INTESTINAL MOTILITY

# Inhibition of Rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression

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**Background:** Late radiation enteritis in humans is associated with accumulation of extracellular matrix and increased connective tissue growth factor (CTGF) expression that may involve intestinal muscular layers.
**Aims:** We investigated the molecular pathways involved in maintenance of radiation induced fibrosis by gene profiling and postulated that alteration of the Rho pathway could be associated with radiation induced fibrogenic signals and CTGF sustained expression.
**Patients and methods:** Ileal biopsies from individuals with and without radiation enteritis were analysed by cDNA array, and primary cultures of intestinal smooth muscle cells were established. Then, the effect of pharmacological inhibition of p160 Rho kinase, using Y-27632, was studied by real time reverse transcription-polymerase chain reaction, western blot, and electrophoretic mobility shift assay.
**Results:** Molecular profile analysis of late radiation enteritis showed alterations in expression of genes coding for the Rho proteins. To investigate further the involvement of the Rho pathway in intestinal radiation induced fibrosis, primary intestinal smooth muscle cells were isolated from radiation enteritis. They retained their fibrogenic differentiation in vitro, exhibited a typical cytoskeletal network, a high constitutive CTGF level, increased collagen secretory capacity, and altered expression of genes coding for the Rho family. Rho kinase blockade induced a simultaneous decrease in the number of actin stress fibres, α smooth muscle actin, and heat shock protein 27 levels. It also decreased CTGF levels, probably through nuclear factor κB inhibition, and caused decreased expression of the type I collagen gene.
**Conclusion:** This study is the first showing involvement of the Rho/Rho kinase pathway in radiation fibrosis and intestinal smooth muscle cell fibrogenic differentiation. It suggests that specific inhibition of Rho kinase may be a promising approach for the development of antifibrotic therapies.

Late intestinal toxicity is one of the most common complications of pelvic radiation therapy. It may occur several months to years after radiation therapy and may significantly alter the quality of life of cancer survivors. Radiation enteritis is characterised by severe transmural fibrosis associated with extracellular matrix remodelling.[1 2] Tissue stricture is responsible for loss of the compliant relationship between the mucosa and muscularis layers and the ensuing loss of intestinal function.

Intestinal function depends on both its transport capacity and its motility, which ensures peristalsis. The contraction process is mainly controlled by the enteric nervous system and is achieved by smooth muscle cells. The structural and also the functional role of intestinal smooth muscle cells in intestinal connective tissue homeostasis, repair, remodelling, and fibrosis is increasingly recognised.[3 4] During fibrogenesis, intestinal function is dramatically altered due to impaired motility[5] and excessive transmural deposition of collagen secreted by fibrosis activated subepithelial myofibroblasts and smooth muscle cells.[1] The fibrogenic phenotype of intestinal smooth muscle cells has been poorly investigated[6] but differential isoactin expression (α smooth muscle actin (α-sm actin) *v* γ smooth muscle actin (γ-sm actin)) has been found to be associated with synthetic or contractile smooth muscle cells in vitro.[7] In radiation enteritis, we found a high expression level of α-sm actin associated with increased collagen deposition and increased expression of the fibrogenic growth factor connective tissue growth factor (CTGF) in the muscularis propria.[1] This suggests that CTGF could be associated with radiation induced fibrogenic differentiation in intestinal smooth muscle cells. Thus understanding the mechanisms responsible for CTGF over-expression in intestinal smooth muscle cells may give new insights into the maintenance of radiation enteritis.

In the present study, we investigated regulation of CTGF gene expression in intestinal radiation induced fibrosis by cDNA array and found specific alteration of genes coding for proteins of the Rho family. Rho proteins belong to a family of small GTPases (RhoA, B, C, Rac-1, cdc 42) that control a wide range of cellular functions including cell adhesion, formation of stress fibres, and cellular contractility through reorganisation of actin based cytoskeletal structures.[8 9] Modulation of these cellular functions by Rho proteins largely depends on activation of their downstream effector, Rho kinase (ROCK).[10] Furthermore, Heusinger-Ribeiro *et al* showed that CTGF gene expression depends on the Rho signalling

**Abbreviations:** CTGF, connective tissue growth factor; α/γ-sm actin, α/γ smooth muscle actin; HSP, heat shock protein; ROCK, Rho kinase; N/RE SMC, normal/radiation enteritis smooth muscle cells; COL1A1, type I collagen alpha 1; MLCK, myosin light chain kinase; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; NFκB, nuclear factor κB; TNF-α, tumour necrosis factor α; TGF-β1, transforming growth factor β1

pathway during kidney fibrogenesis.[11] Thus we hypothesised that both overexpression of CTGF and appearance of an immature cytoskeleton in intestinal fibrosis activated smooth muscle cells may be regulated by the Rho/ROCK pathway. We analysed the involvement of the Rho/ROCK pathway in the regulation of CTGF gene expression and actin cytoskeleton using physiologically relevant primary cultures of intestinal smooth muscle cells from individuals with and without radiation enteritis, together with a specific inhibitor of ROCK, Y-27632.

## PATIENTS AND METHODS

### Tissue sampling and immunohistochemistry

Tissue sampling was performed as previously described[1] and patient characteristics are shown in table 1. Procurement of tissue samples received prior approval from our institution's ethics committee and was performed according to the guidelines of the French Medical Research Council. Immunostaining was performed on fixed paraffin embedded samples sectioned at 5 µm, using an automated immunostainer (Ventana Medical Systems, Illkirch, France) with the avidin-biotin-peroxidase complex method. Collagen deposition was assess by Sirius red staining and adjacent sections were incubated with antibodies against vimentin (1:50; Sigma, St Quentin Fallavier, France) and CTGF (1:100; a gift from AC de Gouville).

### Cells, immmunofluorescence, and confocal laser microscopy

Primary intestinal smooth muscle cells were isolated from the muscularis propria by complete enzymatic digestion at 37°C (0.2% type II collagenase and 0.1% soybean trypsin inhibitor), subcultured in SmGM2 (Cambrex, Emerainville, France), and used between P3 and P4. Three cell lines were isolated from normal ileal muscularis propria and two cells lines from fibrotic muscularis propria. Confluent monolayers of normal (N SMC) and fibrotic (RE SMC) smooth muscle cells were incubated with 10, 50, and 100 µM Y-27632 (Bioblock, Illkirch, France) and subsequently analysed. After fixation (0.5% paraformaldehyde) and permeabilisation (0.1% triton X-100), cells were incubated with phalloidin-FITC (Sigma) or with primary antibodies and FITC conjugated antibody, rinsed, and incubated in Rnase A/propidium iodide. Stained cells were imaged by laser scanning confocal microscopy.

### Gene array analysis

Total RNA was extracted from tissue (n = 6 normal ileum and n = 6 radiation enteritis) and confluent cells (n = 3 N SMC and n = 2 RE SMC) by the method of Chomczynski and Sacchi,[12] quantified by absorption spectrometry, and treated with RNase free DNase (0.5 unit/µl) to remove contaminating genomic DNA. Atlas Human 1.2 (1176 genes + nine housekeeping genes) and Cell Interaction (265 genes + nine housekeeping genes) expression arrays from Clontech Laboratories (Ozyme, St Quentin en Yvelines, France) were used, as previously described.[13] (A list of all of the genes included in these two arrays as well as their functions can be found at www.clontech.com/atlas and is deposited in the GEO database (www.ncbi.nih.gov/geo) under GEO accession numbers GPL127 and GPL135.) Duplicate radiolabelled probes were generated from a single preparation of RNA. Hybridisation intensities were obtained using the Atlas Image 1.5 software, converted into ratios, and adjusted for background and housekeeping gene expression:

(Gene × intensity − background) / (average intensity for housekeeping gene − background)

Baseline gene expression was established by averaging the arrays obtained from six control samples; 25–35% variation in gene expression was observed in the control group. This allowed us to create a single ''normal composite array'' used to compare the set of normal samples with each radiation enteritis sample. A change in gene expression greater than twice that of the averaged control group was considered significant and data were used only when signal intensities were above background (that is, 50% or more).

### mRNA expression analysis using quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Real time RT-PCR was performed as previously described.[1] CTGF FAM probe was purchased from PE Biosystems (Courtaboeuf, France). CTGF, 5′-TGT GTG ACG AGC CCA AGG A-3′ (forward) and 5′-TCT GGG CCA AAC GTG TCT TC-3′ (reverse); 5′-FAM, CTG CCC TCG CGG CTT ACC GA-3′; type I collagen alpha 1 (COL1A1), 5′-CCT CAA GGG CTC CAA CGA G-3′ (forward) and 5′-TCA ATG ACT GTC TTG CCC CA-3′ (reverse); γ-sm actin, 5′-GCC CTC AGT CAC TGG GAG-3′ (forward) and 5′-TGT GTG GCT GAG TGA GCT GG-3′ (reverse); RhoB, 5′-GTC CCA ATG TGC CCA TCA TC-3′ (forward) and 5′-CTG TGC GGA CAT BCT CGT C-3′ (reverse). Optimised PCR used the ABI PRISM 7700 detection system in the presence of 135 nM specific forward, reverse primers, and fluorogenic probe. Both water and genomic DNA controls were included to ensure specificity. The purity of each PCR product was checked by analysing the amplification plot and dissociation curves. Relative mRNA quantitation was performed using the comparative ΔΔCT method.

### Procollagen type I secretion

Confluent cells were cultured for 24 hours under serum free conditions and procollagen type I secretion was determined using the Procollagen Type I C-Peptide EIA kit (Takara Biomedicals, Cambrex).

### Western blot analysis

Expression of heat shock protein (HSP) 27 (SPA-800; Stressgen Biotechnologies, Victoria, BC, Canada), α-sm actin, RhoA (sc-418; Santa-Cruz), and CTGF (sc-14939, Santa-Cruz

**Table 1** Characteristics of the patient population

| Time after RT (months) | Tumour site | Age (y) | Sex | Treatment |
|---|---|---|---|---|
| Patients with ileal stricture (n = 6) | | | | |
| 1–3 months | Rectal cancer (n = 2) | 44–68 | M/F | n = 2: RT 45 Gy/S/CT |
| 4–16 months | Gynaecological cancer (n = 3) | 37–49 | F | n = 1: S/RT 45 Gy/CT;<br>n = 1: S/RT 45 Gy/CT/BT 10–15 Gy;<br>n = 1: S/BT 60 Gy |
| 75 months | Hodgkin disease (n = 1) | 51 | M | RT 40 Gy/CT |
| Patients with normal ileum (n = 6) | Colon adenocarcinoma | 29–81 | 3M/3F | Non irradiated,<br>Right hemicolectomy |

RT, pelvic radiotherapy; BT, brachytherapy; CT, chemotherapy; S, surgery.

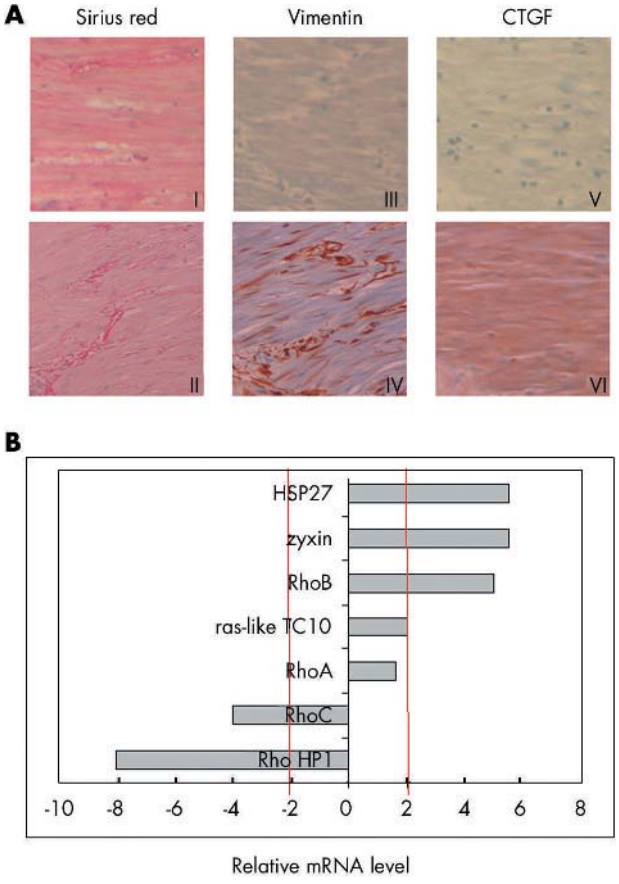


**Figure 1** (A) Intestinal smooth muscle cells exhibited fibrogenic differentiation in vivo. In the muscularis propria, Sirius red staining showed collagen infiltration within smooth muscle bundles in radiation enteritis (II, ×200) versus normal bowel (I, ×200) that colocalised with vimentin positive cells (IV, ×200). Connective tissue growth factor (CTGF) immunostaining was negative in normal muscularis propria (V, ×200) whereas strong staining was observed in radiation enteritis (VI, ×200). (B) Gene array analysis revealed induction of genes coding for the Rho family and for actin polymerisation control in radiation enteritis samples (n = 6) compared with normal bowel samples (n = 6).

distributed by Tebu-Bio SA, Le Perray en Yvelines, France) were assessed by western blot analysis on total protein extracts from tissue or cells ($2–3\times10^6$) incubated or not with Y-27632 (10, 50, and 100 µM for 18 hours). Furthermore, nuclear and cytoplasmic protein extracts were prepared using the method of Schreiber and colleagues[14] from cells ($1\times10^6$) incubated or not with Y-27632 (10 µM for 30 and 120 minutes) and sodium salicylate (25 mM for 45 minutes). Nuclear extracts were used in electrophoretic mobility shift assay (EMSA) experiments. Cytoplasmic extracts were used to measure IκB-α (sc-371; Santa-Cruz) and p65 (sc-8008; Santa-Cruz) protein levels by western blot. Proteins (5–15 µg) were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a 0.45 µm nitrocellulose membrane. The membrane was incubated with the primary antibody, washed, and probed with the peroxidase labelled secondary antibody. Detection was achieved by enhanced chemiluminescence (ECL Amersham Pharmacia, Orsay, France). After dehybridisation, control loading was achieved by anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:2000; H86504M, Biodesign, Maine, USA). Densitometric analyses were performed using an image analyser (Biocom, Les Ulis, France) interfaced with the Phoretix image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

### Electrophoretic mobility shift assay (EMSA)

PAGE purified double stranded oligodeoxynucleotides containing nuclear factor κB (NFκB) binding elements (5′-GAG GAA TGT CCC TGT TTG-3′) were 5′ end labelled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Life Technology, Cergy Pontoise, France). End labelled probes were purified using a G-50 column (Pharmacia, Saclay, France) and $1\times10^5$ cpm were incubated with 2–5 µg nuclear extract for 30 minutes at room temperature in a final volume of 20 µl containing 25 mM Tris HCl, pH 8, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, and 1 µg/µl poly(dI-dC). For competition experiments, 10-fold excess cold competitor was added to the reaction mixture before incubation. Complexes were then resolved by 6% PAGE in 0.5× Tris-Borate-EDTA buffer. Gels were dried and complexes were visualised and quantified using an intensifying screen and a phosphorimager (Image Gauge software, FLA-3000, Fuji Ray Test, France).

### Statistical analysis

All values are reported as mean (SEM). Data were analysed using one way ANOVA and the Student-Newman-Keuls test.

## RESULTS

### Intestinal smooth muscle cells exhibited fibrogenic differentiation in vivo

Fibrogenic differentiation of intestinal smooth muscle cells was investigated in radiation enteritis muscularis propria by histological staining of collagen and immunohistochemical detection of cytoskeleton markers (α-sm actin, vimentin, desmin) as well as CTGF expression. Compared with normal bowel, collagen infiltration was observed in radiation enteritis (fig 1A), associated with accumulation of vimentin positive cells (fig 1A). Strong CTGF immunoreactivity was also observed in the muscularis propria smooth muscle cells from radiation enteritis (fig 1A).

### Genes coding for Rho family small GTPases and genes involved in actin polymerisation are altered in radiation enteritis samples

The global cDNA array approach revealed alterations in the expression profile of genes coding for intracellular signalling molecules of the Rho family. A significant and reproducible fivefold increase in RhoB gene expression was found in radiation enteritis samples (fig 1B) and confirmed by real time RT-PCR (×2.5, $p<0.05$). mRNA level of the gene coding for the ras-like protein TC10 reached a twofold increase whereas that of Rho HP1 and Rho C showed an eightfold and a fourfold decrease, respectively. Rho A mRNA level slightly increased in radiation enteritis samples (1.6-fold) but this difference was not confirmed at the protein level (data not shown). Expression of Cdc42 and Rac genes was not detected by cDNA array analysis nor were the genes coding for the LIM kinase and MLCK (myosin light chain kinase), which are involved in the control of actin polymerisation and act downstream of Rho. Conversely, gene expression of the actin filament assembly regulator zyxin and of the actin chaperone HSP27 significantly increased (5.5-fold) in radiation enteritis samples (fig 1B).

### Primary smooth muscle cells isolated from radiation enteritis biopsies exhibit a fibrogenic phenotype

In order to study the molecular mechanisms involved in the maintenance of radiation induced fibrogenic differentiation in intestinal smooth muscle cells, primary cells were derived from normal (N SMC) and fibrotic (RE SMC) muscularis propria.

Primary N SMC exhibited a typical phenotype with a characteristic spindle shaped morphology and the presence of

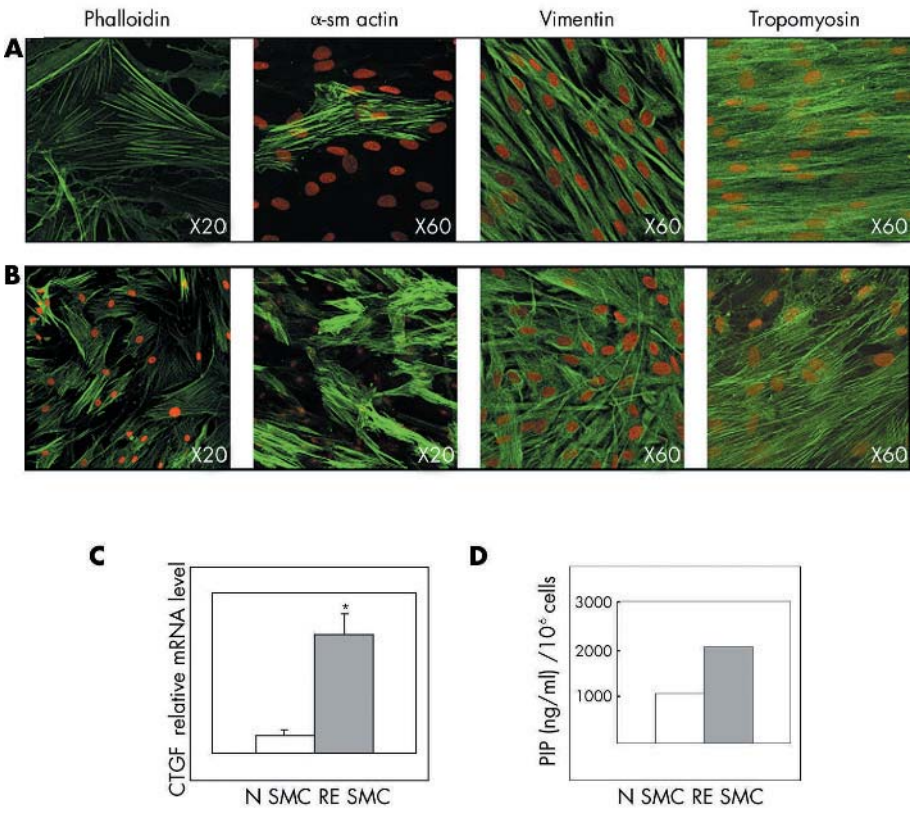


**Figure 2** (A) Immunofluorescence experiments showed that normal smooth muscle cells (N SMC) exhibited typical intestinal differentiation markers in vitro. They were positive for vimentin and tropomyosin whereas only a few cells expressed α smooth muscle actin (α-sm actin). (B) Smooth muscle cells derived from radiation enteritis (RE SMC) showed greater densities of stress fibres and a strong constitutive α-sm actin protein expression. RE SMC (two cell lines) exhibited a prosecretory phenotype with a higher constitutive connective tissue growth factor (CTGF) mRNA level (experiments done in triplicate; C) and procollagen type I (PIP) secretion (experiments done in duplicate; D) than their normal counterpart (three cell lines). *$p<0.05$ compared with untreated N SMC.

actin stress fibres. At confluency, spontaneous retraction occurs and produces ''hill and valley'' pictures, as previously described.[7 15] RE SMC exhibited a more compact morphology and higher density of stress fibres than their normal counterparts (fig 2A and B, phalloidin). Cellular differentiation was assessed using the markers proposed by Graham and colleagues[16] and Brittingham and colleagues.[7] No differences were found between N and RE SMC regarding vimentin, tropomyosin protein expression (fig 2A, B), and γ-sm actin mRNA levels (data not shown) whereas high levels of α-sm actin were found in RE SMC, suggesting an immature and synthetic phenotype. Semi quantitative western blot analysis confirmed the high α-sm actin constitutive level in RE SMC that was barely detected in N SMC (see fig 4C, lane 0).

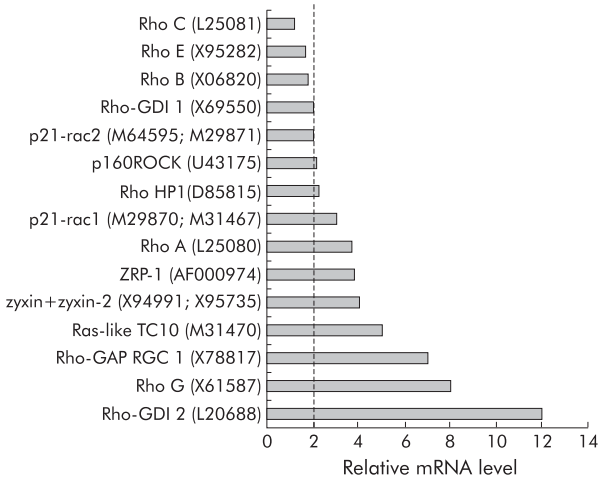


**Figure 3** Gene array analysis revealed induction in the expression profile of genes coding for the Rho pathway in radiation enteritis smooth muscle cells (RE SMC) compared with normal smooth muscle cells (N SMC). Hybridisation intensities were obtained using the Atlas Image 1.5 software, converted into ratios, and adjusted for background and housekeeping gene expression. A ''normal composite array'' was established by averaging the three arrays obtained from the three N SMC lines and compared with the two RE SMC lines.

The synthetic phenotype of RE SMC was confirmed by the CTGF and type I procollagen study. Constitutive CTGF mRNA level was higher in RE SMC versus N SMC, as assessed by cDNA array analysis (×2.5) and real time RT-PCR (×7) (fig 2C). Furthermore, RE SMC secreted twofold more type I procollagen than their normal counterparts, as measured by ELISA (fig 2D).

The global cDNA array approach confirmed induction of genes coding for the Rho pathway in RE SMC (fig 3). Expression of genes coding for Rho A, B, C, and p21Rac increased, together with that of the gene coding for the p160 Rho kinase and for zyxin. A threefold increase in RhoB mRNA level in RE SMC versus N SMC was observed by real time RT-PCR analysis ($p<0.05$). Conversely, genes coding for the LIM kinase and MLCK were not detected, and HSP27 mRNA remained unchanged. Levels of endogenous Rho protein inhibitors however simultaneously increased (Rho GDI -1, -2, Rho E).

## Rho kinase inhibition regulates the fibrogenic phenotype

To study the involvement of the Rho pathway in the maintenance of radiation induced fibrogenic differentiation, we used Y-27632, a pyrimidine derivative inhibitor of ROCK.

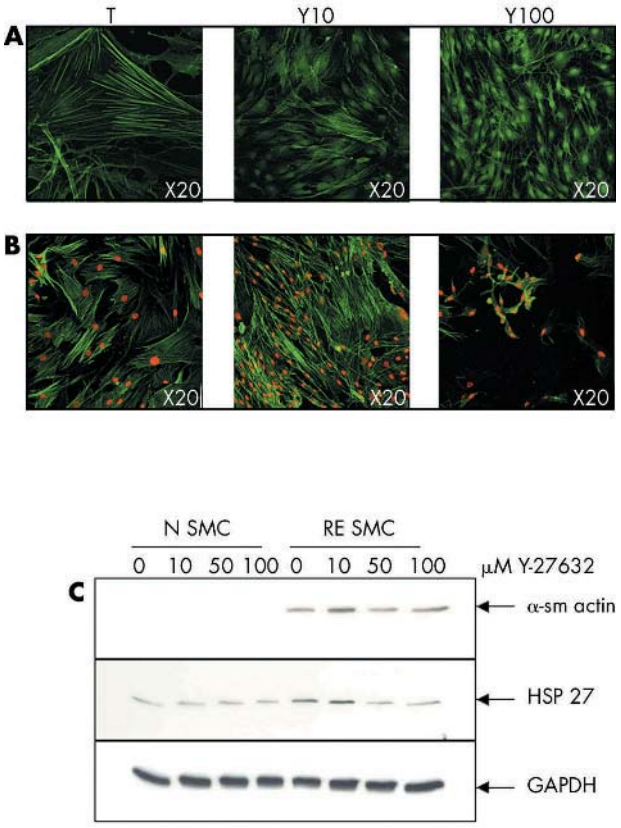


**Figure 4** Alteration of actin stress fibre network by Rho kinase inhibition. F-actin was determined by FITC-phalloidin staining after Y-27632 incubation in normal smooth muscle cells (N SMC) (A) and radiation enteritis smooth muscle cells (RE SMC) (B). Rho kinase inhibition decreased heat shock protein (HSP)27 and α smooth muscle actin (α-sm actin) protein expression. (C) HSP27 and α-sm actin protein levels were assessed by western blot. Values were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels. The blot is representative of three independent experiments.

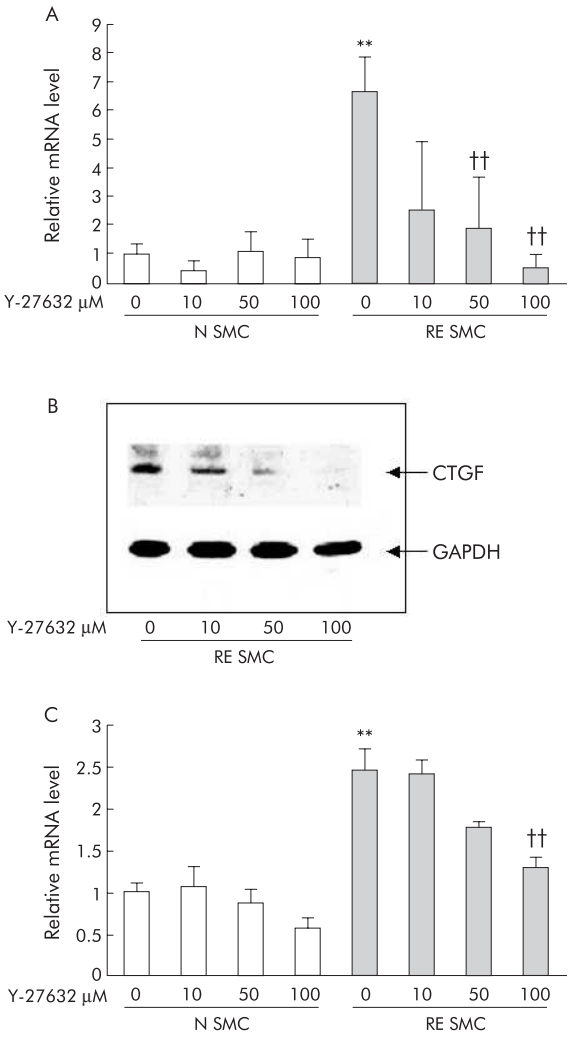


**Figure 5** (A, B) Regulation of connective tissue growth factor (CTGF) expression by Rho kinase inhibition. (A) CTGF mRNA level was assessed by real time reverse transcription-polymerase chain reaction (RT-PCR). Values are mean (SEM); n = 4. **p<0.01 compared with untreated normal smooth muscle cells (N SMC); ††p<0.01 compared with untreated radiation enteritis smooth muscle cells (RE SMC). (B) CTGF protein level was assessed by western blot in RE SMC. The blot is representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) Regulation of type I collagen alpha 1 (COL1A1) expression by Rho kinase inhibition. COL1A1 mRNA levels were assessed by real time RT-PCR. Values are mean (SEM); n = 4. **p<0.01 compared with untreated N SMC; ††p<0.01 compared with untreated RE SMC.

Similar qualitative and quantitative modifications of the stress fibre network were observed after 18 and 24 hours of Y-27632 incubation, thus subsequent analyses were performed after 18 hours of incubation except for COL1A1 gene expression. With the smallest doses (10 and 50 µM Y-27632), the originally flat and confluent cells had assumed a more rounded morphology, and F-actin staining became sparse, especially in the central cell body. With the higher dose (100 µM Y-27632), cells were found to lack stress fibres and had a rounded morphology with very few cytoplasmic processes (fig 4A, B). In RE SMC, the morphological modifications induced by high doses of Y-27632 suggested apoptotic features and were associated with a dose dependent decrease in α-sm actin and HSP27 protein levels (fig 4B, C). Analysis of CTGF expression levels in RE SMC after incubation with Y-27632 showed a significant dose dependent decrease in CTGF mRNA to levels detected in untreated N SMC (fig 5A). This was further confirmed by western blot (fig 5B). In order to investigate the CTGF inhibition cascade further downstream, we studied COL1A1 gene expression and showed that COL1A1 mRNA levels decreased significantly in RE SMC after 24 hours of incubation with 100 µM Y-27632 (fig 5C). In N SMC, Y-27632 had no significant effect on α−sm actin or HSP27 protein expression or on CTGF or COL1A1 mRNA levels.

## Rho kinase inhibition decrease NFκB DNA binding activity

Next we investigated the effect of ROCK inhibition on nuclear protein binding activity to NFκB consensus sequence located in the CTGF promoter. Incubation of cells with Y-27632 or sodium salicylate, an NFκB inhibitor, decreased NFκB DNA binding activity in RE SMC but not in N SMC (fig 6A). Western immunoblotting was used to determine whether inhibition of NFκB DNA binding activity occurs through stabilisation of the IκBα isotype. We found increased IκBα levels in cytoplasmic extracts of RE SMC treated with Y-27632 and sodium salicylate (fig 6B) which was not associated with increased levels of the p65 subunit in RE SMC (fig 6B).

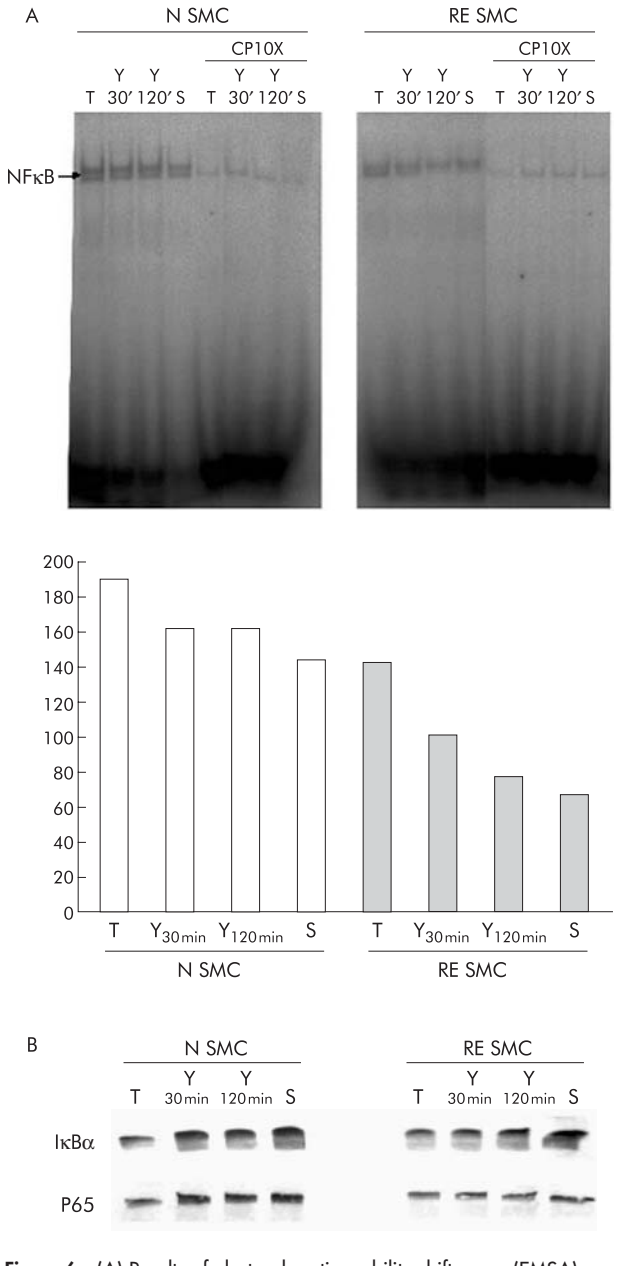


**Figure 6** (A) Results of electrophoretic mobility shift assay (EMSA) protein binding to the nuclear factor κB (NFκB) consensus site after incubation with 10 μM Y-27632 (Y) for 30 minutes and 120 minutes, and with 25 mM sodium salicylate (S) for 45 minutes. Non-radioactive NFκB oligo (CPX10) blocked NFκB DNA binding. The blot is representative of two independent experiments. (B) Western blot analysis of IκBα and p65 in cytoplasmic extract of normal smooth muscle cells (N SMC) and radiation enteritis smooth muscle cells (RE SMC) incubated with Y-27632 (Y) and sodium salicylate (S). The blot is representative of two independent experiments.

## DISCUSSION

The main finding of our study was that the small GTPase Rho/ROCK signalling pathway regulates the radiation induced fibrogenic programme. This conclusion was based on two observations: firstly, expression of the genes coding for proteins of the Rho/ROCK pathway was enhanced both in tissues and primary smooth muscle cells derived from radiation enteritis patients. Secondly, p160 ROCK blockade altered the actin network and decreased CTGF constitutive expression, most probably through inhibition of NFκB. Finally, CTGF inhibition led to decreased type I collagen

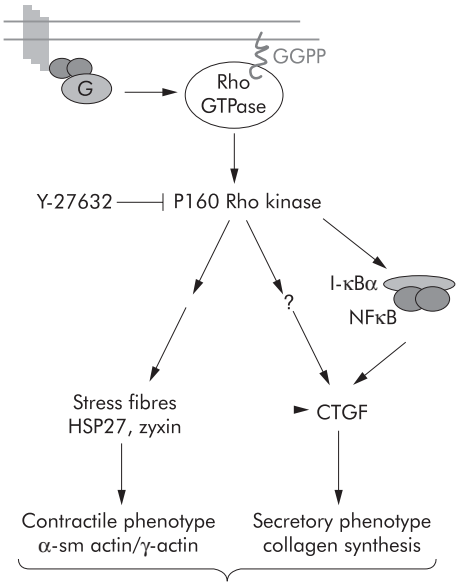


**Figure 7** Chronic activation of the Rho/Rho kinase (ROCK) cascade in radiation enteritis is involved in intestinal smooth muscle cell differentiation towards an immature phenotype with altered prosecretory and contractile properties. CTGF, connective tissue growth factor; α/γ-sm actin, α/γ smooth muscle actin; HSP, heat shock protein; NFκB, nuclear factor κB.

synthesis. Our results suggest that p160 ROCK blockade tends to reverse fibrogenic differentiation in vitro, and provides new insight into the molecular mechanisms involved in maintenance of radiation induced fibrosis in the intestine (fig 7).

In an effort to characterise the cellular phenotype involved in maintenance of late radiation induced fibrosis, we developed a useful in vitro model of radiation fibrosis. Here we showed that primary smooth muscle cells derived either from normal or radiation enteritis samples retained their respective phenotype after isolation and prolonged culture, as previously described in other culture models.[17–20] Intestinal smooth muscle cells derived from radiation enteritis samples maintained an immature (α-sm actin expression and prominent stress fibres) and synthetic phenotype (procollagen and CTGF expression) in vitro. Furthermore, our ex vivo and in vitro studies showed concomitant enhanced expression of CTGF, Rho proteins, and p160 ROCK in smooth muscle cells isolated from radiation enteritis, suggesting that alteration of the Rho/ROCK pathway may be associated with the activation network involved in the maintenance of radiation induced fibrogenic differentiation.

In smooth muscle cells derived from radiation enteritis samples, inhibition of p160 ROCK using Y-27632[21] elicited disruption of the actin cytoskeleton and decreased expression of α-sm actin. Furthermore, we observed concomitant decreased expression of the actin chaperone HSP27, suggesting that regulation of cell morphology and stress fibre formation may be mediated by HSP27. Indeed, HSP27 has been proposed as a molecular link between the Rho signal transduction cascade and the cytoskeleton.[22 23] HSP27 is required for orientation of the cytoskeletal network composed of actin, tropomyosin, myosin, and caldesmon,[24] and acts in conjunction with zyxin to mediate actin assembly.

Regulation of the intracellular actin network in fibrosis activated smooth muscle cells may affect the mechanical tension within the tissue and modulate tissue stricture. Furthermore, regulation of the cytoskeleton organisation affects gene expression. Indeed, Goppelt-Struebe's group

recently found that changes in the microtubular and actin fibre network regulated CTGF expression in immortalised human renal fibroblasts.[25] They showed that inhibition of Rho mediated signalling using various pharmacological agents, including Y-27632, prevented upregulation of CTGF induced by microtubule disrupting agents. Our results extend these observations to cellular models that are physiologically relevant to intestinal fibrosis, as the modulation obtained after Y-27632 incubation reached significance only in cells derived from radiation enteritis. Our data further showed that inhibition of ROCK reversed the established phenotype (that is, sustained high expression of CTGF). These observations indicate that the Rho/ROCK pathway may be involved in sustained overexpression of CTGF in radiation induced fibrosis and that it may contribute to maintenance of the fibrogenic phenotype.

The molecular mechanisms involved in the Rho/ROCK dependent control of CTGF expression remain to be investigated but one attractive hypothesis concerns the transcription factor NFκB.[26] Segain and colleagues[27] recently demonstrated that blockade of ROCK with Y-27632 prevented production of proinflammatory cytokines (tumour necrosis factor α (TNF-α), interleukin 1β) through inhibition of IκB kinase and NFκB activation in Crohn's disease. As the CTGF promoter includes a NFκB consensus binding site,[28 29] we tested this hypothesis in our primary cells and found that incubation with Y-27632 inhibited NFκB DNA binding activity and induced cytosolic stabilisation of IκBα. This suggests that a regulatory cascade is activated after incubation with Y-27632: inhibition of p160 ROCK prevents activation of IκB kinase, which in turn stabilises IκBα, and inhibits NFκB nuclear translocation and CTGF transcriptional activation. This hypothesis seems consistent with the findings of Segain *et al* but does not concur with prior findings by Abraham and colleagues.[30] The latter showed that TNF-α suppresses transforming growth factor β1 (TGF-β1) induced CTGF expression and proposed that this inhibition may be directly or indirectly mediated by NFκB activation. These discrepancies could be explained by the fact that different cellular models were used (physiological model of fibrosis versus TGF-β1 stimulated cells) and different tissues were targeted. Further studies will however be necessary to fully define how NFκB acts on CTGF transcriptional activation in our model and to determine if NFκB modulation could occur specifically in cells isolated from radiation enteritis. CTGF is involved in maintenance of the fibrogenic phenotype and transactivation of genes coding for components of the extracellular membrane,[31] and as such its inhibition may be a promising novel antifibrotic strategy. In our model, the decrease in type I collagen mRNA levels observed after incubation with Y-27632 further supports this hypothesis. The precise mechanisms involved in maintenance of the fibrogenic phenotype are poorly known but alteration of the Rho pathway may be involved. In cells derived from radiation enteritis samples, we observed a concomitant increase in levels of RhoA and B and their physiological inhibitors, Rho E and Rho-GDI. Rho E inhibits Rho activity by direct binding to ROCK[32] whereas Rho-GDI acts by direct binding to the inactive form of Rho GDP.[9] Although expression of both Rho and Rho inhibitors is enhanced in radiation enteritis, the Rho/ROCK pathway seemed to be more active in cells derived from radiation enteritis samples. This suggests that endogenous control of Rho activity may contribute to maintenance of fibrogenic differentiation.

Taken together, these observations indicate that radiation induced fibrogenic differentiation of intestinal smooth muscle cells does not solely depend on local regulatory mediators but may also involve a genetic programme triggered by alteration of signal transduction pathways. Furthermore, these observations provide evidence that radiation induced fibrogenic differentiation can be modulated, thus opening new perspectives for antifibrotic therapies. Targeting the Rho/ROCK pathway may become a novel therapeutic approach to treat radiation fibrosis. Further studies will however be necessary to investigate the respective contribution of RhoA, B, C, Rac-1, and cdc42 in the fibrogenic phenotype and the effectiveness of inhibition of the Rho/ROCK signalling pathway in vivo.

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# EDITOR'S QUIZ: GI SNAPSHOT

**Robin Spiller,** *Editor*

## An unusual cause of upper gastrointestinal haemorrhage

### Clinical presentation

A 59 year old man presented with melena. There was no history of non-steroidal anti-inflammatory drug use, peptic ulcer, or chronic liver disease. He had a history of iron deficiency anaemia for the past five years that required oral iron supplements intermittently. Previous oesophagogastroduodenoscopy and colonoscopy were negative.

Physical examination disclosed bluish vascular lesions on the upper trunk and undersurface of the tongue (fig 1A, 1B). Laboratory investigations revealed a haemoglobin level of 4.2 mg/dl and haematocrit of 15%, but normal international normalised ratio and platelet count. Oesophagogastroduodenoscopy and colonoscopy on an emergent basis were negative.

### Question

What further investigation should be obtained to make a definitive diagnosis? What is the most likely diagnosis?

*See page 373 for answer*

This case is submitted by:

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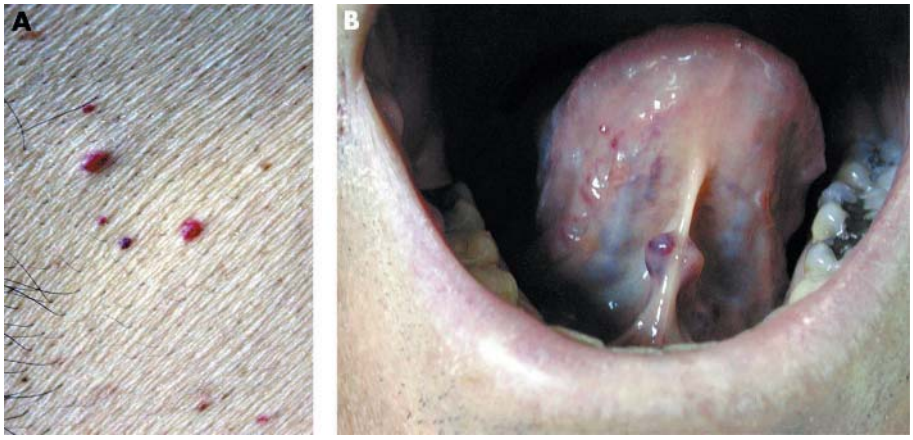


**Figure 1** Physical examination of the patient revealed bluish vascular lesions on the upper trunk (A) and on the undersurface of the tongue (B).