Connective tissue growth factor expression and induction by transforming growth factor-β is abrogated by simvastatin via a Rho signaling mechanism

Keira L. Watts and Monica A. Spiteri
Lung Research, Institute of Science and Technology in Medicine, University Hospital of North Staffordshire/ Keele University, Stoke on Trent ST4 7QN, Staffordshire, United Kingdom

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Watts, Keira L., and Monica A. Spiteri. Connective tissue growth factor expression and induction by transforming growth factor-β is abrogated by simvastatin via a Rho signaling mechanism. Am J Physiol Lung Cell Mol Physiol 287: L1323–L1332, 2004.—Connective tissue growth factor (CTGF), a potent profibrotic mediator, acts downstream and in concert with transforming growth factor (TGF)-β to drive fibrogenesis. Significant upregulation of CTGF has been reported in fibrogenic diseases, including idiopathic pulmonary fibrosis (IPF), and is partly responsible for associated excessive fibroblast proliferation and extracellular matrix deposition, but no effective therapy exists for averting such fibrogenic events. Simvastatin has reported putative antifibrotic actions in renal fibroblasts; this study explores such actions on human IPF-derived and normal lung fibroblasts and examines associated driving mechanisms. Simvastatin reduces basal CTGF gene and protein expression in all fibroblast lines, overriding TGF-β induction through inhibition of the cholesterol synthesis pathway. Signaling pathways driving simvastatin’s effects on CTGF/TGF-β interaction were evaluated using transient reporter transfection of a CTGF promoter construct. Inhibition of CTGF promoter activity by simvastatin was most marked at 10 μM concentration, reducing activity by 76.2 and 51.8% over TGF-β-stimulated cultures in IPF and normal fibroblasts, respectively. We also show that geranylgeranylation of proteins, but not farnesylation, induces CTGF promoter activity following simvastatin inhibition by 55.3 and 31.1% over GGPP-negative cultures in IMR90 and IPF-derived fibroblasts, respectively, implicating small GTPase Rho involvement rather than Ras in these effects. Indeed, the specific Rho inhibitor C3 exotoxin significantly (P < 0.05) suppressed TGF-β-induced CTGF promoter activity in transfected lung fibroblasts, a finding further supported by transfection of dominant-negative and constitutively active RhoA constructs, thus demonstrating that simvastatin through a Rho signaling mechanism in lung fibroblasts can modulate CTGF expression and interaction with TGF-β.

idiopathic pulmonary fibrosis; fibroblasts; statins; small GTPase

IDIOPATHIC PULMONARY FIBROSIS (IPF) is an insidious fibroproliferative disorder characterized by interstitial alveolar fibrosis (29) resulting from an aberrant response to as-yet-undefined microinsults and involving failure of reepithelialization (2), fibroblast to myofibroblast differentiation (4), and extracellular matrix accumulation (26). The presence of fibroblastic foci is recognized to be central to the pathogenesis of the condition (27), but the mechanisms driving the phenomena are still unclear. However, increasing evidence suggests that fibroblast and epithelial cell responses to transforming growth factor (TGF)-β and the level of expression of connective tissue growth factor (CTGF) may be crucial factors in IPF disease progression/outcome (9, 22, 25). CTGF is a known fibroblast mitogen (24) and promoter of collagen deposition (7) known to work in concert with TGF-β to promote fibrogenesis. CTGF is rapidly induced in fibroblasts following treatment with TGF-β, believed to be secondary to a TGF-β response element located within the CTGF promoter (14). The regulation of CTGF appears to be controlled primarily at the level of transcription, and a brief exposure of fibroblasts to TGF-β is sufficient to induce prolonged, high levels of CTGF expression (13). As conventional anti-inflammatory/immunosuppressive treatments in IPF are invariably inefficacious, targeted disruption of critical TGF-β/CTGF interactions may prove beneficial in combating fibrogenic disease.

Statins are widely used as antilipidemic agents due to their efficacy in lowering low-density lipoprotein; they also have excellent tolerability and safety (23). The statins are known to interfere with the rate-limiting step of cholesterol biosynthesis; hence, they are first-line therapies for treatment and/or prevention of hypercholesterolemia. However, statins also appear to have a myriad of other cellular effects, including immunomodulation (6) and anti-inflammatory actions (20), which occur independently of their cholesterol-lowering abilities.

The statins abrogate the cholesterol synthesis pathway by inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. However, the cholesterol pathway is also involved in small guanosine triphosphatase (GTPase) signaling. The isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylation of proteins (GGPP), intermediates of the cholesterol synthesis pathway, are important lipid attachments for posttranslational modification of proteins (32). Indeed, the small GTPases of the Rho and Ras family require posttranslational modification to enable them to bind to the cell membrane and exert signaling activities (18). Thus disruption of GTPase signaling could partly explain the emerging modulatory actions of statins on immune effector cells.

Data are now emerging that could implicate HMG CoA reductase inhibitors, otherwise known as statins, as having potential beneficial effects in the pathogenesis of fibrosis (8). The antifibrotic actions may result from disruption of small GTPase signaling, arising from inhibition of isoprenylation. This is supported by the observation that the small GTPases of the Rho family are important for basal as well as induced ccn2 (CTGF) expression in human renal mesangial cells (12). Indeed, HMG CoA reductase inhibitors were shown to interfere with the isoprenylation and subsequent activation of Rho

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proteins, resulting in modulation of CTGF expression, a downstream target of small GTPase activity. Consequently, we rationalize that such concepts could be applied to the overexpression of CTGF associated with IPF, although neither the in vitro nor in vivo use of statins in the lung has been documented to date.

In this present study, utilizing primary human normal and IPF-derived lung fibroblasts, we explore whether simvastatin can modulate CTGF gene and protein expression and, crucially, its induction by the profibrogenic mediator TGF-β. Simvastatin is an inhibitor of the enzyme HMG CoA reductase, a key enzyme in the cholesterol synthesis pathway. As modulation of CTGF expression is thought to depend on the inhibition of the cholesterol synthesis pathway, specifically the signaling function of the small GTPases (16), we deliberately explore the role for the small GTPase Rho. Experimental culture models will incorporate transient transfection of a CTGF promoter reporter construct to establish the mechanisms used by simvastatin to exert its actions on the CTGF molecule. Accordingly, selective inhibitors (C3 exotoxin) and intermediates (GGPP and FPP) of the signaling pathways downstream of HMG CoA reductase, the enzyme known to be inhibited by simvastatin, will be utilized. Final confirmational experiments involve the transient transfection of dominant-negative and constitutively active RhoA constructs.

METHODS

Cell Culture

Experiments were carried out in normal human lung fibroblasts (IMR90, obtained from the American Type Culture Collection, Rockville, MD) and three separate human lung fibroblasts cell lines isolated from IPF patients [LL29 and LL97a from ATCC, Manassas, VA; and HIPF, a generous gift from R. J. McAnulty, University College London (UCL), London, UK]. IPF-derived cell lines in culture exhibit a more flattened dendritic morphology and enhanced expression of α-smooth muscle actin (SMA) (3). Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM). Medium was supplemented with penicillin-streptomycin (100 U/ml; GIBCO-BRL, Paisley, UK) and t-glutamine (2 mM, GIBCO-BRL) with 10% fetal calf serum (FCS; Labtech, Sussex, UK). For experiments, medium was replaced with serum-free DMEM (SF-DMEM) for 48 h to induce quiescence before treatment. Human recombinant TGF-β1 (R&D Systems, Oxford, UK) was used at a dose of 5 ng/ml.

pLuc-CTGF Plasmid

pLuc-CTGF reporter vector (generous gift of Professor Eugene Chen, Morehouse School of Medicine, Atlanta, GA) encodes the CTGF promoter sequence, which is used to control the expression of the luciferase gene. With the CTGF gene sequence (GenBank accession no. AL354866), primers were designed to amplify the 2-kDa region relating to the CTGF promoter (bp −2,065 to +72) and were subsequently cloned into the luciferase reporter plasmid PGL3 (11) (Promega, Madison, WI).

Transient Transfections and CTGF Promoter Activity Assays

Transfections of human CTGF promoter-reporter constructs into human lung fibroblasts (HIPF) (at 90% confluence) were performed using the Transfast mammalian transfection system (Promega, Southampton, UK) following the manufacturer’s recommendations. Cells were transfected with 0.75 μg of DNA per well (18-mm diameter) using a 1:1 ratio of DNA/Transfast reagent in serum-negative cultures. Confluent cells were incubated in the transfection mix containing the pLuc-CTGF plasmid for a 1 h; DMEM containing 10% FCS was added up to a volume of 1 ml, and cultures were left for 4 h. After this, the transfected cells were serum deprived for 48 h before treatment. After appropriate time points the cells were lysed, and luciferase activity was measured as chemiluminescence with a Berthold Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany) using the Steady Glo luciferase assay system (Promega). These data are presented as relative light units ± SE. Transfection efficiency was optimized and assessed using transfection of a plasmid construct containing green fluorescent protein (pEGFP-C1; Clontech, Palo Alto, CA). The cell density, DNA/Transfast ratio, and time course were optimized in repeated separate experiments; the optimized protocol was then used in subsequent transfection studies.

Dominant-Negative RhoA Transfections

Transfection of dominant-negative and constitutively active human RhoA (accession no. L25080) constructs into human lung fibroblasts (IPF-derived and IMR90 cells) were performed using the Transfast mammalian transfection system (Promega) as described above. RhoA G14V (a construct containing a mutation at G14V to render it constitutively active) and RhoA T19N (a construct containing a mutation at T19N, giving it a dominant-negative phenotype) constructs were utilized in a cDNA3.1+ vector and were obtained from the Guthrie Research Institute (www.cdna.org).

Treatment of Transfected HIPF Lung Fibroblasts

Simvastatin (Merck Sharp and Dohme, Hertfordshire, UK) was dissolved and filter sterilized before use (21). Quiescent, transfected HIPF lung fibroblasts were incubated for 16 h with simvastatin in SF-DMEM at a range of concentrations of 0.1–10 μM. After simvastatin preconditioning, cells were stimulated with TGF-β1 (5 ng/ml) for 1 h. GGPP and FPP (Sigma Aldrich, Dorset, UK) were used at a concentration of 10 μM. Transfected cells were incubated for 16 h with these intermediates with and without the presence of simvastatin before 5 ng/ml TGF-β treatment for 1 h. Clostridium botulinum C3 exotoxin (Upstate Biotechnologies, Lake Placid, NY) was used at concentrations ranging from 0.5 to 5 μg/ml. Transfected cells were incubated with C3 exotoxin for 16 h before TGF-β (5 ng/ml) stimulation for 1 h. The concentration of TGF-β used in our experiments was determined from ongoing studies within the laboratories, in which 5 ng/ml were found to show significant induction of CTGF.

CTGF Gene Detection by Competitive RT-PCR

Quiescent, near-confluent IMR90 and HIPF lung fibroblasts were treated for up to 24 h with 5 ng/ml TGF-β1 (R&D Systems). mRNA abundance was quantified by an established competitive RT-PCR procedure (5), using the primers shown in Table 1. The primers for the

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequences</th>
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<tr>
<td>CTGF Forward</td>
<td>GGCCTCTCTCTTGACTTG</td>
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<tr>
<td>CTGF Reverse</td>
<td>TGGAGGACCCCTGTACCT</td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>AAATGCTGACACACCTTACA</td>
</tr>
<tr>
<td>β-Actin Reverse</td>
<td>GTGATCTCCTTCTGAGTCAA</td>
</tr>
<tr>
<td>Competitor primer CTGF</td>
<td>TGGAGAGGCCGTTCATGACCACCTTCTG</td>
</tr>
<tr>
<td>Competitor primer β-Actin</td>
<td>GTGATCTCCTTCTGAGTCAA</td>
</tr>
<tr>
<td>Table 1. Primer sequences used for amplification of CTGF and β-Actin</td>
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CTGF, connective tissue growth factor.
target genes were designed with computer assistance using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers were amplified within the coding region of the gene and spanned introns from the genomic template.

Briefly, PCR reactions consisted of 25 pmol of primers, 200 μM deoxynucleotide triphosphates, 1.5 mM MgCl₂, 5 μl of 10× PCR buffer, and 2.5 units of AmpliTag Gold (Applied Biosystems, Warrington, UK). To this, 5 μl of the appropriate competitor molarity and 2 μl of cDNA were added in 50 μl of total volume. PCR conditions were 95°C enzyme activation for 12 min, then 1 min 15 s at 94°C, 1 min at 55°C (for both CTGF and β-actin transcripts) followed by 1 min at 72°C for 35 cycles, and then a final extension of 10 min at 72°C.

**CTGF Gene Detection by Quantitative Real-Time RT-PCR**

cDNA samples isolated from normal and IPF-derived lung fibroblasts transfected with the T19N and G14V RhoA constructs were used to assess CTGF gene expression. Two microliters of undiluted cDNA were used per reaction; a standard curve for CTGF and β-actin was generated from serial dilutions of positively expressing cDNA. The probe and primer sets were “predesigned assay on demand” probes (Applied Biosystems, Foster City, CA); these predesigned primers are tested and standardized for reproducible expression analysis. Primer and cDNA were added to Taqman universal master mix (Applied Biosystems, Foster City, CA) containing all the reagents for PCR. Absolute quantification of the PCR products was carried out using the ABI prism 7000 (Applied Biosystems, Foster City, CA).

**CTGF Protein Detection by Western Blotting**

Total cell proteins were extracted in lysis buffer comprising 1% (vol/vol) Triton X-100, 20 mM Tris-HCl (pH 8.0), 10% (vol/vol) glycerol, 1 mM sodium orthanadate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin, and 0.15 U/ml aprotinin. Briefly, cells recovered by trypsinization were lysed in 30 μl of buffer and placed on ice for 20 min. The lysates were then centrifuged at 10 000 g for 15 min at 4°C to pellet cell debris. Supernatant containing the protein was recovered and assayed for total protein using a commercial microplate assay (Bio-Rad, Hemel Hempstead, UK). We combined 25 μg of total protein with sample buffer and boiled it for 5 min before gel loading. Proteins were resolved on a 12.5% polyacrylamide gel by electrophoresis at 120 V for 90 min in reducing buffer, and transfer was carried out at 100 V for 4 h. Membranes were blocked with 5% (wt/vol) BSA in Tris-buffered saline-Tween (20 (TBS-T) buffer overnight at 4°C. For detection of the CTGF protein a rabbit monoclonal anti-CTGF antibody (provided by Fibrogen) was used at 2 μg/ml in TBS-T and 1% BSA for 1 h with gentle agitation. Secondary detection was carried out with a peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch, Philadelphia, PA) at a dilution of 1:25 000 in TBS-T and 1% (wt/vol) BSA for 1 h. The CTGF protein band was visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) exclusion and accumulation of lactate dehydrogenase (DAPi; Vector Laboratories, Peterborough, UK). Slides were viewed under epifluorescence with a filter set at 450–490 nm for FITC and 340–380 nm for DAPI. Images were obtained using a Leica DC200 digital camera and software (Leica Microsystems, Heerbrugg, Switzerland). Camera exposure setting remained constant throughout the experiment; for each treatment, the total number of cells positive for CTGF expression was counted according to the published procedure (33). Five separate random fields of view were selected and imaged for each treatment, allowing the mean number of positively stained cells per field of view ± SE to be calculated.

**Cell Viability**

Cell viability was determined by Trypan blue (Sigma Aldrich, Dorset, UK) exclusion and accumulation of lactate dehydrogenase (LDH). LDH (a stable cytosolic enzyme that is released upon cell damage) was measured using a sensitive cytotoxicity detection kit (Roche, East Sussex, UK) following the manufacturer’s recommendations.

**Statistical Analysis**

Data are shown as means ± SE. An unpaired Student’s t-test was employed for comparing two groups of data. Multiple comparisons were made using analysis of variance, followed by Tukey’s pair-wise comparison. All P values < 0.05 were considered significant.

**RESULTS**

**Effect of Simvastatin on CTGF Gene Expression in Unstimulated Lung Fibroblasts**

The expression of the CTGF gene was quantified in IMR90 and HIPF lung fibroblasts by competitive RT-PCR in response to treatment with simvastatin (0.1, 1, and 10 μM) (Fig. 1). Under exponential growth there is a 33.3% increase in basal
preconditioning with simvastatin for 16 h, before the TGF-
β/H9252 cells treated with TGF-
β was shown to override this TGF induction and
downregulate CTGF gene expression in a concentration-depen-
ting manner to subbasal levels. This inhibition of CTGF was
expected, these levels of expression decrease following serum
depetration for 48 h; during this time the cells become quies-
cent. Serum-deprived cells were exposed to simvastatin; in
both cell lines the drug induced further substantial decreases in
CTGF gene expression at all concentrations. These results
show that simvastatin can significantly downregulate (P <
0.05) CTGF gene expression even under basal conditions. Al-
though the results shown are those from HIPF fibroblast
cultures, the same pattern of simvastatin downregulation was
also seen in the other two IPF-derived (LL29 and LL97a)
fibroblast lines.

**Effect of Simvastatin on CTGF/TGF-β Interaction**

**CTGF gene expression.** The effect of cell preincubation with
varying concentrations of simvastatin (0.1, 1, 5, and 10 µM) on
TGF-β (5 ng/ml) induction of CTGF expression was evaluated
in IMR90 and HIPF lung fibroblasts by competitive RT-PCR
(Fig. 2). Compared with the untreated controls, TGF-β induced
abundant upregulation in CTGF gene expression in both cell
lines, more so in the HIPF (P < 0.05). However, fibroblast
preconditioning with simvastatin for 16 h, before the TGF-
stimulation, was shown to override this TGF induction and
downregulate CTGF gene expression in a concentration-depend-
ent manner to subbasal levels. This inhibition of CTGF was
significant in both fibroblast lines compared with equivalent
cells treated with TGF-β alone at 1, 5, and 10 µM simvastatin
(P < 0.05). Indeed levels of CTGF expression at the higher
drug concentrations were decreased to below those exhibited in
untreated resting cells. These observations suggest that simva-
statin can maintain its downregulation of the CTGF molecule
and abolish the potent stimulatory capacity of TGF-β in lung
fibroblasts. Comparable results were also observed in the
additional IPF-derived lung fibroblast lines (LL29 and LL97a).

**CTGF protein expression.** To confirm that the effects of
simvastatin treatment on CTGF gene profile were reflected at
the protein level, Western blot analysis was performed. Cell
lysates were collected, and 25 µg of total protein were loaded
from each sample. CTGF protein was detected with a specific
monoclonal antibody directed against the protein and was
labeled by a secondary horseradish peroxidase-conjugated an-
tibody. Representative scans of Western blots (Fig. 3A) derived
from IMR90 and HIPF cell lysates were analyzed by densi-
ometric analysis. Data for CTGF protein expression (Fig. 3B)
show that TGF-β alone induces CTGF protein expression in
the cell lysates of both IPF-derived and normal fibroblasts;
however, TGF-β induction appears to be comparably higher and
significant in the fibrotic cell lines (P < 0.05). The presence of simvastatin (0.1–10 µM), upregulatory effects of
TGF-β are overridden, and CTGF protein levels are downregu-
lated, again best observed in the IPF-derived fibroblasts. There
is a 27.9 and a 43.4% reduction in IMR90 and HIPF, respec-
tively, in CTGF protein when preconditioned with 10 µM
simvastatin compared with those treated with TGF-β alone
(P < 0.05). We observed detectable double-banded CTGF
protein by Western blotting; this is not uncommon and relates
to different glycosylation products of the CTGF protein. It is

![Fig. 2](image-url)
currently unknown how changes in the production of each glycosylation product relate to function.

The ability of simvastatin to suppress the induction of CTGF protein by TGF-β was supported by separate experiments that selectively analyzed protein with immunofluorescence staining and confocal microscopic analysis. In both IMR90 and IPF-derived lung fibroblasts, CTGF expression was enhanced following TGF-β exposure (5 ng/ml) for 24 h, with higher induction observed in the fibrotic cell lines. However, preconditioning of these cells in simvastatin (0.1–10 μM) induced a dose-dependent downregulation of CTGF protein irrespective of TGF-β exposure (data not shown).

The viability of lung fibroblasts following treatment remained uncompromised by simvastatin treatment as determined by trypan blue exclusion, with 89% of cells remaining viable even at the highest simvastatin concentration. In addition, measurement of the stable cytoplasmic enzyme LDH that is released rapidly into the supernatant upon cellular damage revealed that cytotoxicity remained <16% throughout our experiments (data not shown).

**Effect of Simvastatin on CTGF Promoter Activity**

To examine the mechanisms driving the actions of simvastatin on the CTGF molecule, cells from both IPF-derived and normal lung fibroblast lines were transfected with the pLuc-CTGF reporter construct. We performed all transfections using an in-house optimized procedure. The effects of varying concentrations of simvastatin on CTGF induction alone and in the presence of TGF-β (5 ng/ml) were analyzed.

We observed that transfection of the pLuc-CTGF plasmid itself enhances the activity of the CTGF promoter by 80.7 and 90.2% in IMR90 and HIPF, respectively; these levels thus represented basal CTGF expression in the lung fibroblasts. Fibroblast preconditioning with simvastatin (0.1–10 μM) reduced the activity of the CTGF promoter. This was most pronounced at a drug dose of 10 μM, where a 36.8 and 34.9% reduction in luciferase release was observed in IMR90 and HIPF, respectively, compared with equivalent transfected, untreated cells; these changes are not significant (P < 0.08, Fig. 4A).

In contrast, the basal levels of luciferase activity exhibited by untreated transfected cells, and hence CTGF promoter activity, are enhanced by 64.8 and 61.7% on exposure to TGF-β (P < 0.05) in IMR90 and HIPF cultures, respectively (Fig. 4B). However, when the same cells are preconditioned with simvastatin (0.1–10 μM), a sustained reduction in CTGF promoter activity is observed in a concentration-dependent manner, with significant downregulation best observed at 1 and 10 μM (P < 0.05). These findings support the concept that simvastatin overrides the potent upregulatory effects of TGF-β on the CTGF promoter and implicates a direct test drug action on the CTGF molecule, capable of abrogating CTGF promoter induction. Reproducible results have been obtained in two additional IPF-derived lung fibroblast lines (LL29 and LL97a).

**Effect of GGPP on CTGF Promoter Activity**

GGPP is an intermediate of the cholesterol synthesis pathway and is involved in the postmodification of the small GTPase Rho. GGPP was employed to elucidate whether the cholesterol pathway was involved in simvastatin-induced responses of the CTGF gene. A significant increase (P < 0.05) in luciferase release from cells transfected and exposed to 5 ng/ml TGF-β compared with transfected, untreated cells is observed (Fig. 5). However, as previously shown, pretreatment of cells with 10 μM simvastatin decreases luciferase release (P < 0.05); consequently, CTGF promoter activity is inhibited in the presence of simvastatin. GGPP alone was able to significantly elevate luciferase output from transfected cells compared with those preconditioned with simvastatin (10 μM) in all fibroblast cell lines used. GGPP was able to restore CTGF promoter activity in simvastatin-treated cells at all simvastatin concentrations used (0.1 and 1 μM simvastatin,
Effect of FPP on CTGF Promoter Activity

In the above experiments, GGPP was able to effectively reverse the inhibitory effects induced by simvastatin on the CTGF gene. We concluded that geranylation of the small GTPase Rho is implicated in the signaling of CTGF. However, we also reasoned that the observed restoration of CTGF activity may not be specific to geranylation of Rho and could represent a generic action associated with the small GTPase family. To determine this, FPP (an intermediate involved in the farnesylation of the small GTPase Ras) was analyzed for its ability to restore CTGF promoter activity following simvastatin cell preconditioning. The addition of FPP was not able to produce significant induction of the CTGF promoter in all fibroblast lines used, suggesting that FPP, as well as the farnesylation with which it is involved, is not a critical activity in CTGF induction (data not shown). These findings confirmed that geranylation and therefore Rho signaling are essential to CTGF activity, whereas farnesylation and the associated signaling of Ras are not.

Use of C3 Exotoxin to Confirm the Role of Rho Signaling Pathway in CTGF Modulation

Clostridium botulinum C3 exotoxin (Upstate Biotechnologies) is an enzyme that specifically ADP ribosylates and inactivates Rho. The data presented previously revealed that geranylation of Rho and not farnesylation of Ras is important in induction of the CTGF promoter and therefore CTGF activity. The selective Rho inhibitor C3 exotoxin was employed to elucidate whether inhibition of Rho is able to influence CTGF promoter activity, modulating CTGF/TGF-β interaction.

All tested concentrations (0.5–5 μg/ml) of C3 exotoxin significantly suppressed the luciferase release from TGF-β in transfected cells (P < 0.05, Fig. 7A). At 5 μg/ml C3 exotoxin treatment, luciferase levels return to basal levels as seen in transfected, untreated cells. The results suggest that this selective inhibitor of Rho isoforms A, B, and C is able to inhibit the CTGF promoter activity, a response that is TGF-β independent. Importantly, C3 exotoxin itself does not show any significant alteration in the luciferase release compared with the control untransfected cells; therefore, treatment with this toxin does not alter the plasmid activity. In addition, levels of CTGF protein expression following C3 exotoxin treatment are also reduced (as shown by immunofluorescent cytology, Fig. 7B). Compared with samples treated to 5 ng/ml of TGF-β alone, there is a 24.9 and 14.8% reduction in the percentage number of IMR90 and HIPF cells expressing CTGF protein compared with unstimulated controls in IMR90 and HIPF, respectively (Fig. 6A). Independently of this, GGPP alone is equally capable of inducing CTGF protein by 52.1 and 58.8% in IMR90 and HIPF, respectively. However, in contrast to the simvastatin effects on TGF-β, we observed that GGPP does override the inhibitory influence of simvastatin and provides supporting evidence for the role of Rho signaling in the induction of CTGF (Fig. 6C). Similar CTGF expression profiles have been observed in additional IPF-derived cell lines (LL29 and LL97a).

Dominant-Negative and Constitutively Active RhoA Constructs Confirm Role of Rho in CTGF Regulation

Transient transfection of dominant-negative RhoA (RhoA T19N) and constitutively active RhoA (RhoA G14V) constructs was utilized to confirm the regulatory role of Rho in

Fig. 5. IMR90 and HIPF lung fibroblasts transiently transfected with pLac-CTGF reporter plasmid. After transfection, cells were preincubated with Sim (10 μM) and/or geranylgeranylpolyphosphate (GGPP, 10 μM) for 16 h. Cells were then exposed to TGF-β (5 ng/ml) for 1 h. Results represent the mean RLU ± SE. Significant P values (P < 0.05) are denoted by * for IMR90 and † for HIPF cultures. Data are representative of the mean of triplicate transfections obtained from 3 independent experiments.

Effect of FPP on CTGF Promoter Activity

In the above experiments, GGPP was able to effectively reverse the inhibitory effects induced by simvastatin on the
CTGF induction (Fig. 8). These data reveal that there is no significant difference between untransfected cells stimulated with TGF-β alone compared with those transfected with the constitutively active RhoA construct. When constitutively active RhoA cultures are conditioned with TGF-β, there is a small but insignificant rise in CTGF gene expression. These data support a role for RhoA in CTGF induction; this is further confirmed by the use of a dominant-negative RhoA construct. Transfection of the RhoA T19N construct induces significant reduction (P < 0.05) in CTGF gene expression, producing a...
Fig. 7. A: luciferase release was examined from HIPF lung fibroblasts transiently transfected with pLuc-CTGF reporter plasmid. After transfection, cells were preincubated with C3 exotoxin (0.5–5 μg/ml) for 16 h. Cells were then exposed to TGF-β (5 ng/ml) for 1 h. Results represent mean RLU ± SE. Significant P values (P < 0.05) are denoted by * for IMR90 cultures and † for HIPF cultures. Data are representative of transfections carried out in triplicate and performed in 3 independent experiments. B: quiescent serum-deprived lung fibroblasts (normal IMR90 and IPF-derived HIPF) were stained for the presence of CTGF protein. Cells were preconditioned for 16 h with C3 exotoxin (0.5–5 μg/ml) and then stimulated with TGF-β (5 ng/ml) for 24 h; CTGF protein was detected by immunofluorescent cytology. The mean percentage number of cells positively expressing the protein ± SE is presented; significant increase in expression is denoted by *P < 0.05, TGF-β-stimulated compared with control. Data are representative of images obtained from 3 independent experiments. C: representative images of IMR90 and HIPF lung fibroblasts stained for CTGF protein by immunofluorescent cytology.
DISCUSSION

CTGF is a fibroblast mitogen and promoter of collagen deposition (10) that has been implicated in the pathogenesis of tissue fibrosis including that of the lung (1), liver (17), and kidney (31). The potent profibrogenic activities of TGF-β are directly involved in the induction of CTGF (14); disruption of this interaction may have beneficial antifibrotic potential and was one of the objectives of this study. For this purpose, we focused on the actions of simvastatin in disrupting critical interaction of TGF-β with CTGF within human lung fibroblasts.

Simvastatin is a member of the statin group, which are widely used in clinical practice because of their efficacy in the prevention of cardiovascular events (28), resulting in improved lipid profiles. However, it is increasingly recognized that statins have pleiotropic effects beyond that of cholesterol lowering. Recent reports have described the potential use of simvastatin in blocking CTGF action (12) and its antifibrotic potential (8) in renal disease. Hitherto such actions and associated underlying mechanisms of simvastatin on lung-derived fibroblasts have not been explored.

In this paper, we report that simvastatin can significantly inhibit CTGF gene expression in primary human lung fibroblasts; this was observed to occur parallel to a reduction in CTGF protein levels. Interestingly, the finding that basal CTGF expression in fibroblasts can be inhibited suggests that simvastatin can exert inhibitory effects on CTGF gene transcription.

TGF-β, a recognized potent inducer of CTGF, produced a 73.1% induction of the CTGF gene in IMR90 normal lung fibroblasts and an 83.9% induction in HIPF lung fibroblasts. However, preconditioning of the same cells with simvastatin dose dependently inhibited TGF-β stimulatory effects, thereby lowering CTGF gene expression to below basal levels at 5- and 10-μM simvastatin concentrations. The data demonstrate the ability of simvastatin to modulate the TGF-β/CTGF interaction and specifically to override the potent induction of CTGF by TGF-β. In further support we have demonstrated that by using transient transfections of a plasmid construct containing the CTGF promoter sequence, simvastatin (0.1–10 μM) reduced the activity of the promoter irrespective of the presence of TGF-β in both normal and IPF-derived lung fibroblasts.

The cholesterol synthesis pathway is known to be inhibited by simvastatin. However, this pathway also possesses important signaling properties involving the small GTPases, which include the Ras, Rho, and Rac family. The small GTPase Rho has been implicated in normal wound healing (15); in addition, it has been suggested that guanine nucleotide-binding regulatory proteins (G proteins) are a novel signaling pathway able to induce CTGF in rat mesangial cells (16). Further elucidation of this pathway suggests that HMG CoA reductase inhibitors (statins) can inhibit the expression of the profibrotic mediator CTGF by interfering with the isoprenylation of small GTPases, specifically the isoprenylation of Rho (8). We incorporated intermediates of the cholesterol synthesis pathway in our series of experiments to establish the role of the small GTPases in the simvastatin-induced responses of the CTGF gene. The ability of GGPP to reverse the simvastatin-induced inhibition of the CTGF promoter activity, whilst FPP was unable to do so, suggests that the effects were not generic to the GTPases family and implicates a specific, crucial role for Rho. This was further substantiated by the use of C3 exotoxin (a specific inhibitor of Rho). The use of this reagent significantly inhibited CTGF promoter activity, irrespective of TGF-β action; in addition C3 exotoxin was able to lower protein expression, thereby suggesting that Rho is essential for TGF-β-induced elevation of CTGF. To further clarify this link, dominant-negative and overexpression technology was utilized. Transient transfection of dominant-negative RhoA construct T19N resulted in a significant decrease in CTGF gene expression, whereas overexpression of RhoA using the G14V construct increased CTGF gene expression to levels comparable to those seen with TGF-β alone. We conclude that the modulatory effects of simvastatin on CTGF/TGF-β interactions (including its ability to inhibit CTGF expression) depend upon blocking Rho prenylation and its subsequent signaling.
Dysregulation of CTGF expression may be a key factor in influencing disease progression in IPF and consequently causing severe, irreversible lung parenchymal remodeling associated with fibrogenesis. It is possible that such events could be averted by the use of simvastatin. Indeed, utilizing a specifically designed in vitro cell model, we have carried out separate preliminary studies that have explored functional effects of simvastatin on α-SMA gene and protein expression and collagen gel contraction. We have observed that simvastatin modulation of the CTGF molecule is associated with inhibitory changes in α-SMA expression and decrease in collagen gel contraction (K. L. Watts, unpublished observations). α-SMA and contractile properties are known to be associated with the myofibroblasts phenotype, a key element in fibrogenesis (19).

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