Original article

Simvastatin impairs smad-3 phosphorylation and modulates transforming growth factor β1-mediated activation of intestinal fibroblasts

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Background: Transforming growth factor (TGF) β1, acting through the smad pathway, is critical to fibroblast-mediated intestinal fibrosis. Simvastatin exhibits antifibrotic properties. This study assessed the effects of simvastatin on TGF-β1-mediated intestinal fibroblast activation.

Methods: Human intestinal fibroblasts were activated with TGF-β1 with or without simvastatin or the cholesterol pathway intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Collagen-Iα2 expression was assessed by reverse transcriptase–polymerase chain reaction. Connective tissue growth factor (CTGF) and smad phosphorylation were evaluated by western blot, and plasminogen activator inhibitor (PAI) 1 activity by enzyme-linked immunosorbent assay. Fibroblast filamentous (F)-actin accumulation was assessed by confocal microscopy and contraction by a fibroblast-populated collagen lattice (FPCL) model.

Results: TGF-β1 treatment of fibroblasts induced smad-2/3 phosphorylation, CTGF and collagen-Iα2 production, F-actin bundling, FPCL contraction and PAI-1 activation. Pretreatment with simvastatin inhibited the induction of CTGF and collagen-Iα2, PAI-1 activation, F-actin bundling and FPCL contraction. The inhibitory effect of simvastatin on PAI-1 activation was reversed by GGPP and FPP. Simvastatin pretreatment inhibited TGF-β1-mediated phosphorylation of smad-3.

Conclusion: Simvastatin abrogates TGF-β1-mediated intestinal fibroblast activation by inhibition of smad-3 phosphorylation. These findings offer a mechanism for the antifibrotic effects of simvastatin and a therapeutic entry point in the treatment of intestinal fibrosis.

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Introduction

Intestinal fibrosis is a multifactorial disorder resulting from diverse aetiologies such as chronic inflammation, radiation enteritis, infection and ischaemia[1–3]. In Crohn's disease more than one-third of patients develop a fibrostenosing phenotype that results in recurrent intestinal stricture formation[4], the end-product of chronic transmural inflammation, dysregulated wound healing and abnormal deposition of extracellular matrix[1,5]. Contraction of extracellular matrix leads to scar formation, tissue distortion and, ultimately, intestinal obstruction. Currently there are no effective preventive or therapeutic interventions for intestinal fibrosis.

Transforming growth factor (TGF) β1 plays a central role in fibrosis[1]. It is highly expressed in areas of intestinal stricture and is overproduced by fibroblasts isolated from enteric strictures[6,7]. The pathways that mediate TGF-β signalling are complex and include the central smad pathway, and interaction with the Ras/mitogen-activated protein kinase kinase (MEK) extracellular signal-regulated protein kinase (ERK) and Rho/Rho-associated

coiled kinase (ROCK) pathways[8,9]. TGF-β signalling is initiated by ligand binding to the transmembrane receptors TβR-I and TβR-II, and is transduced predominantly by phosphorylation of receptor-associated smad proteins (smad-2 and -3)[10,11]. Smad-2 and -3 combine with smad-4, and translocate into the nucleus where they function as transcription factors alone or in association with other DNA-binding factors[11]. Fibroblasts isolated from areas of intestinal stricture express increased levels of extracellular matrix genes such as connective tissue growth factor (CTGF, CCN2), fibronectin and collagen-I[9,12]. The production of these promoters and constituents of extracellular matrix is greatly enhanced by TGF-β1[9,12].

Statins are widely used in both primary and secondary management of cardiovascular disease owing to their lipid-lowering and directly beneficial cardiovascular effects[13,14]. However, they also have immunomodulatory and anti-inflammatory effects[15,16]. These additional effects occur independently of cholesterol-lowering properties and are due to inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in the cholesterol biosynthetic pathway[15]. The HMG-CoA reductase inhibitor simvastatin inhibits the synthesis of isoprenoid intermediates of the cholesterol biosynthetic pathway, including the 15-carbon farnesyl pyrophosphate (FPP) and the 20-carbon geranylgeranyl pyrophosphate (GGPP), both of which are necessary for post-translational modification of small guanosine 5′-triphosphate (GTP)-bound molecules such as RhoA (*Fig. 1*)[15].

Statins exhibit antifibrotic properties in models of fibrotic disease in the kidney, heart, lung and skin[17–20] owing to modulation of TGF-β1 signalling through inhibition of the Rho/ROCK pathway[21]. This results in reduced expression of growth factors such as CTGF[17], reduced transcription of collagen[20] and reduced collagen contraction[19]. However, whether this effect relies on inhibition of canonical TGF-β/smad signalling has not been explored previously.

The aims of the present study were to determine the effect of direct Rho/ROCK pathway inhibition on TGF-β1-mediated activation of human intestinal fibroblasts and to investigate the effect of simvastatin, a known inhibitor of the Rho/ROCK pathway, on TGF-β1-mediated CTGF, fibronectin and collagen-Iα2 production, and plasminogen activator inhibitor (PAI) 1 activation, in these fibroblasts. The effect of simvastatin on intracellular actin filament accumulation and *in vitro* collagen contraction was also examined, as were the intracellular signalling mechanisms underlying the observed effects.

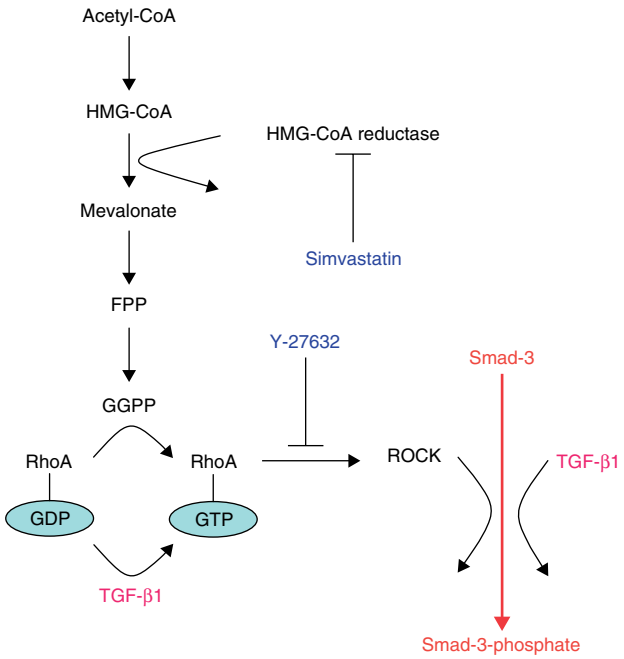


Fig. 1 Proposed intracellular mechanism by which simvastatin modulates transforming growth factor (TGF) β1 signalling. CoA, coenzyme A; HMG, 3-hydroxy-3-methylglutaryl; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; ROCK, Rho-associated coiled kinase; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate

Methods

The study protocol was approved by the Mater Misericordiae University Hospital Ethics Committee and written informed consent obtained from all patients. Eight patients who were undergoing intestinal resection for colorectal cancer (median age 68 (interquartile range 55–81) years) were recruited.

Materials

RPMI medium, penicillin, streptomycin, L-glutamine and fetal calf serum (FCS) were purchased from Gibco Life Technologies (Paisley, UK). Human TGF-β1 was purchased from R&D Systems (Oxford, UK). Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hemel Hempstead, UK). Antihuman CTGF was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antihuman smad-2 and smad-3, phospho-smad-2 and phospho-smad-3, ERK-1/2 and phospho-ERK-1/2 were purchased from Cell Signaling Technology (Boston, Massachusetts, USA). Rat tail collagen type I was purchased from Becton Dickinson Biosciences (Cambridge, UK). The cell-permeable selective

ROCK inhibitor, Y-27632, was purchased from Sigma Aldrich (Poole, UK). The chemiluminescent detection kit was from Amersham (Amersham, UK). Reagents for complementary (c) DNA synthesis and Lipofectamine 2000© were purchased from Invitrogen Life Technologies (Paisley, UK). Primers for real-time polymerase chain reaction (PCR) were purchased from Applied Biosystems (Warrington, UK). The N19Rho.dn3 dominant negative Rho plasmid was a kind gift from Professor C. Widmann (Institut de Biologie Cellulaire et de Morphologie, Université de Lausanne, Lausanne, Switzerland)[22]. Simvastatin (Merck, Hoddesdon, UK) was dissolved in deionized water and filter-sterilized before use. The simvastatin concentrations used (1–5 μmol/l) are within physiologically relevant levels of the drug, as used in clinical practice (in which doses vary from 10 to 80 mg/dl)[19]. All other reagents were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise.

Fibroblast isolation and culture

Intestinal fibroblast cultures were established using a primary explant technique as described previously[6]. Serosal biopsies were obtained from patients undergoing resection for colorectal cancer. All biopsies were taken before bowel devascularization from a segment of healthy colon remote from the tumour but to be included in the resection. Confluent cells were characterized by morphological features, and immunohistochemical staining properties for vimentin, α-smooth muscle actin and desmin[23,24]. For all experiments fibroblasts were used between passages 2 and 5, and maintained in low-serum medium (1 per cent FCS).

The human intestinal fibroblast cell line CCD-18Co was obtained from the American Type Culture Collection (Rockville, Maryland, USA), and used between passages 8 and 15 for plasmid transfection experiments. Cells were maintained in Dulbecco's modified Eagle medium–F12 (culture medium) containing 10 per cent fetal bovine serum, 2 mmol/l glutamine and 1 per cent penicillin–streptomycin. Incorporation of the control empty vector pcDNA3 or N19Rho.dn3 plasmids was achieved using Lipofectamine 2000© according to the manufacturer's instructions. The cells were plated in antibiotic-free medium for 24 h, followed by transfection for 6 h with 1 μg plasmid DNA the evening before experimentation.

Protein extraction and western blot analysis

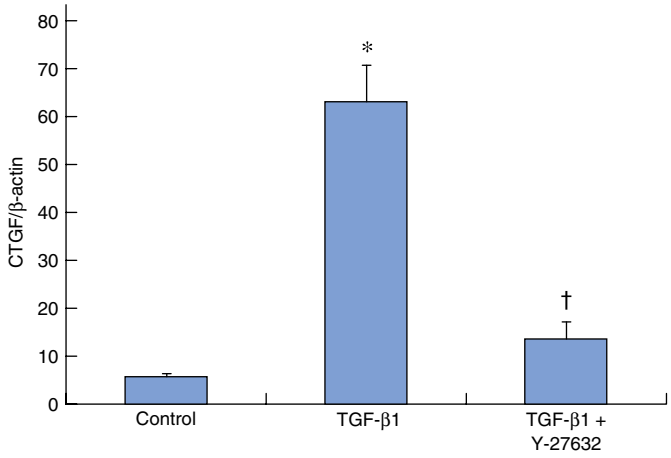
Total protein was isolated from 2×10^6 fibroblasts using NP-40 isolation solution (0·5 per cent NP-40, 10 mmol/l Tris, pH 8·0, 60 mmol/l potassium chloride, 1 mmol/l EDTA, pH 8·0, 1 mmol/l dithiothreitol plus protease inhibitor mix) and western blot analysis performed as described previously[6]. Aliquots containing 30 μg were resolved on a 12 per cent sodium dodecyl sulphate gel (75 min at 140 V) before transfer at 100 V for 80 min. Blots were incubated with the appropriate primary antibody (dilution 1 in 100 for CTGF; 1 in 500 for ERK-1/2, phospho-ERK-1/2, smad and phospho-smad-2 and -3). Appropriate secondary antibodies were incubated at a concentration of 1 in 5000. The primary and secondary antibody concentrations were determined from dose–response experiments. Equal protein loading was confirmed by β-actin or unphosphorylated protein expression. Protein density on scanned western blots was determined using the Un-Scan-It™ gel automated digitizing system version 5.1 (Silk Scientific, Orem, Utah, USA).

Quantitative real-time reverse transcriptase–polymerase chain reaction

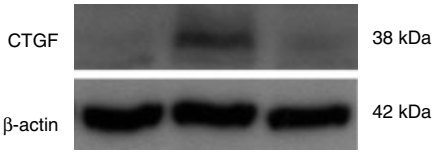
RNA was extracted using TRIzol® reagent (Invitrogen, Dublin, Ireland) according to the manufacturer's protocol. RNA concentration and quality were assessed by ultraviolet spectrophotometry (NanoDrop™ ND-1000 spectrophotometer; NanoDrop Technologies, Wilmington, Delaware, USA). Complementary DNA for real-time PCR was prepared from RNA following treatment with DNAse I. Total RNA (1 μg) was used to synthesize cDNA using random primers and the cDNA template amplified by PCR in a thermal cycler (Perkin Elmer 7700; Perkin Elmer, Norwalk, Connecticut, USA). Real-Time PCR TaqMan® assay was used to quantitate the relative gene expression levels of CTGF, collagen-Iα2 and fibronectin, and was performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). The primers and probes for these targets were supplied as a preoptimized single tube primer Gene Expression Assay (Applied Biosystems) as described previously[6]. The endogenous control 18S RNA was purchased from Applied Biosystems as a predeveloped assay reagent. Results were analysed using the relative delta ct (threshold cycle) method of analysis with candidate gene expression normalized to that of the housekeeping gene 18S.

Enzyme-linked immunosorbent assay

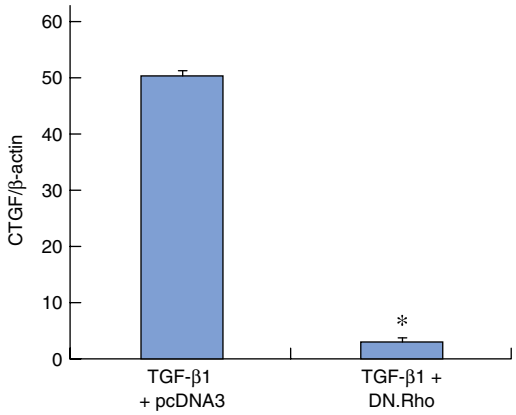
The concentration of total human PAI-1 in conditioned media was determined by enzyme-linked immunosorbent assay (Innovative Research, Novi, Michigan, USA) according to the manufacturer's instructions. The assay reacts with functionally active human PAI-1; latent



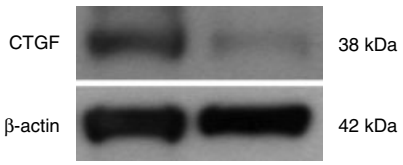
a CTGF



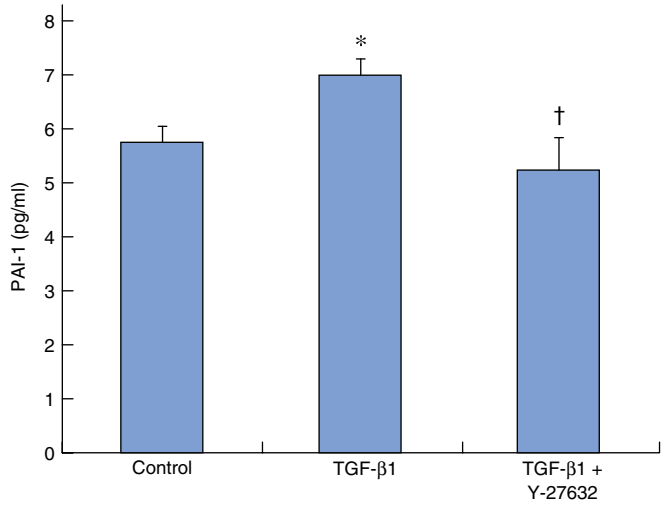
b CTGF



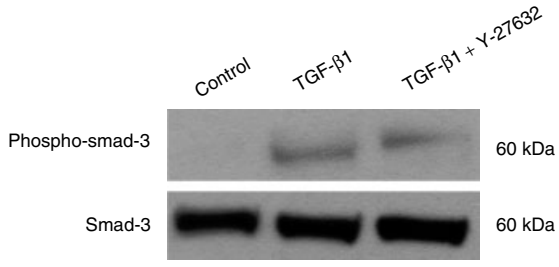
c CTGF



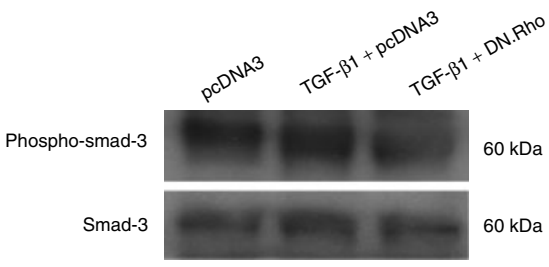
d CTGF



e PAI-1



f Smad phosphorylation



g Smad phosphorylation

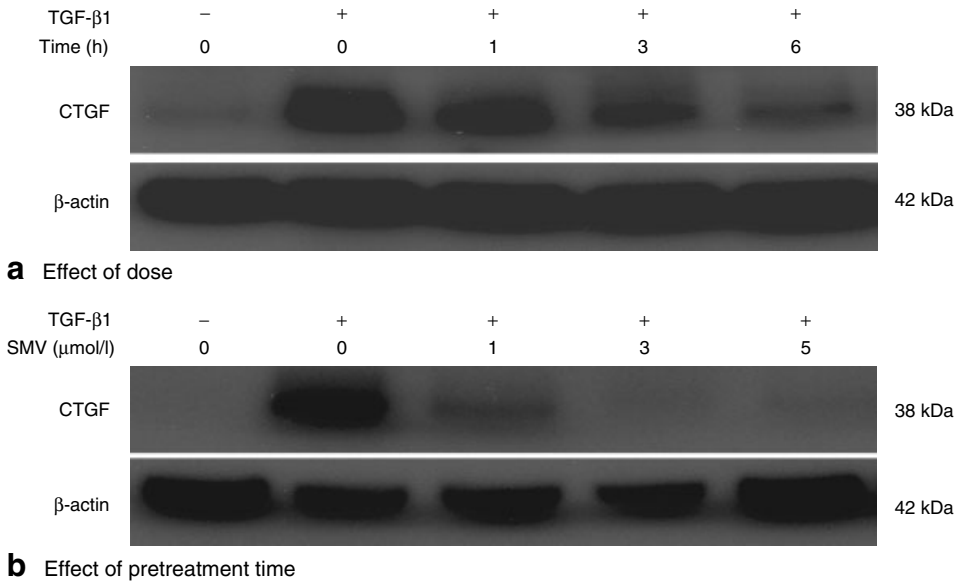


Fig. 3 Effect of simvastatin (SMV) pretreatment on transforming growth factor (TGF) β1-mediated induction of connective tissue growth factor (CTGF). Pretreatment with **a** 1 μmol/l for different lengths of time and **b** escalating doses. Fibroblast CTGF expression was determined by western blot analysis; blots are representative of three independent experiments

or complexed PAI-1 was not detected. Results were normalized according to cell number in the monolayer by determining total protein content of the corresponding sample.

Confocal microscopy for actin microfilaments

Cells were fixed in 3·7 per cent paraformaldehyde, permeabilized in 0·2 per cent Triton X-100 in phosphate-buffered saline (PBS), and blocked in PBS containing 5 per cent goat serum for 1 h at room temperature. Filamentous (F) actin was labelled with Texas red phalloidin (Molecular Probes, Eugene, Oregon, USA), at a concentration of 3 in 100 for 1 h at room temperature, and the nuclei were labelled with 4′,6-diamidino-2-phenylindole at a concentration of 2 ηg/ml by immersion. The fluorescence images were acquired with an LSM 510 confocal microscope (Zeiss, Hamburg, Germany) equipped with argon and helium/neon lasers, using an inverted ×100, 1·4 numerical aperture, oil-immersion objective.

Fibroblast-populated collagen lattice

Free-floating fibroblast-populated collagen lattices (FPCLs) were prepared as described previously in 24-well sterile culture plates[25]. Intestinal fibroblasts were mixed with rat tail collagen type I and RPMI containing 1 per cent FCS to give final concentrations of $6{\cdot}25 \times 10^4$/ml fibroblasts and 1·25 mg/ml collagen in a

Fig. 2 Effect of Rho/Rho-associated coiled kinase (ROCK) inhibition with Y-27632 on transforming growth factor (TGF) β1-mediated induction of connective tissue growth factor (CTGF), activation of plasminogen activator inhibitor (PAI) 1 and phosphorylation of smad-3. Intestinal fibroblasts were pretreated with Y-27632 before addition of TGF-β1. Values are mean(s.e.m.).
a Fibroblast CTGF expression determined by densitometric analysis of western blots and **b** representative western blots. *$P = 0{\cdot}002$ *versus* control, †$P = 0{\cdot}004$ *versus* TGF-β1; $n = 3$ per group (Student's *t*-test). **c** CTGF expression determined by densitometric analysis of western blots and **d** representative western blots after transfection of CCD-18Co cells with an empty vector (pcDNA3) or a plasmid encoding the dominant negative N19.Rho mutant (DN.Rho) 24 h before addition of TGF-β1. *$P = 0{\cdot}004$ *versus* pcDNA3; $n = 3$ per group (Student's *t*-test). **e** Fibroblast PAI-1 expression determined by enzyme-linked immunosorbent assay. *$P = 0{\cdot}022$ *versus* control, †$P = 0{\cdot}032$ *versus* TGF-β1; $n = 3$ and $n = 5$ per group respectively (ANOVA). **f** Primary intestinal fibroblasts were pretreated with Y-27632, or **g** CCD-18Co cells were transfected with either pcDNA3 or a DN.Rho plasmid, before the addition of TGF-β1, and smad-3 phosphorylation was assessed by western blot; all blots are representative of three independent experiments

total volume of 0·5 ml. Incubation at 37°C, 5 per cent carbon dioxide for 25 min caused the mixture to set, and the resulting gel was freed from the well by gentle manipulation with a pipette tip and refloated in 0·5 ml RPMI culture medium containing 1 per cent FCS and test substances. Contracting lattices were photographed at 24 h over a light box from a fixed height using a mounted digital camera (Powershot A70; Canon, Lake Success, New York, USA). Analysis of calibrated images allowed area determination (ImageJ Software, version 1.26t; National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

All data are expressed mean(s.e.m.). For western blot experiments, densitometric values for protein bands were adjusted for β-actin expression, and statistical analysis performed on the resulting values. For contraction studies statistical analysis was performed on percentage reduction in gel surface area. Statistical significance between groups was determined using Student's *t* test or ANOVA, as appropriate. $P < 0{\cdot}050$ was considered significant. Data were analysed using SPSS® version 12.0 (SPSS, Chicago, Illinois, USA).

Results

Effect of Rho-associated serine–threonine protein kinase inhibition

Following 24 h of stimulation with 1 ηg/ml TGF-β1 fibroblasts demonstrated an 11-fold increase in CTGF expression (5·6(0·6) *versus* 63·0(7·5) relative density (RD); $P = 0{\cdot}002$) (*Fig. 2a,b*). Inhibition of the Rho/ROCK pathway with 10 μmol/l Y-27632 for 30 min before addition of TGF-β1 significantly attenuated the effect of TGF-β1 on CTGF induction (63·0(7·5) *versus* 13·5(3·5) RD; $P = 0{\cdot}004$). Cells transfected with 1 μg of a plasmid encoding a dominant negative RhoA vector (N19Rho.dn3) produced less CTGF in response to TGF-β1 stimulation than those mock-transfected with 1 μg of the empty vector pcDNA3 and subsequently stimulated with TGF-β1 (50·3(4·1) *versus* 2·9(0·3) RD; $P = 0{\cdot}004$) (*Fig. 2c,d*).

PAI-1 inhibits the breakdown of extracellular matrix. Steady-state activity of PAI-1 was significantly increased in fibroblasts exposed to TGF-β1 (5·76(0·29) *versus* 6·99(0·32) pg/ml; $P = 0{\cdot}022$). Pretreatment of fibroblasts with Y-27632 abrogated the effect of TGF-β1 on PAI-1 activation (6·99(0·32) *versus* 5·25(0·59) pg/ml; $P = 0{\cdot}032$) (*Fig. 2e*).

Smad-3 is the primary effector of the profibrotic responses to TGF-β1. Stimulation with 1 ηg/ml TGF-β1 for 30 min induced smad-3 phosphorylation which was reduced by inhibition of the Rho/ROCK pathway with Y-27632 (*Fig. 2f*) or transfection with the dominant negative Rho vector (*Fig. 2g*).

Effect of simvastatin pretreatment on markers of intestinal fibrogenesis

Intestinal fibroblasts preincubated in 1 per cent FCS with simvastatin at increasing concentrations (0–5 μmol/l) for

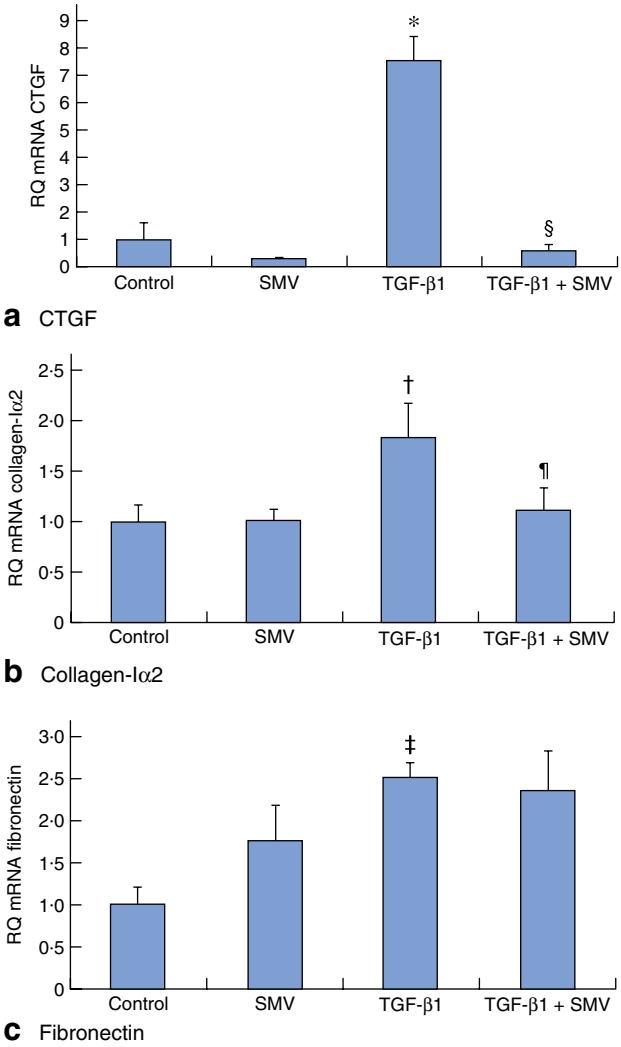


Fig. 4 Effect of pretreatment with 5 μmol/l simvastatin (SMV) for 6 h on transforming growth factor (TGF) β1-mediated induction of **a** connective tissue growth factor (CTGF), **b** collagen-Iα2 and **c** fibronectin mRNA in intestinal fibroblasts. The relative quantity (RQ) mRNA expression of candidate genes was examined. Values are mean(s.e.m.). *$P < 0{\cdot}001$, †$P = 0{\cdot}015$, ‡$P = 0{\cdot}004$ *versus* control; §$P < 0{\cdot}001$, ¶$P = 0{\cdot}014$ *versus* TGF-β1; $n = 3$ per group (ANOVA)

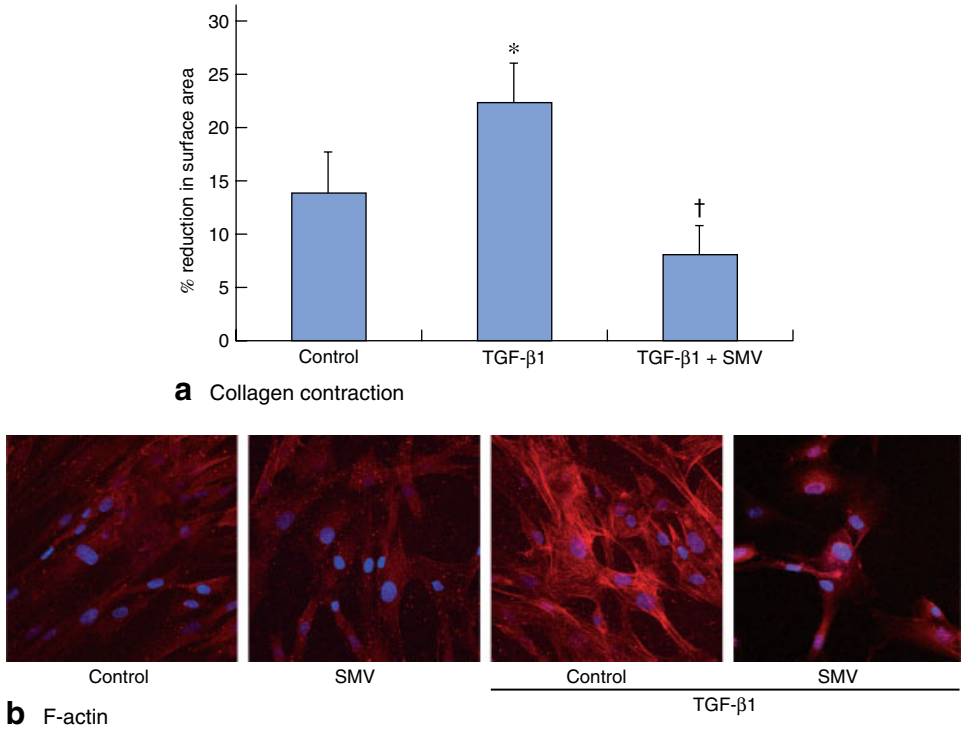


Fig. 5 Effect of pretreatment with 5 μmol/l simvastatin (SMV) for 6 h on transforming growth factor (TGF) β1-mediated **a** collagen contraction and **b** filamentous (F) actin accumulation. Collagen contraction was assessed in fibroblast-populated collagen lattices. Values are mean(s.e.m.). *$P = 0{\cdot}04$ *versus* control, †$P = 0{\cdot}005$ *versus* TGF-β1; $n = 3$ per group (Student's *t*-test). F-actin, stained with rhodamine–phalloidin, was visualized by confocal microscopy; nuclei were stained with 4′,6-diamidino-2-phenylindole. Photographs were taken under identical conditions and data shown are representative of three experiments for each group (original magnification × 400)

increasing lengths of time (0–6 h) demonstrated maximal inhibition of TGF-β1-mediated CTGF protein induction following pretreatment for 6 h (*Fig. 3a*) at a concentration of 5 μmol/l (*Fig. 3b*).

TGF-β1 significantly induced CTGF mRNA following 4 h exposure (1·00(0·58) *versus* 7·55(0·88) relative quantity (RQ) mRNA; $P < 0{\cdot}001$). Simvastatin pretreatment reduced TGF-β1-induced CTGF expression (7·55(0·88) *versus* 0·58(0·24) RQ; $P < 0{\cdot}001$) (*Fig. 4a*). TGF-β1 also induced the extracellular matrix component collagen-Iα2 following 4 h of exposure (1·00(0·17) *versus* 1·84(0·34) RQ; $P = 0{\cdot}015$). Simvastatin pretreatment attenuated TGF-β1-mediated induction of collagen-Iα2 (1·84(0·34) *versus* 1·11(0·23) RQ; $P = 0{\cdot}014$) (*Fig. 4b*). Furthermore, TGF-β1 induced fibronectin following 4 h of exposure (1·00(0·21) *versus* 2·51(0·18) RQ; $P = 0{\cdot}004$). However, simvastatin pretreatment did not prevent TGF-β1-mediated induction of fibronectin (2·51(0·18) *versus* 2·35(0·47) RQ; $P = 0{\cdot}625$) (*Fig. 4c*).

Effect of simvastatin pretreatment on collagen contraction and F-actin accumulation

In an *in vitro* FPCL model, TGF-β1 enhanced fibroblast-mediated collagen contraction (13·8(2·3) *versus* 22·3(2·2) per cent reduction in lattice surface area at 24 h; $P = 0{\cdot}040$). Pretreatment with simvastatin prevented TGF-β1-stimulated fibroblast contraction (22·3(2·2) *versus* 8·0(1·6) per cent reduction in lattice surface area at 24 h; $P = 0{\cdot}005$) (*Fig. 5a*).

Confocal microscopy showed that simvastatin treatment for 6 h resulted in F-actin stress fibre dissolution. TGF-β1 treatment stimulated F-actin polymerization which was inhibited by simvastatin pretreatment (*Fig. 5b*).

Effect of simvastatin in combination with isoprenoid intermediates on plasminogen activator inhibitor 1 activity

TGF-β1 significantly increased PAI-1 activity in intestinal fibroblasts (5·76(0·29) *versus* 6·99(0·32) pg/ml; $P = 0{\cdot}022$).

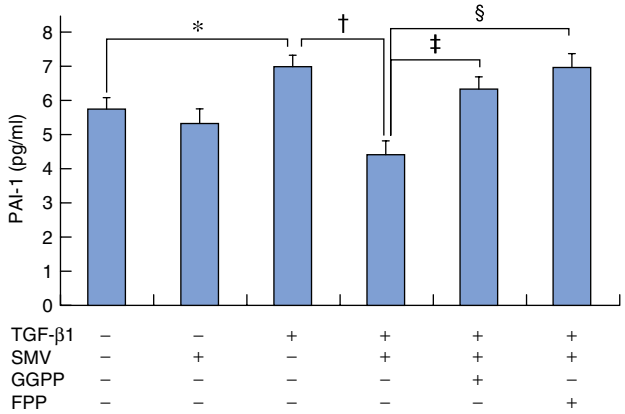


Fig. 6 Effects of pretreatment with 5 μmol/l simvastatin (SMV) for 6 h, in combination with the isoprenoid intermediates geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), on transforming growth factor (TGF) β1-mediated changes in fibroblast plasminogen activator inhibitor (PAI) 1 activity. Values are mean(s.e.m.). *$P = 0{\cdot}022$, †$P = 0{\cdot}001$, ‡$P = 0{\cdot}007$ and §$P = 0{\cdot}002$; $n = 5$ per group (ANOVA)

Pretreatment with simvastatin reduced basal (5·76(0·3) *versus* 5·32(0·43) pg/ml; $P = 0{\cdot}417$) and prevented TGF-β1-induced (6·99(0·32) *versus* 4·41(0·42) pg/ml; $P = 0{\cdot}001$) PAI-1 activity. However, addition of the isoprenoid cholesterol pathway intermediates GGPP (10 μmol/l) (4·41(0·42) *versus* 6·32(0·34) pg/ml; $P = 0{\cdot}007$) and FPP (10 μmol/l) (4·41(0·42) *versus* 6·98(0·38) pg/ml; $P = 0{\cdot}002$) reversed the inhibitory effects of simvastatin on TGF-β1-mediated PAI-1 activation (*Fig. 6*).

Effect of simvastatin pretreatment on intracellular transforming growth factor β signalling

Intestinal fibroblasts were stimulated for 30 min with TGF-β1, and phosphorylation of smad-2 and smad-3 was assessed by western blot. TGF-β1 induced smad-2, smad-3 and ERK-1/2 phosphorylation. Simvastatin pretreatment had no effect on basal or stimulated smad-2 or ERK-1/2 phosphorylation, but it reduced constitutive

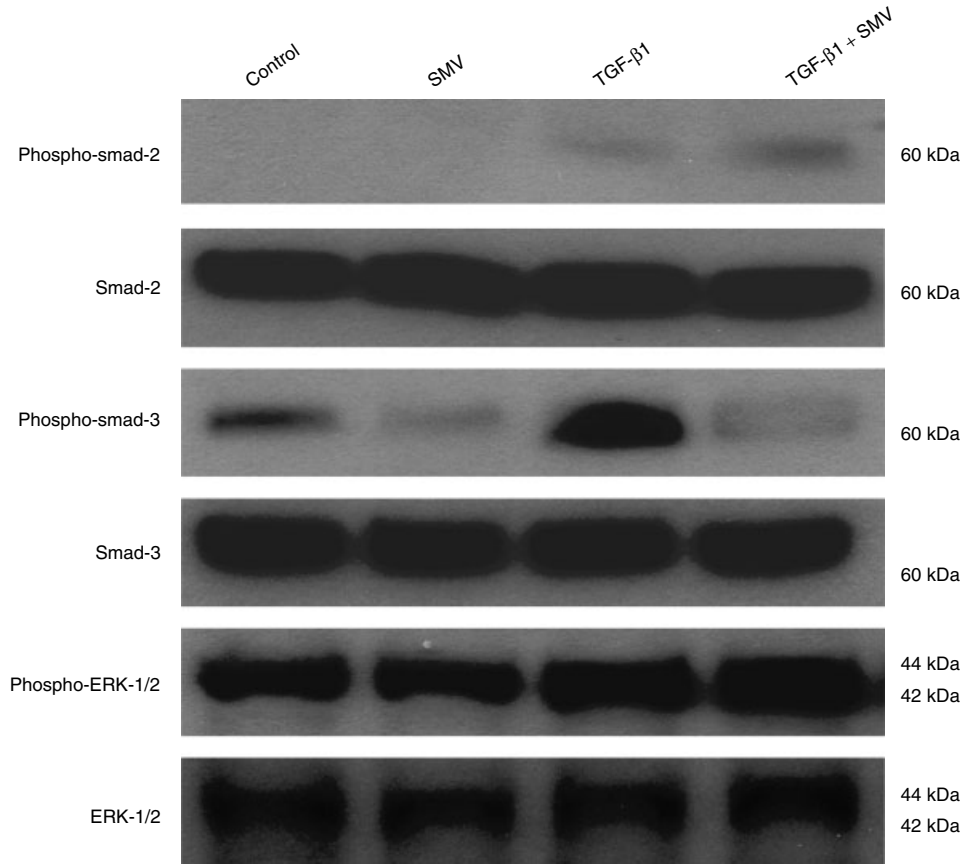


Fig. 7 Effects of pretreatment with 5 μmol/l simvastatin (SMV) for 6 h on transforming growth factor (TGF) β1 signalling in intestinal fibroblasts. Unphosphorylated and phosphorylated smad-2, smad-3 and extracellular signal-regulated protein kinase (ERK) 1/2 were assessed by western blot; all blots are representative of three independent experiments

smad-3 phosphorylation and completely abrogated TGF-β1-induced smad-3 phosphorylation (*Fig. 7*).

Discussion

In this study, inhibition of the Rho/ROCK pathway reduced TGF-β1-mediated activation of intestinal fibroblasts, coincident with inhibition of smad-3 phosphorylation. This raises the potential for the modulation of this pathway as a treatment in fibrostenosing intestinal diseases such as Crohn's disease. However, some inhibitors of the Rho/ROCK pathway (such as Y-27632) have undesirable systemic effects[26]. Simvastatin, a known inhibitor of Rho/ROCK signalling, was shown in the present study to reduce TGF-β1-mediated induction of CTGF, collagen-Iα2 and activation of PAI-1, all established components of fibrogenesis. Simvastatin pretreatment also reduced intracellular F-actin accumulation and subsequent collagen contraction.

The inhibitory effects of simvastatin on PAI-1 activation were reversed by the addition of FPP or GGPP, isoprenoid pyrophosphates necessary for post-translational prenylation of Rho[27], confirming the involvement of the cholesterol biosynthetic pathway in this process (*Fig. 1*). The inhibitory effects of simvastatin were not observed in TGF-β1-mediated induction of fibronectin transcription, because fibronectin transcription is an ERK-1/2-dependent response to TGF-β[28]. No change in basal or stimulated ERK-1/2 pathway activity was observed in response to simvastatin.

Up to now it has been believed that the effects of statins on the profibrotic response to TGF-β are dependent on Rho/ROCK pathway inhibition[21]. Impaired F-actin stress fibre stabilization and an increase in G-actin leading to reduced CTGF production[29] may be important in this, there being an inverse relationship between CTGF gene expression and monomeric actin[30]. The present study showed that simvastatin directly inhibits TGF-β1 signalling by inhibiting smad-3 phosphorylation.

The observation that simvastatin pretreatment prevents TGF-β1-mediated smad-3 phosphorylation while preserving smad-2 phosphorylation is interesting when the emerging roles of smad-2 and smad-3 in TGF-β1 signal transduction are considered. Smad-2 specifically is required for the anti-inflammatory actions of TGF-β in intestinal epithelial cells[31]. Furthermore, smad-3 may be the primary mediator of TGF-β1-associated fibrotic and proinflammatory responses[32–35]. Studies using smad-3 knockout mice have shown that most of the profibrotic actions of TGF-β are mediated by smad-3. Smad-3 null inflammatory cells and fibroblasts do not respond to the chemotactic effects of TGF-β and do not autoinduce TGF-β. Loss of smad-3 also interferes with TGF-β-mediated induction of collagens and PAI-1[33]. Smad-3 null mice are resistant to radiation-induced cutaneous fibrosis, bleomycin-induced pulmonary fibrosis and carbon tetrachloride-induced hepatic fibrosis[33].

Phosphorylation at the Ser203 and Ser207 residues is required for the full transactivation potential of smad-3 and these residues are targets of the Rho/ROCK pathway[36]. RhoA signalling is critical to TGF-β-induced smooth muscle cell differentiation. Blockade of RhoA inhibits nuclear translocation of smad-2 and -3 through inhibition of phosphorylation[37]. TGF-β1-mediated stress fibre restructuring requires interaction between smad and Rho GTPase signalling pathways[38]. Taken together, these data point to a role for RhoA as a modulator of canonical TGF-β1 signalling. The redundancy that exists between canonical smad signalling and the Rho kinase pathway presents an ideal opportunity for pharmacotherapeutic intervention.

Molecular inhibitors of smad-3 phosphorylation represent an attractive therapeutic target in fibrogenesis. In the present study simvastatin, a HMG-CoA reductase inhibitor, was shown to modulate smad-3-mediated gene transcription. Future investigation using *in vivo* murine models of intestinal fibrosis[39] may provide a platform for clinical studies in patients with intestinal fibrostenotic diseases for which there are currently no effective preventive or non-surgical therapeutic strategies.

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