneous hyperplasia by reducing the level of collagen message via an inhibitory effect on promoter activity of the type-1 collagen genes.

The effect of halofuginone on TGF-β-induced collagen gene expression As we found that the halofuginone inhibited the synthesis of collagen by fibroblasts, in further experiments we studied its effect on TGF-β-induced collagen synthesis because it has been shown that TGF-β plays a major role in the induction of fibrosis (McCormick *et al*, 1999; Nakao *et al*, 1999; McGaha *et al*, 2001). We evaluated the effect of halofuginone on TGF-β-induced upregulation of collagen synthesis. In this experiment fibroblasts were treated with 10^{-8} M halofuginone for 16 h prior to the addition of 10 ng per ml of TGF-β. Twenty-four hours after stimulation with TGF-β, the amount of collagen in fibroblast cultures was assayed as described above. In the absence of halofuginone we observed an approximately 3-fold induction in the levels of extracellular collagen after stimulation with TGF-β (**Fig 6*A***), which is in agreement with the findings from previous studies. In contrast, pretreatment with halofuginone decreased the basal levels of collagen protein synthesis in a manner similar to that seen in **Fig 2** and blocked TGF-β-induced upregulation of collagen production fibroblasts (**Fig 6*A***), demonstrating that halofuginone blocks TGF-β induction of collagen synthesis. A similar inhibitory effect was observed on promoter activity in fibroblasts transfected with the reporter constructs (**Fig 6*B***, ***C***). In cultures transfected with the −3200 to +54 bp α2(I) collagen promoter construct there was no change in luciferase activity when the fibroblasts were treated with 10^{-10} halofuginone. Unlike the effect on basal activity, however, we observed a 50% decrease in luciferase activity when the transfected fibroblasts were treated with 10^{-9} M halofuginone (2432 ± 400 RLU *vs* 1137 ± 256 RLU, respectively, **Fig 6*B***), and a 7-fold decrease in the luciferase levels after treatment with 10^{-8} M halofuginone (2432 ± 400 RLU and 352 ± 85 RLU, respectively, **Fig 6*B***). Therefore it appears that fibroblasts are more sensitive to the effects of halofuginone after stimulation with a pro-fibrotic agonist such as TGF-β.

Although this suggests that halofuginone can directly block TGF-β responsiveness of the COL1A2 gene it is possible that these results illustrate an overall inhibitory effect and the lack of responsiveness to TGF-β is due to a general suppression and not to specific interference with TGF-β stimulation. To test this hypothesis we transfected fibroblasts with the CAT reporter

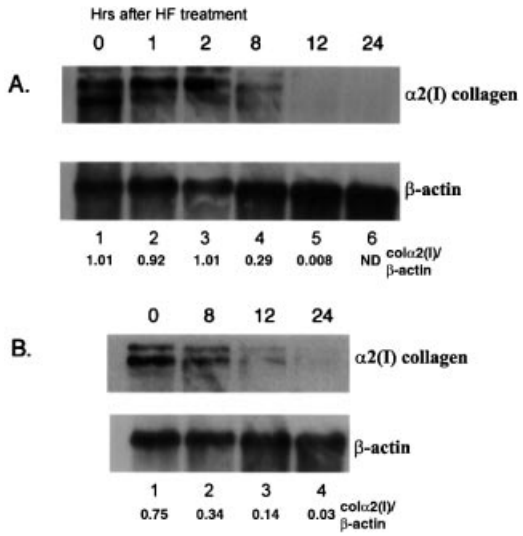

Figure 4. The effect of halofuginone on collagen type I mRNA levels in fibroblasts. Primary TSK fibroblasts were incubated with 10^{-8} M halofuginone either in the absence (*A*) or presence (*B*) of 100 ng per ml cyclohexamide. RNA was extracted over a 24 h period and 5 μg were probed via northern blotting assay for the presence of α2(I) collagen and β-actin mRNA. Relative intensities of the bands in (*A*) and (*B*) were determined on digital image files using Quantity One software (Biorad) according to the manufacturer's instructions. The experiment was repeated twice with similar results.

construct under the control of the $(A + B)_3$ box of the α2(I) collagen promoter. This construct, as stated earlier, contains three tandem repeats of the COL1A2 A + B box. This region is known to be critical for TGF-β inducibility of the COL1A2 gene and contains a Smad3 binding site. Therefore, inhibition of this construct is more likely to reflect a direct effect on the TGF-β response. We found that like the results with the luciferase construct there was a dose-dependent reduction in CAT protein levels when fibroblasts were treated with halofuginone (**Fig 6*C***, a 1.5-fold decrease after 10^{-10} M halofuginone pretreatment and a 3.5-fold decrease after treatment with 10^{-9} M or 10^{-8} M halofuginone). So it appears that halofuginone blocks TGF-β-induced upregulation of the A + B box suggesting that halofuginone is specifically blocking the TGF-β induction of the COL1A2 gene.

Lack of effect of halofuginone on the expression of TGF-β receptors I or II on fibroblasts We wished to determine if the decrease in responsiveness to TGF-β was due to an inhibitory effect of halofuginone on the level of expression of the type I and II TGF-β receptors. Western blot analysis of lysates from fibroblasts showed that treatment with 10^{-8} M halofuginone had no effect on expression of either TGF-βR1 or TGF-βRII (**Fig 7*A***) demonstrating that halofuginone blocks TGF-β-induced upregulation of collagen synthesis by some mechanism other than receptor downregulation.

We further evaluated the effect of halofuginone on the activation of the TGF-βR complex. To accomplish this fibroblasts were pretreated with 10^{-8} M halofuginone for 24 h and then stimulated with 10 ng per ml TGF-β for 10 or 30 min. Immunoprecipation and western blot analysis of the TGF-βRI showed that 10^{-8} M halofuginone had no effect on the kinetics or level of serine phosphorylation (**Fig 7*B***) demonstrating that halofuginone most probably does not affect TGF-β-mediated upregulation of collagen synthesis at the receptor level. Similarly, no effect was observed on the expression or phosphorylation of the PDGF-RAA after stimulation with its ligand, PDGF-AA, another growth factor for fibroblasts (**Fig 7*C***).

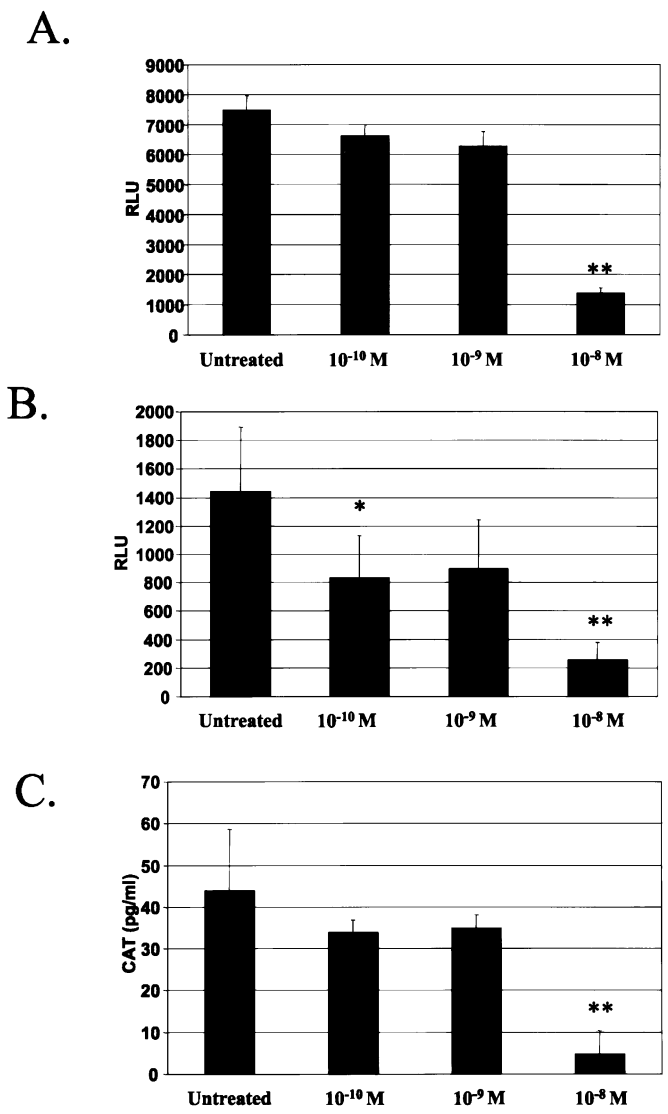

Figure 5. Transcriptional activity of the α2(I) collagen promoter in the presence of halofuginone. Primary fibroblasts were transiently transfected with a luciferase reporter construct under control of the –300 to +54 bp α2(I) collagen promoter (*A*), the –3200 to +54 bp α2(I) collagen promoter (*B*), or three tandem repeats of the A + B box of the α2(I) collagen promoter controlling expression of a CAT gene. Forty-eight hours after the addition of halofuginone cells were assayed for luciferase activity or CAT protein levels as described in *Materials* and *Methods*. Values were normalized for transfection efficiency and the bars represent the mean value for triplicate samples ± SD. *$p \leq 0.05$, **$p \leq 0.02$ as determined by the unpaired Student's *t* test. These experiments were repeated four times with similar results.

The effect of halofuginone on the TGF-β signaling pathway The results described above clearly demonstrate that halofuginone exhibited a strong inhibitory effect on TGF-β-induced collagen synthesis without affecting the expression or activation of the TGF-β receptor. This suggests that the effect of halofuginone may be related to intracellular events of TGF-β signaling. Smad2 and Smad3 are considered to be the primary signaling molecules involved in TGF-β signal transduction; however, Smad3 has been suggested to be the primary transcription factor associated with the regulation of type-I collagen protein production by TGF-β (Chen *et al*, 1999, 2000; Zhang *et al*, 2000). Therefore, we investigated the effect of halofuginone on the activation of Smad3 and Smad2 in response to TGF-β stimulation.

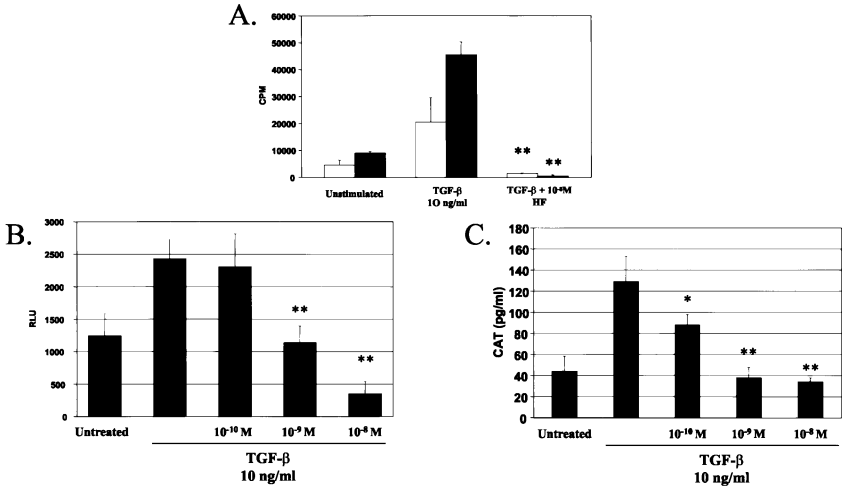

Figure 6. The effect of halofuginone on TGF-β-induced collagen protein synthesis and promoter activity. (*A*) Primary TSK and C57BL/6 fibroblast cultures were incubated in serum-free medium in the presence or absence of 10^{-8} M halofuginone and 10 ng per ml rhTGF-β1 for 24 h. Extracellular collagen synthesis was then assayed as described in *Materials and Methods*. *Y* axis values represent CMP of collagenase-digestible protein per 10^5 fibroblasts. For this figure open bars represent C57BL/6 fibroblasts and filled bars represent TSK fibroblasts. (*B*) Fibroblast cultures transiently transfected with the –3200 to +54 bp α2(I) collagen promoter reporter construct were incubated in serum-free medium in the presence or absence of 10^{-8} M halofuginone and 10 ng per ml rhTGF-β1 for 48 h. The cells were then assayed for luciferase activity. (*C*) Fibroblast cultures transiently transfected with the col α2(I) $(A + B)_3$ CAT reporter construct were incubated in serum-free medium in the presence or absence of 10^{-8} M halofuginone and 10 ng per ml rhTGF-β1 for 48 h. The cells were then assayed for CAT protein levels as described in *Materials and Methods*. For all graphs the bars represent the mean value for triplicate samples ± SD. *p ≤ 0.05, **p ≤ 0.01. All experiments were repeated at least three times with similar results.

We first examined the nuclear translocation of Smad3. As can be seen in **Fig 8**(***A***) nuclear extracts from fibroblasts exposed to TGF-β showed a rapid translocation within 5 min of Smad3 to the nucleus, reaching a maximum 30 min subsequent to TGF-β stimulation. In contrast, pretreatment with halofuginone inhibited the translocation of Smad3 to the nucleus. As the nuclear translocation of Smad3 requires the phosphorylation of the protein, we also studied the effect of halofuginone on phosphorylation of Smad3.

To this end the fibroblasts were transfected with an expression vector encoding FLAG-tagged Smad3. The transfected fibroblasts were then incubated with ^{32}P-orthophosphate to radio-label any activated Smad3. The phosphorylation was increased upon TGF-β stimulation demonstrating the TGF-β inducibility of Smad3 phosphorylation (**Fig 8*B***, *lane 2*). The presence of halofuginone, however, inhibited the upregulation of phosphorylation after stimulation (**Fig 8*B***, *lanes 3*, *4*). This indicates that halofuginone directly inhibits Smad3 phosphorylation thus inhibiting TGF-β activity and fibrosis.

Further we studied whether the effect of halofuginone on TGF-β signaling was specific to Smad3 or whether halofuginone has a more pleiotrophic effect on TGF-β signaling. To address this question TSK fibroblasts were incubated with 10^{-8} M halofuginone for 16 h and then stimulated with TGF-β. The fibroblasts were then analyzed via western blot for the phosphorylation of Smad2. The data in **Fig 8**(***C***) show that 10^{-8} M halofuginone had no effect on the expression or phosphorylation of Smad2 in response to TGF-β stimulation. This suggests that halofuginone has a specific effect on Smad3 activation.

Finally we performed EMSA analysis of nuclear lysates from fibroblasts using an oligonucleotide containing a Smad3 consensus binding site in order to examine the effect of halofuginone on Smad3–DNA interactions. We observed a basal level of DNA binding resulting in major and minor shifted bands in unstimulated fibroblasts (**Fig 9**, *arrows*). The addition of TGF-β greatly increased the intensity of the shifted bands demonstrating TGF-β inducibility of DNA binding by Smad3. Strikingly, the pretreatment of the fibroblasts with halofuginone abrogated DNA binding in the basal state and prevented the upregulation of DNA binding after TGF-β stimulation (**Fig 9**). These data strongly suggested that halofuginone inhibits TGF-β-induced Smad3 activation.

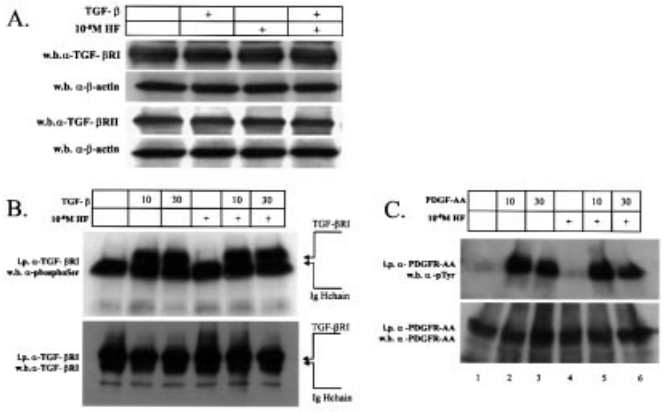

Figure 7. The effect of halofuginone on TGF-β receptor protein levels and phosphorylation. (*A*) Fibroblast cultures were incubated in serum-free medium in the presence or absence of 10^{-8} M halofuginone and 10 ng per ml rhTGF-β1 for 16 h. Cellular lysates were generated and probed via western blot for the presence of TGF-βRI, TGF-βRII, or β-actin as described in *Materials and Methods*. (*B*) Fibroblast cultures were incubated with 10^{-8} M halofuginone for 16 h and stimulated for 10 or 30 min with 10 ng per ml rhTGF-β. The TGF-βRI was then immunoprecipitated using an α-TGF-βRI polyclonal antibody. The immunoprecipitated TGF-βRI was probed via western blot for the presence of phosphorylated serine residues as described earlier. (*C*) Fibroblast cultures were incubated with 10^{-8} M halofuginone for 16 h and stimulated for 10 or 30 min with 10 ng per ml rhPDGF-AA. The PDGF-AA receptor was then immunoprecipitated using an α-PDGF receptor polyclonal antibody. The immunoprecipitated PDGF receptor was probed via western blot for the presence of phosphorylated tyrosine residues as described earlier. These experiments were repeated twice with similar results.

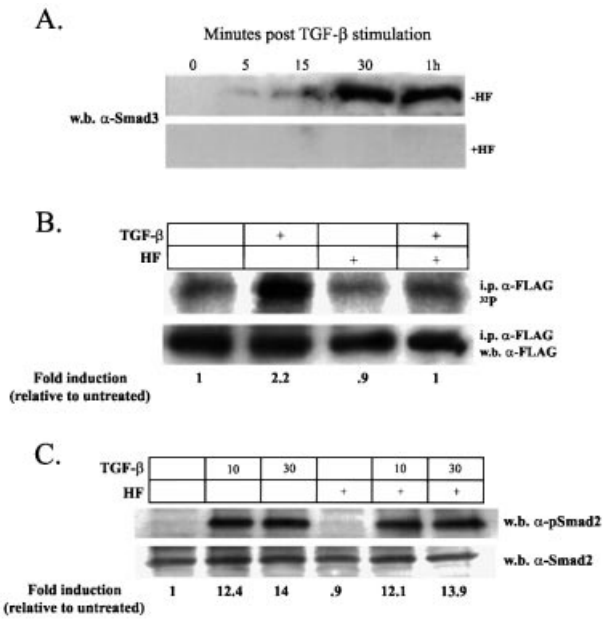

Figure 8. The effect of halofuginone on the activation of Smad3 and Smad2. (*A*) Nuclear extracts from fibroblasts were probed via immunoblot for the presence of Smad3 over a 1 h time course following stimulation with 10 ng per ml rhTGF-β1 in the absence (*top panel*) or presence (*bottom panel*) of 10^{-8} M halofuginone. (*B*) Fibroblasts transfected with a Smad3:FLAG expression vector were labeled with ^{32}P-orthophosphate and stimulated with 10 ng per ml rhTGF-β1 for 30 min after a 16 h pretreatment with medium or 10^{-8} M halofuginone. The Smad3 was immunoprecipitated with an α-FLAG antibody and the level of ^{32}P incorporation was determined via autoradiography. (*C*) Fibroblast cultures were incubated with 10^{-8} M halofuginone for 16 h. The fibroblasts were then stimulated with 10 ng per ml of rhTGF-β1 for 10 or 30 min. The level of phosphorylation of Smad2 was then determined via western blot as described in *Materials and Methods*. Relative intensities of the bands in (*B*) and (*C*) were determined on digital image files using Quantity One software (Biorad) according to the manufacturer's instructions. These experiments were repeated twice with similar results.

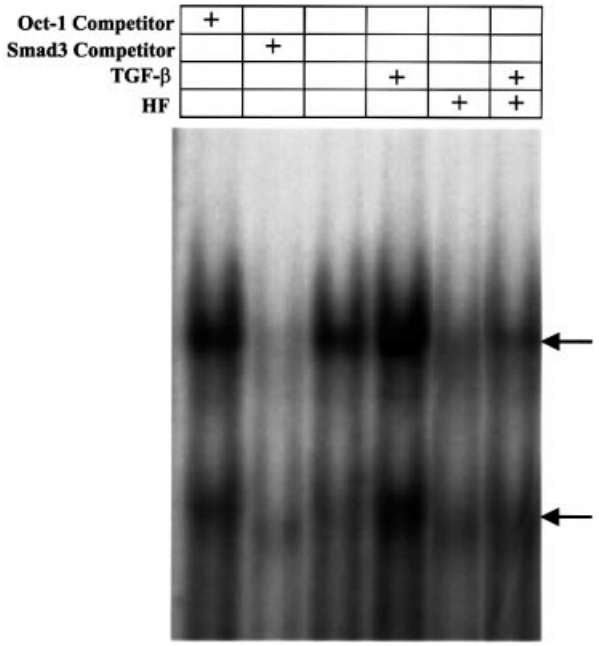

Figure 9. TGF-β inducibility of Smad3–DNA interactions in the presence of halofuginone. Fibroblasts were stimulated for 30 min with 10 ng per ml rhTGF-β1 after pretreatment for 16 h with medium (*lane 4*) or 10^{-8} M halofuginone (*lane 6*). Nuclear lysates were then generated and assayed for DNA binding ability via EMSA as described in *Materials and Methods*. Specificity of the shifted bands (*arrows*) was demonstrated with specific (*lane 2*) and nonspecific (*lane 1*) oligonucleotide competitors. This experiment was repeated twice with similar results.

DISCUSSION

Halofuginone is a drug that has been shown to have antifibrotic activity. Although it has been shown to inhibit collagen synthesis by fibroblasts and fibrosis in several different experimental models, no information on activity has been forthcoming to explain the effector mechanisms. Therefore, in this study we had two goals: (i) to examine the *in vivo* efficacy of halofuginone in the treatment of a spontaneous dermal fibrosis animal model and (ii) to investigate molecular mechanisms responsible for the inhibition of collagen synthesis by halofuginone.

For the *in vivo* studies, we used the TSK mouse, which spontaneously develops a scleroderma-like syndrome consisting of cutaneous hyperplasia, lung emphysema, heart abnormalities, and an autoantibody profile similar to that seen in scleroderma (Green *et al*, 1976; Rossi *et al*, 1984; Bona and Rothfield, 1994). This syndrome is due to a mutation in the fibrillin-1 gene (Siracusa *et al*, 1996), which is an important component of the elastic microfibrils of connective tissue (Sakai *et al*, 1996). The TSK mice were given halofuginone for 2 mo intraperitoneally and assessed for the development of TSK syndrome. We found that halofuginone significantly decreased the skin thickness in TSK mice injected as adults or neonates indicating an efficacy in treating fibrotic conditions such as cutaneous hyperplasia associated with scleroderma. The decrease of fibrosis subsequent to halofuginone treatment has been found to correlate with decreased hydroxyproline content in the skin and numbers of cells expressing type-1 collagen mRNA (McGaha *et al*, 2001) suggesting that halofuginone inhibits dermal fibrosis by affecting type-1 collagen message levels.

In agreement with reported findings (Granot *et al*, 1993) we found that halofuginone inhibits collagen type I synthesis in fibroblast cultures. It is noteworthy that the inhibitory effect was stronger in TSK than normal fibroblasts. This suggests that there is some differential sensitivity to halofuginone via unknown mechanisms probably relating to the level of activity of the fibroblasts, genetic factors, or both. Decreased synthesis of collagen by fibroblasts upon exposure to halofuginone is not related to toxicity as 10^{-8} M halofuginone did not affect viability (data not shown), growth, or overall protein synthesis by fibroblasts. These findings strongly suggest that halofuginone is a selective inhibitor of the collagen type I genes. This concept is supported by our data demonstrating that the decrease of collagen synthesis is paralleled by a decrease in the level of type-I collagen transcript detectable by northern blot.

Although it has been demonstrated that halofuginone inhibits collagen type I mRNA synthesis, no available data exist on the molecular mechanism of this process. Our data showed a definite decrease in α2(I) collagen promoter activity mirroring the decrease in the level of type-I collagen transcript and in collagen production. In the promoter assay we initially used a construct containing the –300 to +54 bp α2(I) collagen promoter. Utilizing this construct we saw a strong inhibition of the promoter when the fibroblasts were treated with 10^{-8} M halofuginone similar to what was observed with protein synthesis; however, there was concern that this may not reflect the true biologic response as it has been reported that the type-1 collagen promoters contain many upstream regulatory sequences (Philips *et al*, 1995; Chung *et al*, 1996; Chen *et al*, 1999; Vergeer *et al*, 2000; Antoniv *et al*, 2001). Therefore we utilized a luciferase construct containing a larger portion of the α2(I) collagen promoter (–3200 to +54 bp). With this construct we saw a similar pattern of inhibition so it appears that halofuginone

inhibits α2(I) collagen synthesis at least in part by reducing the activity of the α2(I) collagen promoter.

Halofuginone was reported to decrease collagen type I mRNA 2 h after the addition of halofuginone (Halevy *et al*, 1996). We did not observe a significant decrease in α2(I) collagen mRNA, however, until 8 h after the addition of halofuginone. The lapse in time between treatment and inhibition of collagen type 1 mRNA indicated that protein synthesis was necessary for inhibition, and some preliminary data supported this notion (data not shown). Conversely, inhibition occurred with a similar time course in the presence of cyclohexamide. Therefore it is likely that halofuginone may directly affect transcription factors essential in collagen type I synthesis.

We further chose to investigate the effect halofuginone had on TGF-β induction of collagen synthesis. This was based on the key role TGF-β is believed to play in fibrosis and collagen synthesis. TGF-β has consistently been found to be upregulated during the onset of fibrotic disease in humans as well as in experimental models. In addition, recent studies have shown that the A + B box of the human COL1A2 gene contains a Tbre (TGF-β responsive element) and a binding site for Smad3/4. Furthermore, it appears that this site is critical for TGF-β responsiveness of the COL1A2 gene and perhaps for overall activity of the type-I collagen promoters (Chen *et al*, 1999, 2000; Zhang *et al*, 2000).

TGF-β is a member of an evolutionarily conserved family of growth factors responsible for regulation of proliferation, differentiation, and numerous other cellular activities including extracellular matrix production (reviewed in Massague, 1998). The receptor for TGF-β consists of a heteromeric complex containing four type I receptors, four type II receptors, and one type III receptor (De Crescenzo *et al*, 2001). Signaling via this activated complex, however, is believed to occur through the action of the type I and II receptors. This assembly of the activated complex allows the phosphorylation of the type I receptor by the constitutively active Ser/Thr-kinase activity of the type II receptor. The receptor phosphorylation allows the subsequent association and activation (via serine residue phosphorylation) of the TGF-β-inducible signaling molecules Smad2/3 in the carboxy-terminal SSXS domain (Nakao *et al*, 1997). The activated Smad2/3 then associates with a cofactor used by several members of the TGF-β superfamily (Smad4/DPC4) and is translocated to the nucleus where the complex binds to a CAGACA promoter sequence and transactivates TGF-β-inducible genes through a poorly understood mechanism (Zhang *et al*, 1998).

We found that TGF-β stimulated a 2–5-fold increase in the level of collagen protein and promoter activity in fibroblast cultures. This is in agreement with previous studies, which demonstrated that TGF-β increases the level of type-I collagen message (Ignotz *et al*, 1987; Raghow *et al*, 1987). Interestingly, treatment with 10^{-8} M halofuginone inhibited the elevation of either protein or promoter activity after stimulation with TGF-β. Therefore it appears that the inhibitory effect cannot be overcome by pro-fibrotic stimuli. It is also of interest to note that the fibroblasts seemed to be more responsive to the effects of halofuginone when they were stimulated with TGF-β resulting in a dose-dependent inhibition.

To see if this inhibitory effect was specific to TGF-β-mediated stimulation we used a CAT reporter construct under control of the A + B box of the human COL1A2 promoter. The results showed an even greater sensitivity to halofuginone with a significant inhibition of promoter activity occurring at 10^{-10} M and reaching maximum inhibition of CAT protein levels at 10^{-9} M halofuginone. These data further illustrate the ability of halofuginone to inhibit the pro-fibrotic effects of TGF-β and suggest that it may elicit its effect, at least in part, by inhibition of a TGF-β-inducible element.

Western blot analysis of the TGF-β type I and II receptors showed that halofuginone had no effect on their expression after a 24 h treatment so any effect halofuginone has on TGF-β signaling is not due to receptor downregulation. It is also not likely that halofuginone is affecting the activation or enzymatic activity of the receptor complex as there was no change in type I receptor or Smad2 phosphorylation.

Analysis of Smad3 induction upon TGF-β stimulation showed that halofuginone inhibited the phosphorylation and subsequent transcriptional activity of Smad3. How this inhibition occurs is not clear. Halofuginone probably does not inhibit the interaction of Smad3 with the activated receptor complex as any mechanism that involved blocking Smad3–TGF-β receptor interactions would also most probably block Smad2 phosphorylation due to their homology in a C-terminal domain called the Mad-homology 2 (MH2) domain, which is responsible for Smad–TGF-β receptor interactions (Wu *et al*, 2000). A more likely explanation is that a post-phosphorylation event is responsible for the deactivation of Smad3. Smad2 and Smad3 differ most in an N-terminal region called the MH1 domain. This difference confers DNA binding ability on Smad3 but not Smad2 (Shi *et al*, 1998) and determines the interactions of these Smads with other transcription factors (Labbe *et al*, 1998; Raman and Chen, 2000). Therefore halofuginone may be affecting some factor that associates with Smad3 and not Smad2 leading to deactivation of the protein.

The analysis of PDGFR-AA receptor activation after incubation with halofuginone confirmed that halofuginone-mediated inhibition of signal transduction is specific to TGF-β signaling and that halofuginone is not a general kinase inhibitor. In future studies we will investigate the ability of halofuginone to block other signaling pathways induced by TGF-β stimulation, such as the MAPK pathway, to gain a better understanding of how this drug promotes such specific inhibition of collagen.

In conclusion, we demonstrated that halofuginone inhibits dermal fibrosis *in vivo* via a translationally independent mechanism. Furthermore, this inhibition appears to be the result of interference in TGF-β-induced Smad activation and for the first time demonstrates an antagonist of TGF-β signal transduction. This suggests that halofuginone is not only a tool to combat fibrotic disease, but potentially possesses great value as a research tool in many areas of cell biology.

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