

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 κ B 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 motility⁵ 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 investigated⁶ 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; NF κ B, nuclear factor κ B; TNF- α , tumour necrosis factor α ; TGF- β 1, transforming growth factor β 1

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 described¹ 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 assessed 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, immunofluorescence, 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 cell 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:

$$\frac{(\text{Gene} \times \text{intensity} - \text{background})}{(\text{average intensity for housekeeping gene} - \text{background})}$$

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; n = 1: S/RT 45 Gy/CT/BT 10–15 Gy; 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, Right hemicolectomy

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

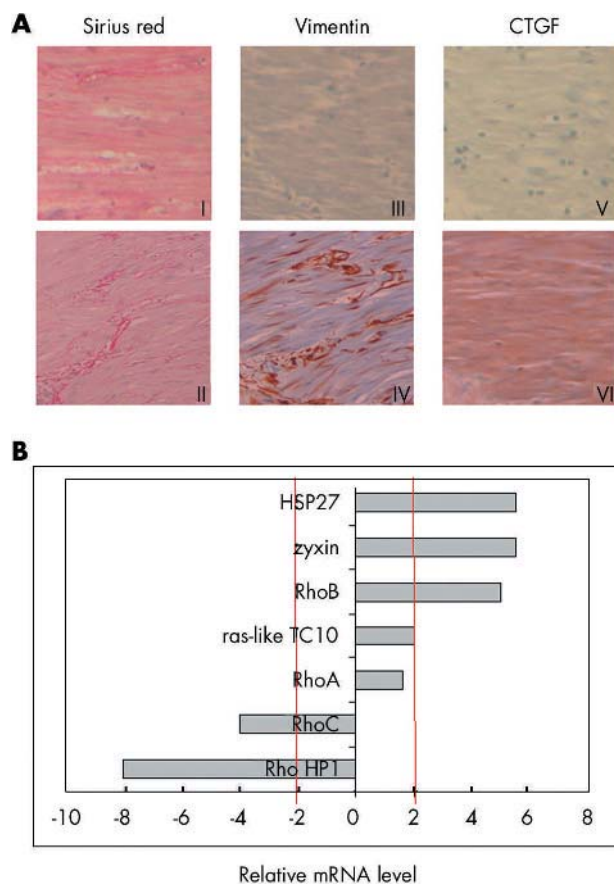


Figure 1 (A) Intestinal smooth muscle cells exhibited fibrogenic differentiation in vivo. In the muscularis propria, Sirius red staining showed collagen infiltration within smooth muscle bundles in radiation enteritis (II, $\times 200$) versus normal bowel (I, $\times 200$) that colocalised with vimentin positive cells (IV, $\times 200$). Connective tissue growth factor (CTGF) immunostaining was negative in normal muscularis propria (V, $\times 200$) whereas strong staining was observed in radiation enteritis (VI, $\times 200$). (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 ($2-3 \times 10^6$) 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 colleagues¹⁴ from cells (1×10^6) 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 I κ B- 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, Bioriginal, 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 (NF κB) binding elements (5'-GAG GAA TGT CCC TGT TTG-3') were 5' end labelled with [γ -³²P]ATP using T4 polynucleotide kinase (Life Technology, Cergy Pontoise, France). End labelled probes were purified using a G-50 column (Pharmacia, Saclay, France) and 1×10^5 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 MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, and 1 $\mu\text{g}/\mu\text{l}$ 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 \times 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 ($\times 2.5$, $p < 0.05$). 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

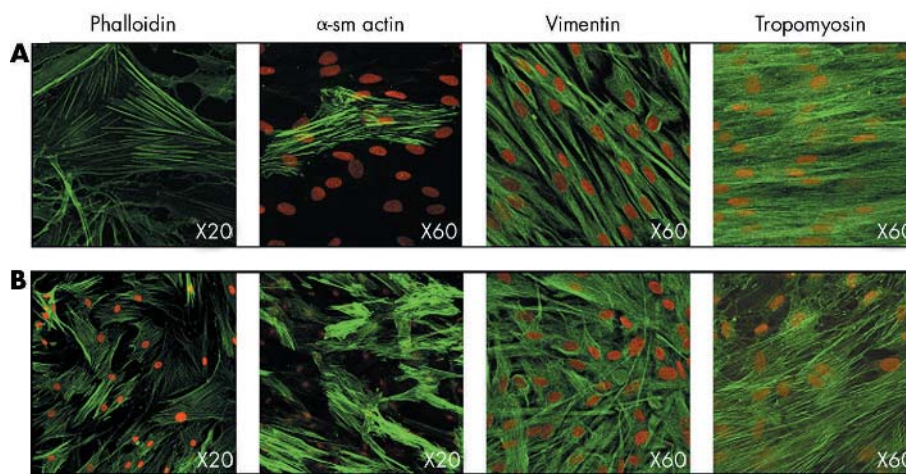
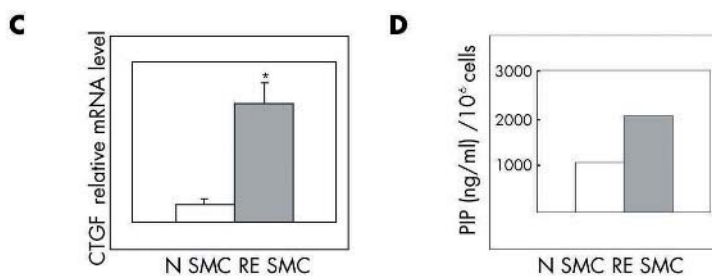


Figure 2 (A) Immunofluorescence experiments showed that normal smooth muscle cells (N SMC) exhibited typical intestinal differentiation markers in vitro. They were positive for vimentin and tropomyosin whereas only a few cells expressed α smooth muscle actin (α -sm actin). (B) Smooth muscle cells derived 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.



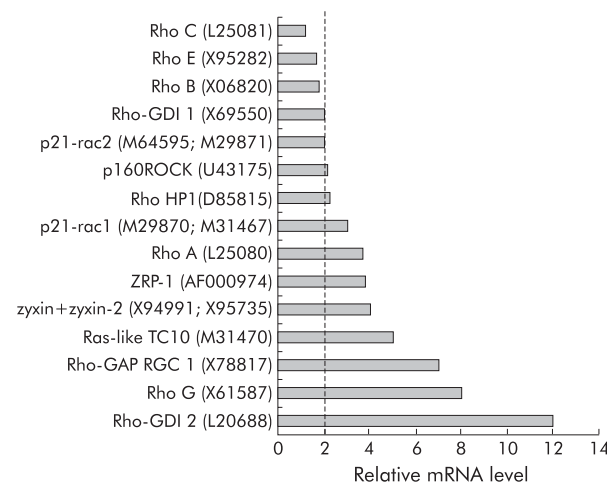
actin stress fibres. At confluency, spontaneous retraction occurs and produces “hill and valley” pictures, as previously described.^{7, 15} RE SMC exhibited a more compact morphology and higher density of stress fibres than their normal counterparts (fig 2A and B, phalloidin). Cellular differentiation was assessed using the markers proposed by Graham

and colleagues¹⁶ 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).

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 ($\times 2.5$) and real time RT-PCR ($\times 7$) (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).

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.



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.

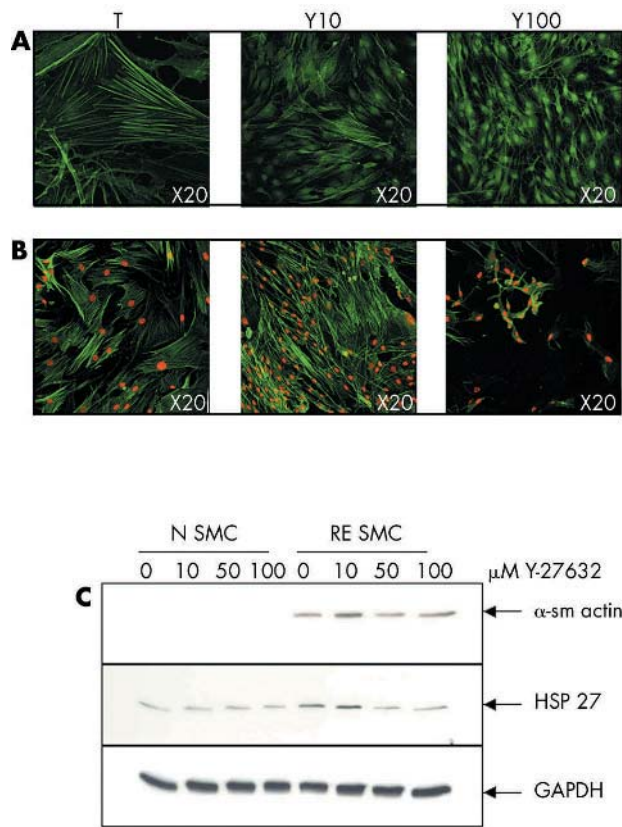


Figure 4 Alteration of actin stress fibre network by Rho kinase inhibition. F-actin was determined by FITC-phalloidin staining after Y-27632 incubation in normal smooth muscle cells (N SMC) (A) and radiation enteritis smooth muscle cells (RE SMC) (B). Rho kinase inhibition decreased heat shock protein (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.

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 μ M 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 μ M 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 μ M 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.

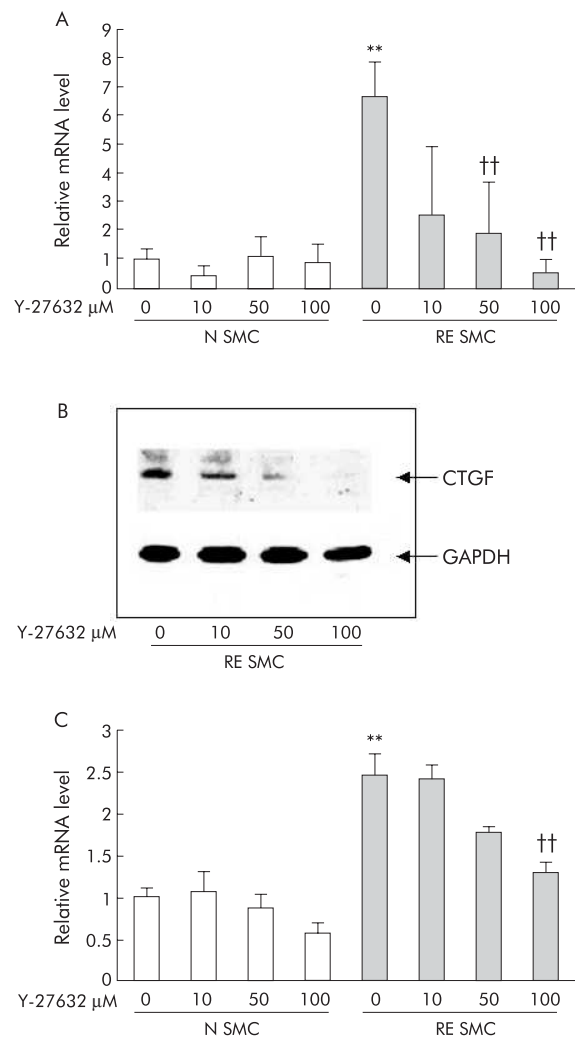


Figure 5 (A, B) Regulation of connective tissue growth factor (CTGF) expression by Rho kinase inhibition. (A) CTGF mRNA level was assessed by real time reverse transcription-polymerase chain reaction (RT-PCR). Values are mean (SEM); $n=4$. ** $p<0.01$ compared with untreated normal smooth muscle cells (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.

Rho kinase inhibition decrease NF κ B DNA binding activity

Next we investigated the effect of ROCK inhibition on nuclear protein binding activity to NF κ B consensus sequence located in the CTGF promoter. Incubation of cells with Y-27632 or sodium salicylate, an NF κ B inhibitor, decreased NF κ B DNA binding activity in RE SMC but not in N SMC (fig 6A). Western immunoblotting was used to determine whether inhibition of NF κ B DNA binding activity occurs through stabilisation of the I κ B α isotype. We found increased I κ B α 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).

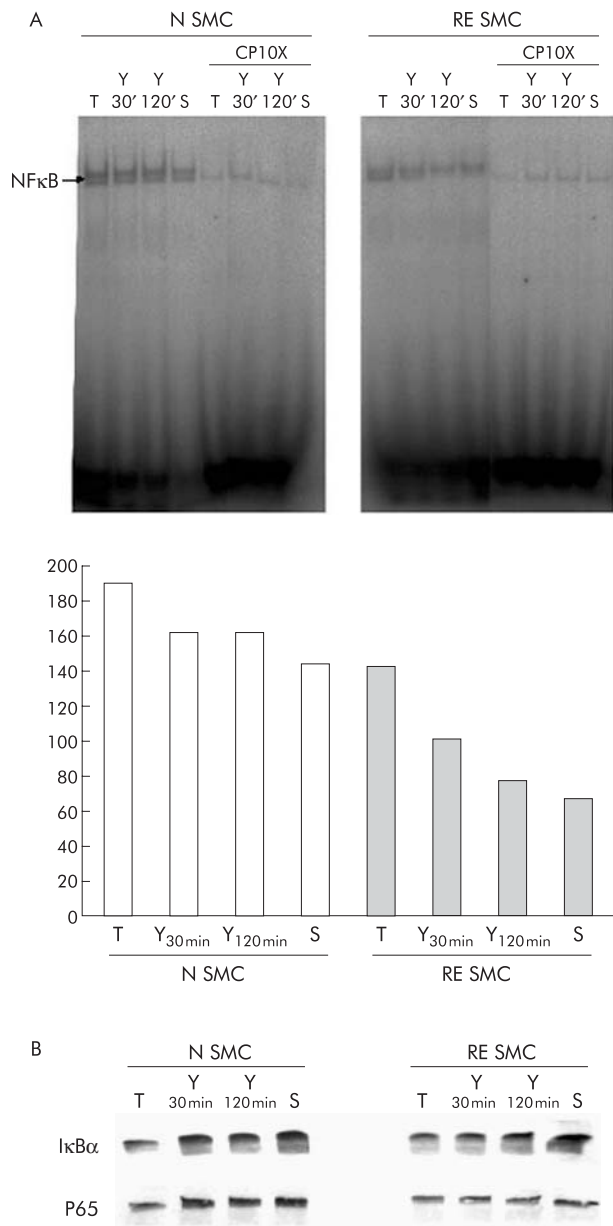


Figure 6 (A) Results of electrophoretic mobility shift assay (EMSA) protein binding to the nuclear factor κ B (NF κ B) consensus site after incubation with 10 μ M Y-27632 (Y) for 30 minutes and 120 minutes, and with 25 mM sodium salicylate (S) for 45 minutes. Non-radioactive NF κ B oligo (CPX10) blocked NF κ B DNA binding. The blot is representative of two independent experiments. (B) Western blot analysis of I κ B α 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 NF κ B. Finally, CTGF inhibition led to decreased type I collagen

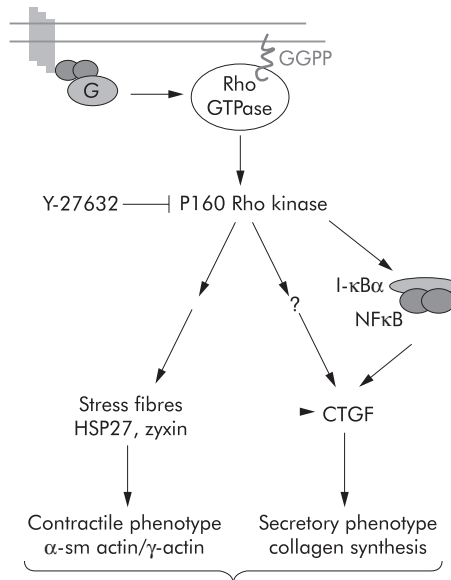


Figure 7 Chronic activation of the Rho/Rho kinase (ROCK) cascade in radiation enteritis is involved in intestinal smooth muscle cell differentiation towards an immature phenotype with altered prosecretory and contractile properties. CTGF, connective tissue growth factor; α / γ -sm actin, α / γ smooth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

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-27632²¹ 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 colleagues²⁷ 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 ROCK³² 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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Conflict of interest: None declared.

REFERENCES

- 1 **Vozenin-Brotans MC**, Milliat F, Sabourin JC, *et al*. Fibrogenic signals in patients with radiation enteritis are associated with increased connective tissue growth factor expression. *Int J Radiat Oncol Biol Phys* 2003;**56**:561-72.
- 2 **Strup-Perrot C**, Mathe D, Linard C, *et al*. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 2004;**287**:G875-85.
- 3 **Graham MF**. Pathogenesis of intestinal strictures in Crohn's disease—An Update. *Inflamm Bowel Dis* 1995;**1**:220-7.
- 4 **Macdonald TT**. A mouse model of intestinal fibrosis? *Gastroenterology* 2003;**125**:1889-92.
- 5 **Husebye E**, Hauer-Jensen M, Kjorstad K, *et al*. Severe late radiation enteropathy is characterized by impaired motility of proximal small intestine. *Dig Dis Sci* 1994;**39**:2341-9.
- 6 **Tomasek JJ**, Gabbiani G, Hinz B, *et al*. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002;**3**:349-63.
- 7 **Brittingham J**, Phiel C, Trzyzna WC, *et al*. Identification of distinct molecular phenotypes in cultured gastrointestinal smooth muscle cells. *Gastroenterology* 1998;**115**:605-17.
- 8 **Ridley AJ**. Rho GTPases and cell migration. *J Cell Sci* 2001;**114**:2713-22.
- 9 **Ridley AJ**. Rho family proteins: coordinating cell responses. *Trends Cell Biol* 2001;**11**:471-7.
- 10 **Riento K**, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003;**4**:446-56.
- 11 **Heusinger-Ribeiro J**, Eberlein M, Wahab NA, *et al*. Expression of connective tissue growth factor in human renal fibroblasts: regulatory roles of RhoA and cAMP. *J Am Soc Nephrol* 2001;**12**:1853-61.
- 12 **Chomczynski P**, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156-9.
- 13 **Vozenin-Brotans MC**, Milliat F, Linard C, *et al*. Gene expression profile in human late radiation enteritis obtained by high-density cDNA array hybridization. *Radiat Res* 2004;**161**:299-311.
- 14 **Schreiber E**, Mathias P, Muller MM, *et al*. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989;**17**:6419.

- 15 **Chamley-Campbell J**, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiol Rev* 1979;**59**:1–61.
- 16 **Graham M**, Glück U, Shah M, *et al*. b-Tropomyosin and α -actin are phenotypic markers for human intestinal smooth muscle cells in vitro. *Mol Cell Differ* 1994;**2**:45–60.
- 17 **Mahida YR**, Beltinger J, Makh S, *et al*. Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. *Am J Physiol* 1997;**273**:G1341–8.
- 18 **Holmes A**, Abraham DJ, Sa S, *et al*. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem* 2001;**276**:10594–601.
- 19 **McKaig BC**, Hughes K, Tighe PJ, *et al*. Differential expression of TGF- β isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. *Am J Physiol Cell Physiol* 2002;**282**:C172–82.
- 20 **McKaig BC**, McWilliams D, Watson SA, *et al*. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* 2003;**162**:1355–60.
- 21 **Breitenlechner C**, Gassel M, Hidaka H, *et al*. Protein kinase A in complex with Rho-kinase inhibitors Y-27632, Fasudil, and H-1152P: structural basis of selectivity. *Structure (Camb)* 2003;**11**:1595–607.
- 22 **Wang P**, Bitar KN. Rho A regulates sustained smooth muscle contraction through cytoskeletal reorganization of HSP27. *Am J Physiol* 1998;**275**:G1454–62.
- 23 **Ibitayo AI**, Sladick J, Tuteja S, *et al*. HSP27 in signal transduction and association with contractile proteins in smooth muscle cells. *Am J Physiol* 1999;**277**:G445–54.
- 24 **Yamada H**, Strahler J, Welsh MJ, *et al*. Activation of MAP kinase and translocation with HSP27 in bombesin-induced contraction of rectosigmoid smooth muscle. *Am J Physiol* 1995;**269**:G683–91.
- 25 **Ott C**, Iwanciw D, Graness A, *et al*. Modulation of the expression of connective tissue growth factor by alterations of the cytoskeleton. *J Biol Chem* 2003;**278**:44305–11.
- 26 **Gnad R**, Kaina B, Fritz G. Rho GTPases are involved in the regulation of NF- κ B by genotoxic stress. *Exp Cell Res* 2001;**264**:244–9.
- 27 **Segain JP**, Raingeard de la Bletiere D, Sauzeau V, *et al*. Rho kinase blockade prevents inflammation via nuclear factor kappa B inhibition: evidence in Crohn's disease and experimental colitis. *Gastroenterology* 2003;**124**:1180–7.
- 28 **Igarashi A**, Nashiro K, Kikuchi K, *et al*. Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 1995;**105**:280–4.
- 29 **Blom IE**, Goldschmeding R, Leask A. Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? *Matrix Biol* 2002;**21**:473–82.
- 30 **Abraham DJ**, Shiwen X, Black CM, *et al*. Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J Biol Chem* 2000;**275**:15220–5.
- 31 **Frazier K**, Williams S, Kothapalli D, *et al*. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 1996;**107**:404–11.
- 32 **Riento K**, Guasch RM, Garg R, *et al*. RhoE binds to ROCK 1 and inhibits downstream signaling. *Mol Cell Biol* 2003;**23**:4219–29.

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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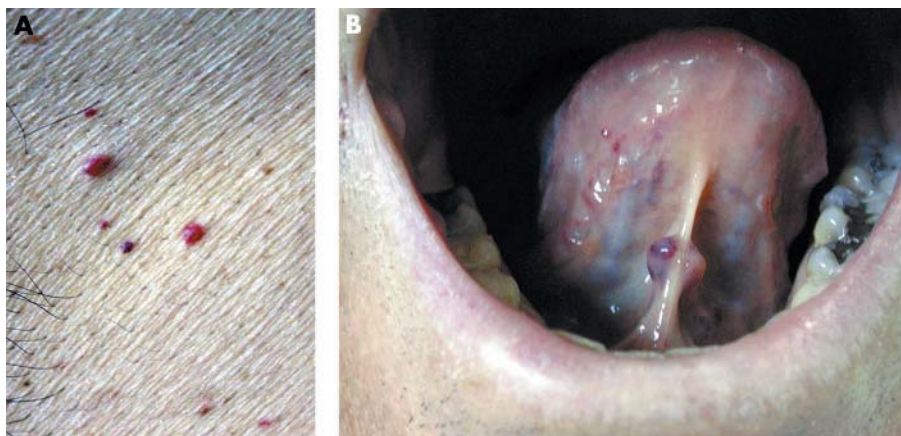


Figure 1 Physical examination of the patient revealed bluish vascular lesions on the upper trunk (A) and on the undersurface of the tongue (B).

carried around hospital wards and outpatient departments.

It is divided into four sections, which have colour-coded pages to aid navigation through the book. The first section covers approaches to 28 common clinical problems. This is an excellent section. Each of the four sections starts with the differential diagnosis, and for conditions with a large differential diagnosis, such as chronic diarrhoea, a pathophysiological framework is given to aid both understanding and memory. I was delighted to see that the old-fashioned prohibitions on the use of antimotility drugs and antibiotics in acute diarrhoea are abandoned but instead wisely discussed. I was rather less convinced that a change in diet has much effect on excessive wind—if only it was that easy! In some areas, this section overlaps with the British National Formulary (BNF). For example, the different treatment protocols for *Helicobacter pylori* are documented far more comprehensively in the BNF.

The second section is an A to Z of all gastroenterological and hepatological conditions, tests and treatments. It is pretty comprehensive, although it took me some time to work out how to follow-up a patient with a colonic adenoma, my problem being the use of the word “adenoma” instead of “polyp”. Detailed recommendations on follow-up protocols for polyps are not provided, but, given the proliferation of guidelines from numerous national gastroenterology societies and bodies, this is inevitable. I looked up some small print problems I have had in my own clinical practice recently. The chapters on solitary rectal ulcers and gastrointestinal stromal tumours are well done. The association of microscopic colitis with proton-pump inhibitors was not mentioned in an otherwise good summary. Gastroparesis does not seem to be covered in this book at all. Coverage of rare diseases is important in books such as this because it is exactly these conditions that catch young clinicians out and such patients often turn up on hospital wards and have lengthy inpatient stays.

There is a short section on drugs used in gastroenterological and hepatological practice. This is brief and punchy, giving the indications, mode of action, dosing regimens, contraindications, side effects and clinical practice points. This duplicates almost exactly information in the BNF, but is much more user friendly and it is easier to find the drug of interest.

The last section deals with gastroenterological and hepatological emergencies. It covers the acute abdomen, diarrhoea, liver failure, gastrointestinal haemorrhage, swallowed foreign bodies, oesophageal obstruction and rupture. There is certainly more than enough information to get a sick patient through the night and a sensible management plan established, which is exactly what the junior doctor wants. The section is also useful for senior clinicians as it includes prognostic scoring systems such as the Rockall Scoring system for upper gastrointestinal bleeding, which can be difficult to remember if one is not using them all the time.

Overall, this is an excellent book taking a nonsense and sensible approach. Most topics are easily found and short enough to be read in less than 10 min. I shall certainly be recommending it to my own junior staff, both for their own benefit and also to remind myself of that annoying fact I've just forgotten!

Alastair J M Watson

The clinician's guide to pancreaticobiliary disorders

Edited by Gregory G Ginsberg and Nuzhat A Ahmad. New Jersey: Published by Slack Incorporated, 2006, pp 400. ISBN 1-55642-694-1

Ginsberg and Ahmad from Philadelphia have edited a compendium on pancreaticobiliary disorders within the Clinician's Guide series. Overall, this is a concise book and makes for good reading. The referencing is somewhat inconsistent from chapter to chapter. Sometimes citations are extensive, and sometimes chapters are rather short on direct references to statements made in the text. Some chapters like chapter 1 recommend further reading instead, whereas others such as chapters 11 and 2 are well referenced.

At the start of the book, congenital abnormalities of the pancreas, such as pancreas divisum, are outlined, and guidelines for interventional therapy are given. Chapter 2 on gallstones and gallbladder disorders is detailed on the pathobiology of gallstone formation. Some facts are repeated in Chapter 3 on choledocholithiasis, which also contains the technical intricacies of various types of sphincterotomies. Unfortunately, no reference is provided for the indicated 25% late complication rate after endoscopic sphincterotomy, a proportion I regard as very high. In Chapter 5, diseases of the ampulla of Vater sphincter of Oddi dysfunction are reviewed in a very pragmatic and helpful way that includes examples like the benefits of using the Milwaukee classification in clinical practice. This chapter also points out where additional randomised multicentre trials would be needed. Chapter 6, dealing with cholangiocarcinoma, goes into detail on stent placement and surgery, but not on chemotherapy or radiation therapy.

Chapter 8 on acute pancreatitis gives a very good overview. The Marshall Score cited should currently be replaced by the Sequential Organ Failure Assessment score. The extensive tables on medication-induced pancreatitis are very useful, but they do not provide the degree of evidence required for each drug, such as whether the information is from a single case report or from rechallenge evidence or from multicentre controlled trials. Although the previous review on the pathogenesis and pathophysiology of gallstone formation in an earlier chapter was excellent, that for pancreatitis is missing. Prophylaxis for endoscopic retrograde cholangiopancreatography-induced pancreatitis is not endorsed, and the approach outlined here is very practical. Also, the approach to CT scan indications in acute pancreatitis is based on personal experience, straightforward and to the point: “The patient usually had one (CT) before we see him. If he doesn't we don't perform one but allow the clinical course to determine the indication.”

I only disagree with the authors' assumption that C-reactive protein levels should be “relegated to the realm of research tools”, since it is widely available and used both in the US and Europe to determine the severity of pancreatitis outside of imaging techniques. Where the chapter sees great promise (trypsinogen activation peptide), the assay is no longer commercially available and was discontinued a few years ago. The chapter is very up to date and practical as far as the use of antibiotics in

necrotising pancreatitis and the use of parenteral nutrition are concerned. The statement on page 169 that “all would agree that an NPO (nil per mouth) status is mandatory to put the pancreas to rest and allow healing to proceed” is now utterly obsolete. Endorsing enteral nutrition over parenteral nutrition in the absence of ileus, on the other hand, is now evidence-based clinical practice. If the authors of the pancreatitis chapter do not use severity scores or C-reactive protein level, the question remains as to how do they follow their own recommendation to determine the severity of pancreatitis other than by means of a CT scan.

Chapter 9 on chronic pancreatitis gives a good overview on pathophysiology and pathology. Unfortunately, the concepts regarding tropical pancreatitis are completely outdated, since neither Cassava nor selenium aetiology hypotheses have stood the test of scientific inquiry, and serine protease inhibitor Kazal 1 mutations are found in 50% of patients with tropical pancreatitis. The statement that “furthermore myriad mutations have been plausibly linked to pancreatitis” is incorrect; also, only a single trypsinogen mutation for hereditary pancreatitis (R117H) is mentioned in this chapter and denoted according to the old chymotrypsinogen classification that was dropped a decade ago (the mutation is now referred to as R122H). The chapter states the varying and conflicting hypotheses about the underlying mechanisms of hereditary pancreatitis as though they are based on experimental evidence—which they are not. The chapter states further that there is a plethora of non-invasive, indirect tests of pancreatic function that do not require passage of collection tubes. However, it lists only five of those, of which two are no longer commercially available. An advantage of the chapter is that it is extensively referenced. Chapter 11 on solid pancreatic tumours has unfortunately no section on chemotherapy or adjuvant therapy. A review on intraductal endoscopic ultrasound appears in this chapter on solid tumours rather than in the preceding chapter on pancreatic ductal complications and their diagnosis. Chapter 12 on pancreatic cystic lesions includes intraductal papillary mucinous neoplasia and is very up to date. Table 11-1 and Table 13-1 are, however, identical. The imaging of the pancreaticobiliary system as covered in Chapter 15 is immensely readable, Chapter 16 on MRI has excellent photo quality and Chapter 17 covers interventional radiology.

Overall, I found this book concise, mostly up to date, very readable and sometimes even entertaining. A more detailed coverage would be found in existing text books. The authors Ginsberg and Ahmad should be congratulated on editing this practical clinician's guide.

Markus M Lerch

CORRECTION

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G D De Palma, M Rega, P Ciamarra, *et al.* An unusual cause of upper gastrointestinal haemorrhage (*Gut* 2005;54:343, 373). In this article the third author's surname appears incorrectly. The correct spelling is Ciamarra.