INFLAMMATORY BOWEL DISEASE

Induction of a fibrogenic response in mouse colon by overexpression of monocyte chemoattractant protein 1

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Background and aims: Monocyte chemoattractant protein 1 (MCP-1) is increased transmurally in inflammatory bowel disease (IBD). Although MCP-1 is considered to play an important role in fibrotic disease in other organs, the role of MCP-1 in gut fibrosis is unknown. We investigated the fibrotic potential of MCP-1 in the gut by overexpressing this chemokine in the mouse colorectal wall.
Methods: Intramural gene transfer by direct injection of adenovector into the mouse rectal wall was established. C57BL/6 and Rag2$^{-/-}$ (B and T cell deficient) mice received 2.5×10^9 plaque forming units of an adenovector encoding murine MCP-1 (AdMCP-1) or control virus (AdDL70) via intramural injection. Mice were killed at various time points and tissues were obtained for histopathological and biochemical analysis.
Results: AdMCP-1 significantly increased collagen production in the colorectum and this was associated with significant elevation of transforming growth factor β (TGF-β) and tissue inhibitor of metalloproteinase (TIMP-1) protein. Transmural collagen deposition was observed after AdMCP-1 administration, and was accompanied by CD3+ T cells, F4/80+ macrophages, and vimentin+ cell infiltrates. Collagen was differentially distributed, with type I deposited in the muscularis mucosa and muscularis propria and type III in the submucosa and myenteric plexus. AdMCP-1 failed to induce collagen overproduction in immunodeficient Rag2$^{-/-}$ mice.
Conclusion: These findings suggest that MCP-1 can induce fibrosis in the gut and that this process involves interaction between T cells and vimentin positive fibroblasts/myofibroblasts, as well as the subsequent upregulation of TGF-β and TIMP-1 production. This model provides a basis for considering MCP-1 in the pathogenesis of strictures in IBD.

Fibrosis is a common occurrence in Crohn's disease (CD), leading to stricture formation in 10–40% of patients.[1 2] Strictures are an important complication of CD as they constitute an indication for surgery in approximately 80% of strictured patients.[1] Benign strictures occur much less commonly in ulcerative colitis[3] but may complicate ileoanal anastomosis in about 5–11% of such patients.[4] Medical therapy has little if any role to play, and immunomodulatory therapy with anti-tumour necrosis factor (TNF) antibody may enhance pre-existing strictures.[5] A better understanding of the mechanisms of fibrosis formation could lead to the development of novel therapeutic approaches, which subsequently may constitute a major advance in the treatment of CD.

Animal models of fibrosis are useful in studying the initial steps leading to fibrosis, as human studies are confined to samples of established fibrosis. Sartor *et al* showed that intramural injection of peptide glycan-polysaccharide complex at laparotomy caused chronic granulomatous enterocolitis and intestinal fibrosis in rats with immunopathological features resembling CD.[6] In this model, they showed bacterial cell wall polymers directly stimulated collagen α1(I), transforming growth factor β1 (TGF-β1), interleukin (IL)-1β, and IL-6 mRNA expression in intestinal myofibroblasts.[7] In the trinitrobenzene sulphonic acid induced model of colitis, rats develop colonic fibrosis and stricture formation but this has not yet been well characterised.[8]

Although mice have been considered to be resistant to fibrosis, Lawrance *et al* recently reported that trinitrobenzene sulphonic acid administration for an extended period induces chronic intestinal inflammation associated fibrosis in the mouse colon.[9] This was accompanied by increased mRNA expression of collagen 1α2, matrix metalloproteinase (MMP) 1, tissue inhibitor of metalloproteinase (TIMP) 1, TNF-α, TGF-β1, and insulin-like growth factor 1 in colonic tissue.[9] SAMP1/YitFc mice spontaneously develop CD-like ileitis with muscular hypertrophy, focal collagen deposition, and stricture formation.[10] It has been shown that interferon γ, TNF, and IL-10 production from mesenteric lymph nodes is increased in the early stages of inflammation in this model.[10] Taken together, observations in these models clearly indicate that chronic inflammation causes intestinal fibrosis which is associated with an increase in proinflammatory cytokines, growth factors, and metalloproteinases. Nevertheless, the precise mechanisms underlying the pathogenesis of fibrosis and the immune mediators involved remain poorly understood.

MCP-1, which belongs to a C-C chemokine superfamily, is considered to play an important role in fibrosis formation in organs such as the lung,[11 12] kidney,[13 14] liver,[15] and pancreas.[16] MCP-1 is produced by several cell types, including mononuclear

Abbreviations: CD, Crohn's disease; ELISA, enzyme linked immunosorbent assay; IBD, inflammatory bowel disease; IL, interleukin; LP, lamina propria; MCP, monocyte chemoattractant protein; MM, muscularis mucosa; MMP, matrix metalloproteinase; MP, muscularis propria; SM, submucosa; SMA, smooth muscle actin; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumour necrosis factor

cells, fibroblasts, endothelial cells, epithelial cells, and smooth muscle cells and is a chemoattractant for monocytes, T lymphocytes, and natural killer cells.[17 18] Although it has been reported that MCP-1 is increased in the submucosa (SM) and muscularis propria (MP) of CD,[19 20] the ability of MCP-1 to induce fibrosis in the gut has not been examined.

In this study, we used a novel method of direct gene transfer into the deeper layers of the gut by intramural injection of an adenoviral vector. We employed this method to transfer the MCP-1 gene in the mouse colon to investigate the fibrotic potential of this chemokine.

MATERIALS AND METHODS

Adenoviral construct

A replication deficient recombinant adenovirus expressing murine MCP-1 (AdMCP-1) was used. cDNA for murine MCP-1 was inserted into the E1 region of Ad5 containing human CMV immediate early promoter and SV40 polyadenylation signal. An adenovirus coding β-galactosidase (AdLacZ) and an empty virus (AdDL70) were also used.[21] Virus preparations were expanded, purified, and plaque titered, as described previously.[22]

Intramural injection of adenoviral vector into mice rectum

Male C57BL/6 mice were obtained from Taconic (Germantown, New York, USA). The protocols employed were in direct accordance with guidelines drafted by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Eight to 10 week old specific pathogen free mice were anaesthetised and injected with 2.5×10^9 plaque forming unit of AdMCP-1 diluted in 30 µl of saline or the same titre of AdLacZ or AdDL70 into the rectum intramurally through the anus using a 30 gauge needle. Mice were killed at various time points, and tissues were collected for histological and biochemical analysis. No mortality caused by injected adenovirus was observed during these experiments.

Histopathology and immunohistochemistry

Cytochemical staining for β-galactosidase was performed on samples obtained from AdLacZ treated animals, as previously described.[23] Total colons were removed and fixed, and then reacted with X-gal staining solution.[23] Sections were counterstained with nuclear fast red.

Tissue samples that were obtained from the rectum of AdMCP-1 treated and control animals on different days were fixed in 10% formaldehyde and paraffin embedded. Sections were stained with haematoxylin-eosin and with Masson's trichrome stain.

Serial paraffin embedded sections were incubated with primary antibodies (table 1) following deparaffinisation, peroxidase blocking, antigen retrieval, and protein blocking. Secondary antibodies shown in table 1 were used. Sections were developed with 3,3′-diaminobenzidine solution, counterstained with methylgreen or Meyer's haematoxylin

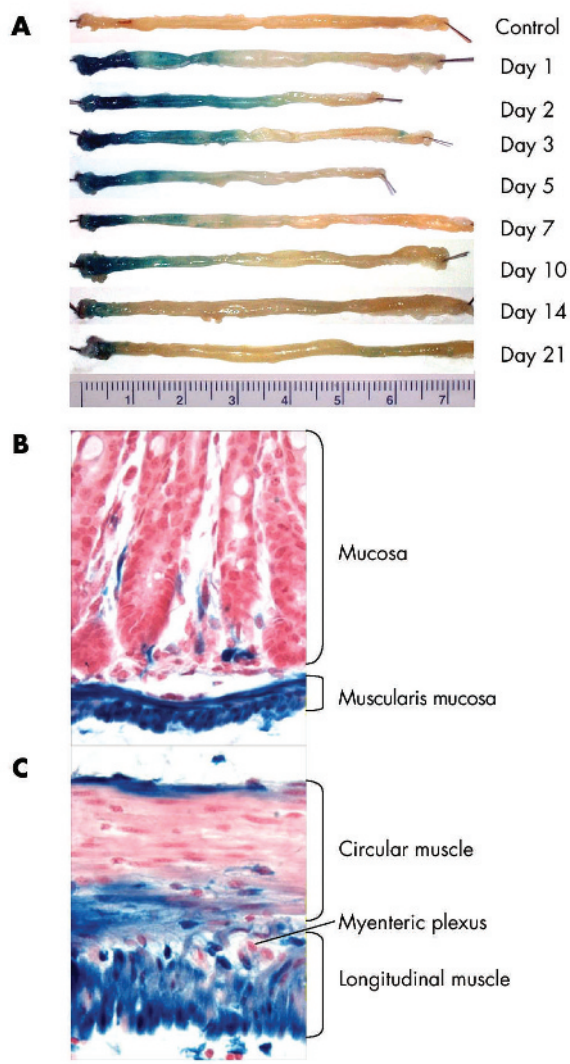

Figure 1 β-Galactosidase expression in the colon after intramural injection of AdLacZ. (A) Macroscopic appearance. Strong expression of β-galactosidase (blue) was observed in the rectum ~1 cm from the anal verge. (B, C) Microscopic appearance of the rectum at day 2 post injection. Staining was seen mainly in the muscularis propria and muscularis mucosa but also in the lamina propria.

Table 1 Antibodies for immunohistochemistry used in this study

Primary antibody				Secondary antibody			
Name	Company	Dilution	Incubation	Name	Company	Dilution	Incubation
Mouse anti-αSMA	Sigma	1:400	1 h RT	Goat anti-mIgG-HRP	Sigma	1:100	30 min
Mouse antidesmin	Sigma	1:20	1 h RT	Goat anti-mIgG-HRP	Sigma	1:100	30 min
Mouse antivimentin	Chemicon	1:200	1 h RT	Rabbit anti-mIgM	MBL	1:300	30 min
				Followed by Envision	Dako	–	30 min
Rabbit anti-col I	RDI	1:400	1 h RT	Envision	Dako	–	30 min
Rabbit anti-col III	RDI	1:500	18 h 4°C	Envision	Dako	–	30 min
Rabbit anti-CD3	Dako	1:500	1 h RT	Envision	Dako	–	30 min
Rat antimouse F4/80	Serotec	1:100	18 h 4°C	Goat anti-rIgG-biotin	Santa Cruz	1:200	1 h
				Streptoavidin-HRP	Dako	1:300	30 min
Goat anti-mMCP-1	Santa Cruz	1:50	18 h 4°C	Mouse anti-g/s IgG-HRP	Sigma	1:100	1 h

RT, room temperature; col, collagen; HRP, horseradish peroxidase; g, goat; s, sheep; m, mouse; r, rat; Envision, HRP coupled antirabbit secondary reagent.

(DakoCytomation, Carpinteria, California, USA), dehydrated, cleared, and mounted. Negative controls were prepared by omission of the primary antibodies. All reactions were performed at room temperature unless otherwise mentioned.

The ratio of collagen III:I was measured by immunostaining based semiquantification. The identical position of the serial sections stained with anticollagen I or III was photographed by digital camera (Olympus Q-Color 3), and the stained area was measured by ImageJ software (NIH). Five different positions from each section were taken. The results are shown as per cent of the whole area for both collagen I and III, and the ratio III:I was calculated.

CD3+ cells infiltrated in the muscle layers were counted. Five different positions in each section were chosen randomly. Results are shown as cell counts per hyper power field. F4/80+ area was measured by ImageJ software as it was difficult to delineate single cells. Five different positions from each section were taken. The results are shown as per cent of the whole area.

Collagen assay

Total rectal collagen content was determined by quantifying pepsin soluble collagen using the Sircol Collagen Assay kit (Biocolor, Newtownabbey, Northern Ireland) according to the manufacturer's instructions. Collagen standard solutions provided by the manufacturer were used to construct a standard curve. Results are expressed as collagen content per millimetre length of colon.

Enzyme linked immunosorbent assay (ELISA)

Homogenised rectal samples were analysed using a human TGF-β1, a mouse JE/MCP-1, MMP-3, and TIMP-1 ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. Results are corrected for protein concentration which was measured by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, California, USA).

Gene knockout mice

To investigate the role of lymphocytes in the development of fibrosis induced by MCP-1, Rag2$^{-/-}$ mice (Taconic) were injected with AdMCP-1 or AdDL70 and killed three days after injection. Rectal tissue was taken and the collagen assay was performed. F4/80 immunostaining was also performed in these mice. C57Bl/6 mice were used as control for Rag2$^{-/-}$ mice.

Statistical analysis

Results are shown as box and whisker plots. Data were analysed using the Mann-Whitney U test between two unpaired groups with $p<0.05$ considered to be significant.

RESULTS

β-galactosidase expression in the colon after intramural injection of AdLacZ

To investigate expression of the transferred gene product, we injected AdLacZ intramurally and tissues were taken on days 1–3, 5, 7, 10, 14, 21, 28, and 35 after injection. Macroscopically, strong expression of β-galactosidase (blue) was observed in the rectum approximately 1 cm from the anal verge (fig 1A). Expression was observed up to the mid colon at the peak of day 2 and lasted until day 21 in the rectum. After day 28, microscopic but not macroscopic expression was observed (data not shown). Microscopically, staining was seen mainly in the longitudinal muscle layer and muscularis mucosa (MM) but also in the circular muscle layer and lamina propria (LP) of the rectum (fig 1B, C). Above the rectum, staining was observed mainly in the MM. Weak expression was observed in the liver during the first few days but not in the spleen or lung (data not shown).

Based on these data, we used rectal tissue for further experiments as the strongest transgene expression was seen in rectum.

Intramural injection of AdMCP-1 causes significant elevation of tissue MCP-1 level, transmural inflammatory cell infiltration, and transmural fibrosis in mice colon

Mice were killed on days 3, 7, 14, 21, 28, 35, and 42 after adenovector injection. In AdMCP-1 treated mice, the rectum became hard and thickened compared with AdDL70 treated

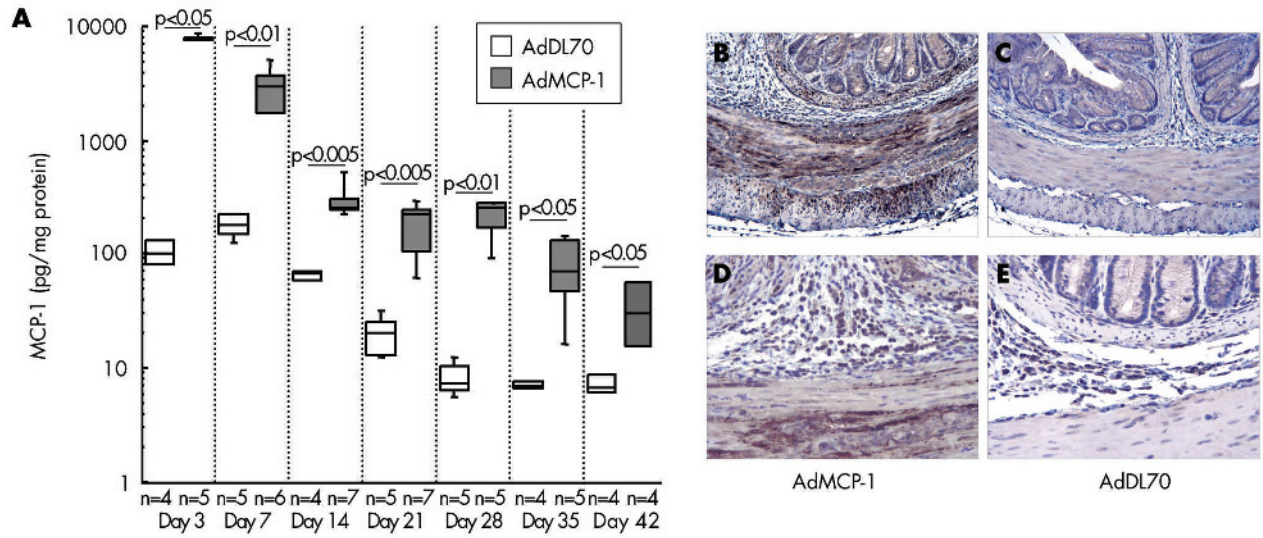

Figure 2 (A) Tissue monocyte chemoattractant protein 1 (MCP-1) levels following virus administration measured by enzyme linked immunosorbent assay. Data are corrected by tissue protein concentration and shown in log scale. A significant elevation in tissue MCP-1 was observed from days 3 to 42 following administration of adenovirus expressing murine MCP-1 (AdMCP-1) compared with control empty virus (AdDL70) treatment. (B–E) MCP-1 immunohistochemistry. (B) Strong MCP-1 immunoreactivity was seen in both the muscularis mucosa and muscularis propria three days after AdMCP-1 injection. (C) There was no expression in the muscularis mucosa or muscularis propria following AdDL70 administration. (D, E) Weak MCP-1 expression was seen in submucosal cells following both AdMCP-1 and AdDL70 injection.

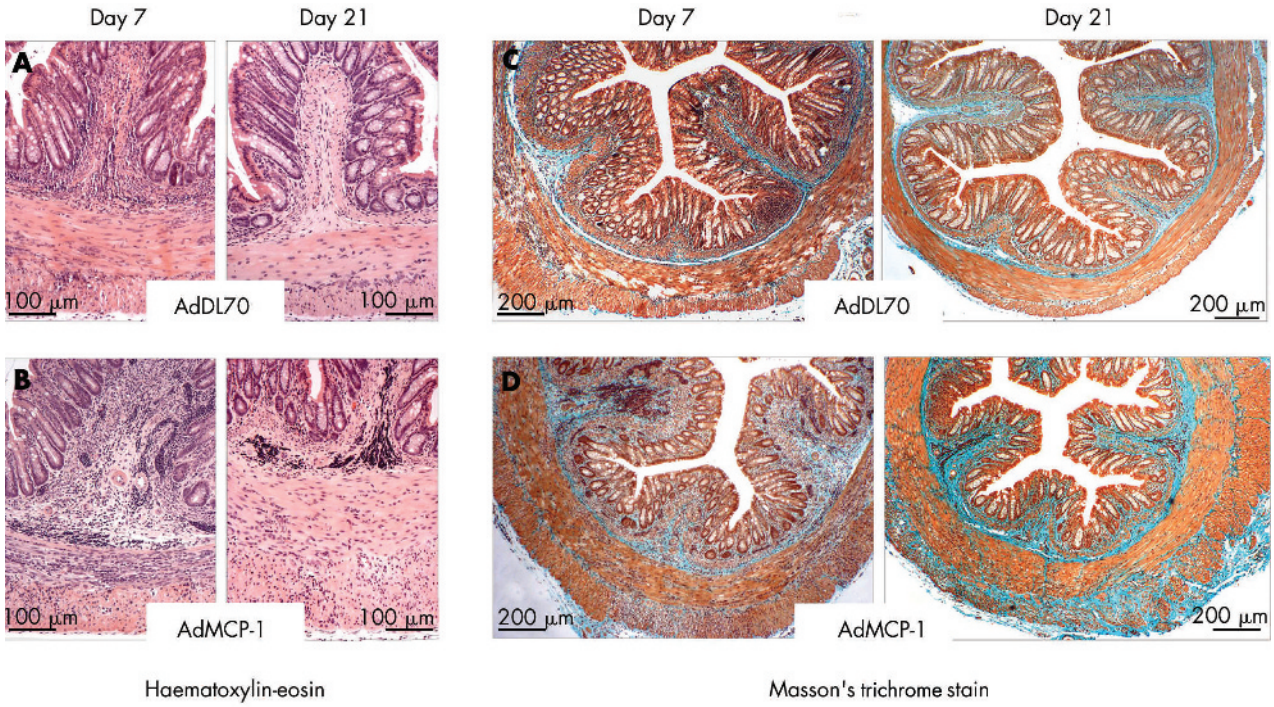

Figure 3 Haematoxylin-eosin (A, B, original magnification ×100) and Masson's trichrome stain (C, D, ×40) of the rectum following adenovector administration. (A, B) Numerous inflammatory infiltrates were observed transmurally in adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) treated rectum compared with empty virus (AdDL70) treated controls. (C, D) Scattered collagen deposition (green) mainly in the submucosa was observed at day 7. At day 21, collagen in the submucosa appeared to be dense, and collagen in the muscularis propria was increased. Collagen deposition between the circular and longitudinal muscle layers and around the myenteric plexus was also increased.

control mice but no ulcerations were observed. Rectal weight was significantly higher compared with control group until day 21 (data not shown).

Significant elevation of tissue MCP-1 levels was observed from day 3 to 42 following administration of AdMCP-1 (fig 2A). Although the peak was on day 3, there was still a significant difference on day 42, suggesting there were still some infected cells that produced the transferred gene product. MCP-1 was slightly increased until day 14 following administration of control virus AdDL70 compared with normal mice that did not receive virus (9.67 (1.27) pg/mg protein). This elevation was considered to be induced by an immune response against the adenovector itself.

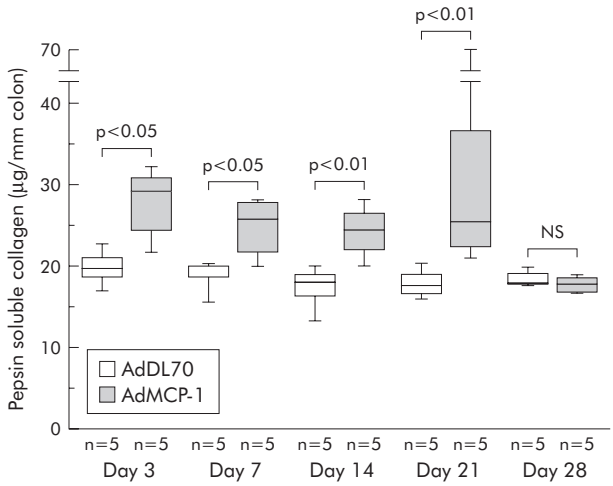

Figure 4 Pepsin soluble collagen, which reflects recently produced collagen, was quantified by the assay. Data are corrected for the length of the sample (mm). Adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) treated mice showed a significant higher level of collagen until day 21 compared with empty virus (AdDL70) treated controls.

MCP-1 expression following adenovector administration was also investigated. Tissue sections (day 3) were stained with anti-MCP-1 antibody. MCP-1 immunoreactivity was seen in mainly MM and MP in AdMCP-1 treated mice colon (fig 2B), which was similar to β-galactosidase expression following AdLacZ administration. There was no such expression in AdDL70 treated colon (fig 2C). Weak expression was seen in submucosal inflammatory cells in both AdMCP-1 and AdDL70 treated colon (fig 2D, E).

Histology showed more inflammatory cells, which were mainly mononuclear cells, in the LP, MM, SM, MP, and adventitia of the rectum obtained from AdMCP-1 treated mice compared with AdDL70 treated controls. In some sections, MP and MM were disrupted by infiltrates. The number of cells appeared to be decreased as the days passed (fig 3A, B).

Scattered collagen deposition was observed mainly in the SM at day 3. At the later time points, collagen deposition in SM became dense, and there was also an increase in collagen deposition in MP (fig 3D). Isolated muscle bundles surrounded by collagen were occasionally seen in the longitudinal muscle layer (fig 3D). Collagen deposition was also increased between the circular and longitudinal muscle layers and around the myenteric plexus but began to decrease at day 28, and returned to normal appearance at day 42 (data not shown). Some mild collagen deposition was observed in the SM in control tissues (fig 3C).

We also quantified rectal collagen content by measuring pepsin soluble collagen, which reflects recently produced

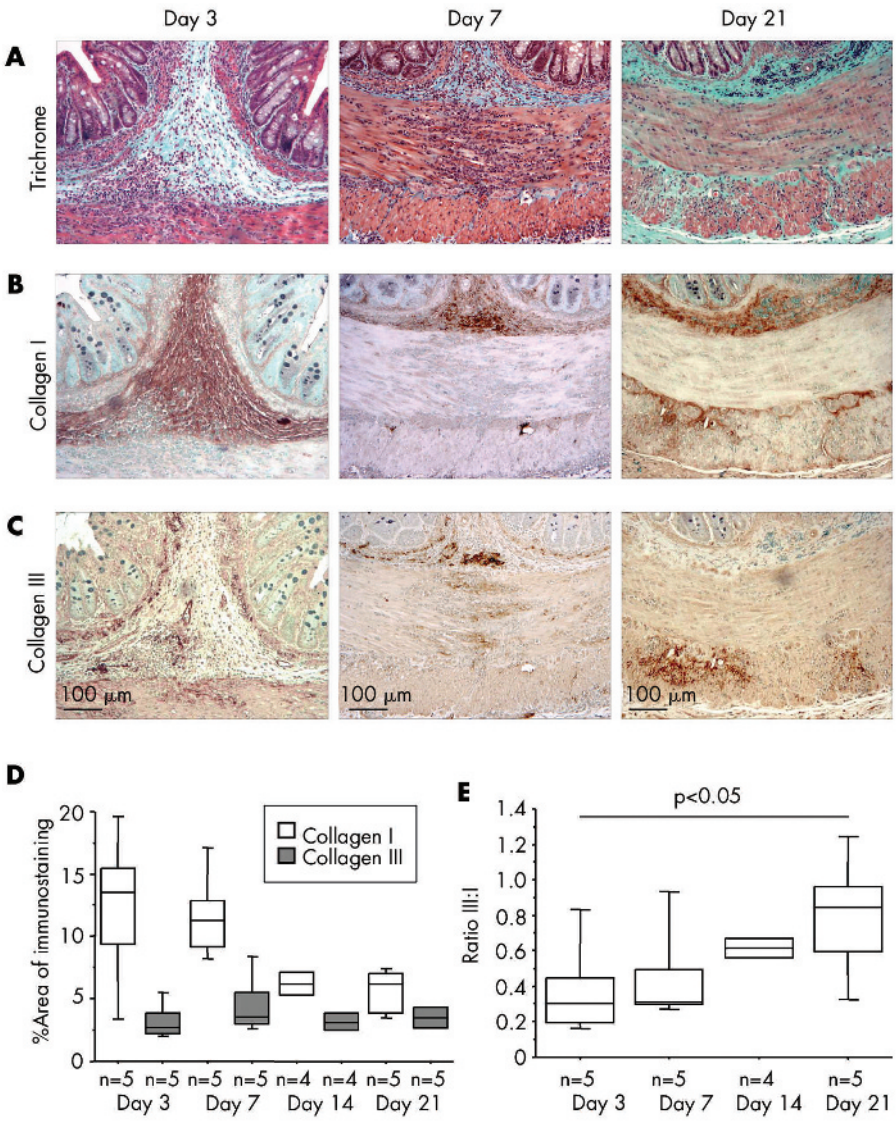

Figure 5 Immunohistochemical staining for collagen types I and III in adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) treated mice. Serial sections were stained with Masson's trichrome (A), anticollagen I (B), and anticollagen III antibodies (C). (A) Collagen I immunostaining was seen in the mucosa, submucosa, and surrounding the myenteric plexus. (B) Collagen III immunostaining was observed in the muscularis mucosa, submucosa, and muscularis propria. Collagen III staining in the muscle layer appeared to be stronger at day 21. Original magnification ×100. (D) Immunostaining based semiquantitative measurement of collagens I and III. The area of collagen I tended to decrease during the time course while type III area did not change. Data are expressed in per cent positive staining area of the total area. (E) The ratio of collagen III:I showed a trend to increase.

collagen. AdMCP-1 treated mice showed a significantly higher collagen level until day 21 compared with AdDL70 treated controls (fig 4).

Different distribution of collagen types I and III, and increased ratio of collagen III:I in AdMCP-1 treated mice

Serial sections were stained with trichrome (fig 5A), anticollagen I (fig 5B), and anticollagen III antibodies (fig 5C). Collagen I immunostaining was seen in the mucosa, SM, and surrounding the myenteric plexus. Staining around the myenteric plexus was more obvious on day 21 than in earlier stages. In contrast, collagen III immunostaining was observed in the MM, SM, and MP. Collagen III staining in the SM was seen on days 3 and 7 but was barely seen after day 14. In the MM and MP, type III collagen staining was observed around the muscle cells and appeared to be stronger on day 21 (fig 5A–C).

Immunostaining based semiquantitative measurement of collagens I and III showed that collagen I tended to decrease throughout the time course while type III area did not change (fig 5D). The ratio of collagen III:I showed a significant increase up to ~0.8 at day 21 (fig 5E).

Immunohistological patterns of mesenchymal cell subtypes in fibrotic colon of AdMCP-1 treated mice

Serial sections were stained with trichrome (fig 6A), antidesmin (fig 6B), anti-α smooth muscle actin (α-SMA) (fig 6C), and antivimentin antibodies (fig 6D). Smooth muscle in MP were both desmin (D^+) and α-SMA positive (A^+), while the MM was weak or negative for desmin. Vimentin immunopositive (V^+) cells were scattered throughout the mucosa, SM, and MP, consistent with the inflammatory infiltrate. However, A^+ cells were barely seen in the SM. Although many of the V^+ cells were $V^+A^-D^-$ in the MP, $V^+A^+D^+$ cells were also seen. V^+ cells were also seen in the myenteric plexus at day 21, but were not clearly evident at day 7, suggesting a phenotypic shift to the fibroblast.

Accumulation of CD3 and F4/80 positive cells after AdMCP-1 administration

To characterize the infiltrate, sections were stained with anti-CD3 and anti-F4/80 antibodies (data not shown). We observed a significant transmural increase in both CD3 and F4/80 positive cells in AdMCP-1 treated mice compared with AdDL70 treated controls until day 21 (fig 7A, B). Control virus AdDL70 caused a slight increase in both CD3+ and F4/80+ cells.

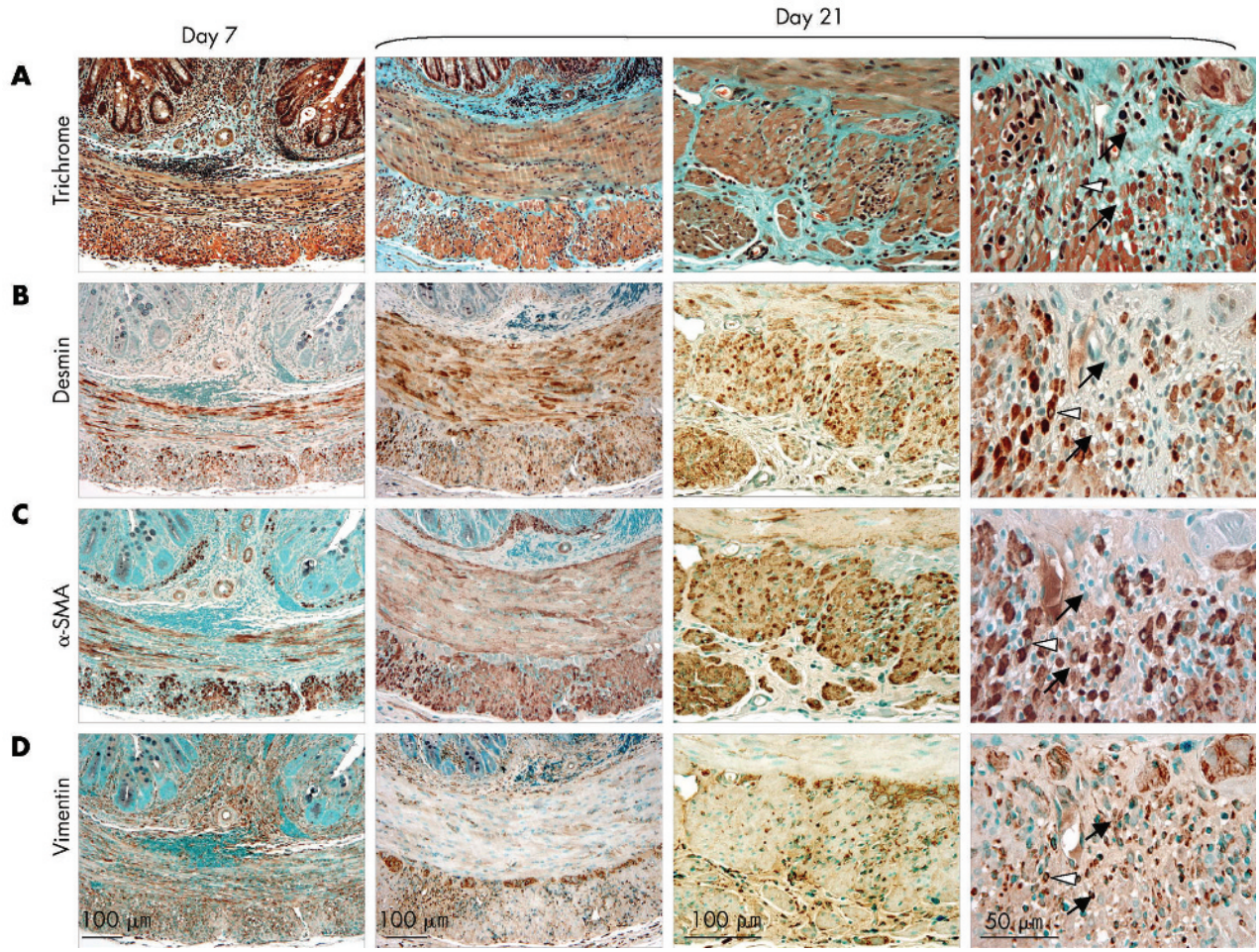

Figure 6 Immunohistological patterns of mesenchymal cell subtypes in fibrotic colon of adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) treated mice. Serial sections were stained with Masson's trichrome (A), antidesmin (B), anti-α smooth muscle actin (α-SMA) (C), and antivimentin antibodies (D). Smooth muscle cells in the muscularis propria were both desmin (D^+) and α-SMA positive (A^+) while the muscularis mucosa was weak or negative with desmin. Vimentin positive (V^+) cells were scattered in the mucosa, submucosa, and muscle layers, consistent with inflammatory infiltrates. Although many of the V^+ cells were $V^+A^-D^-$ in the muscle layer (black arrow), $V^+A^+D^+$ cells were also seen (white arrowhead). The myenteric plexus was also V^+ at day 21.

AdMCP-1 failed to induce collagen overproduction in lymphocytes deficient mice

To investigate the role of lymphocytes in collagen deposition by MCP-1, $Rag2^{-/-}$ mice were injected with AdMCP-1 intramurally, and collagen content in the rectum was measured. $Rag2^{-/-}$ mice showed no collagen overproduction at day 3 (fig 8), suggesting a critical role for lymphocytes in the development of MCP-1 mediated fibrosis.

F4/80 immunostaining was also performed to investigate the role of macrophage in fibrogenesis in this model. There was no significant difference in F4/80 staining between AdMCP-1 treated $RAG2^{-/-}$ mice and wild-type mice (data not shown).

Increased expression of TGF-β1

We also investigated tissue TGF-β1 levels as MCP-1 is known to increase TGF-β1 production. The results show a significant increase in TGF-β1 levels in AdMCP-1 treated mice at days 3 and 7 but not after day 14 (fig 9A).

Expression of tissue inhibitory metalloproteinase 1 and matrix metalloproteinase 3

MMPs and their inhibitors TIMPs are considered to play an important role in tissue remodelling. We investigated protein levels of TIMP-1 and MMP-3. TIMP-1 level showed a significant increase in AdMCP-1 treated mice only on day 7 (fig 9B). We also observed a significant increase in MMP-3 expression in AdMCP-1 treated mice at days 3 and 14. The ratio TIMP-1/MMP-3 was significantly higher in AdMCP-1 treated mice at day 7 compared with AdDL70 treated mice (data not shown).

DISCUSSION

Increased expression of MCP-1 is observed in human pulmonary fibrosis[24] and liver cirrhosis,[15] and in the lung[11] and kidney[13] in animal models of fibrosis. Moreover, it has been shown that anti-MCP-1 gene therapy attenuates fibrosis formation in rat artery,[25] liver,[26] mouse lung,[12] and kidney.[27] Taken together, these studies suggest that MCP-1 plays an important role in fibrosis of these organs.

It has been reported that mRNA expression of MCP-1 is increased in several experimental colitis models.[28 29] Increased expression of MCP-1 has also been shown in human IBD mucosa.[30] McCormack *et al* reported an increase in MCP-1 in human IBD mucosa (ulcerative colitis 175 (45) pg/mg and CD 120 (42) pg/mg).[31] Increased mucosal MCP-1 level in canine colitis has also been reported (4180 (330) to 6550 (460) pg/mg).[32] Several reports have shown

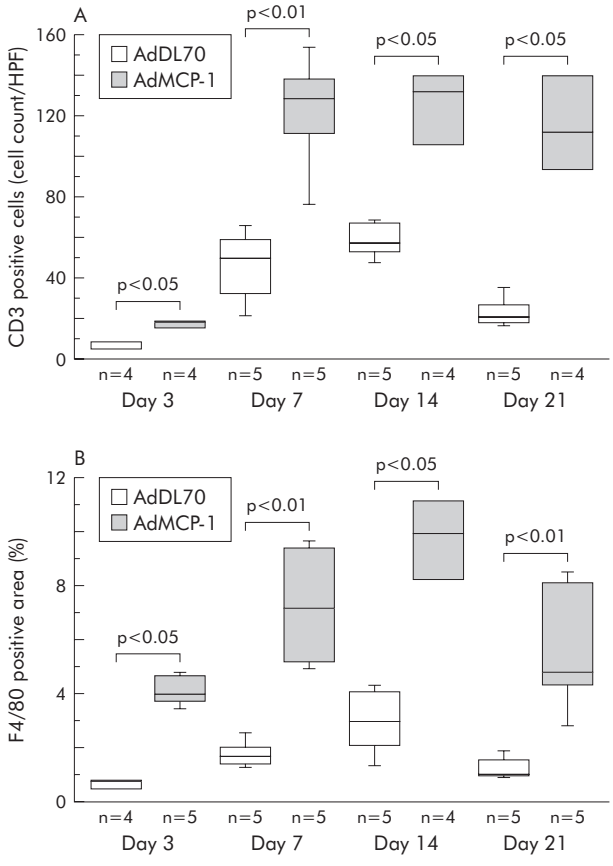

Figure 7 Accumulation of CD3 and F4/80 positive cells in adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) treated mice. (A) Number of positive cells in AdMCP-1 treated mice was significantly higher than in empty virus (AdDL70) treated controls. Data are expressed as cell counts per hyper power field (HPF). (B) F4/80 positive area was significantly larger compared with AdDL70 treated controls. Data are expressed as per cent positive staining area of total area.

increased expression of MCP-1 in the mucosa and deeper layers of the intestine of IBD patients.[19 20] While the amount of MCP-1 protein in the deeper layer of the inflamed gut was not measured in these studies, they provide some justification for considering the role of MCP-1 in the pathophysiology of IBD in general and in the pathogenesis of stricture formation. The fibrogenic potential of MCP-1 in the gut demonstrated in the present study should prompt further consideration of the role of this peptide in stricture formation in IBD.

In this study, we injected the viral vector intramurally to mimic the locus of expression of MCP-1 described in the deeper layer of the CD intestine. Intramural injection of the adenovector carrying *LacZ* produced significant β-galactosidase expression that remained evident for more than 21 days after injection—considerably longer than had been reported by rectal intraluminal administration of the vector.[33] Tissue MCP-1 level after AdMCP-1 injection was significantly higher compared with that in controls and remained elevated until day 42. MCP-1 expression was seen in mainly the MM and MP, which was consistent with the microscopic appearance of β-galactosidase expression following AdLacZ injection.

Collagen deposition, which involves the MP, was seen at later time points (days 14, 21) after AdMCP-1 administration; the muscle layer was disorganised by infiltrating cells, and isolated smooth muscle bundles were surrounded by collagen. This finding is considered to be important as the

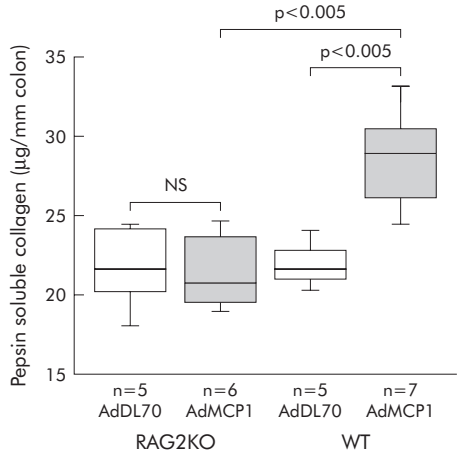

Figure 8 Rag2$^{-/-}$ (RAG''KO) mice were injected with adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) or control empty virus (AdDL70) and killed three days after injection. Tissues were taken and collagen assay was performed. C57Bl/6 mice (WT) were used as controls. AdMCP-1 failed to induce overproduction of collagen.

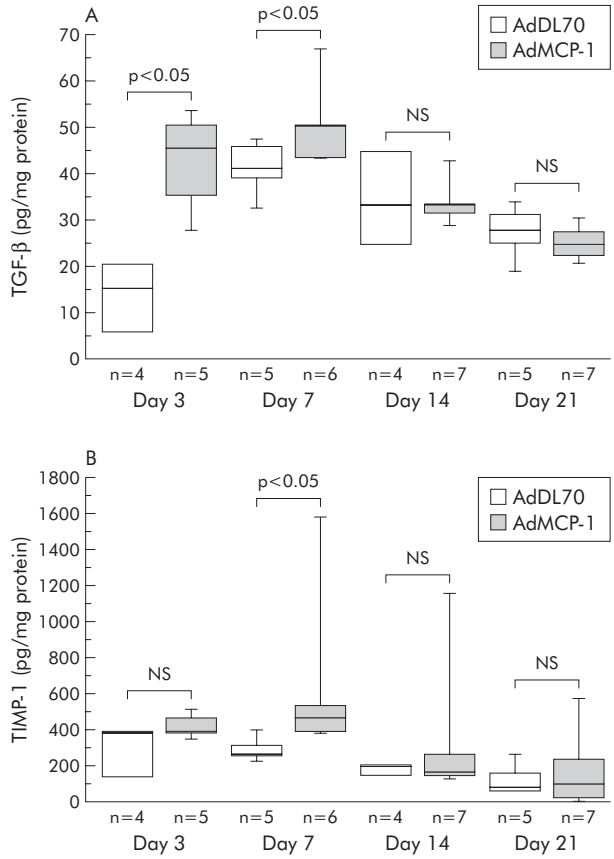

Figure 9 (A) Tissue transforming growth factor β (TGF-β) level after virus administration measured by enzyme linked immunosorbent assay (ELISA). Data are corrected for tissue protein concentration. A significant elevation in total TGF-β level was observed at days 3 and 7 following administration of adenovirus expressing murine monocyte chemoattractant protein 1 (AdMCP-1) compared with control empty virus (AdDL70) treatment. (B) Tissue inhibitor of metalloproteinase (TIMP-1) was measured by ELISA. A significant elevation in TIMP-1 level was observed at day 7 following administration of AdMCP-1. Data are corrected for tissue protein concentration.

muscle layer often involves fibrosis in CD.[34] Collagen deposition started to decrease at day 28 and normalised by day 42. Quantifiable pepsin soluble collagen significantly increased, peaked at day 3, and returned to normal by day 28. As this quantitative assay of pepsin soluble collagen reflects newly produced collagen, the results are considered to reflect overproduction of collagen caused by an imbalance between fibrogenesis and fibrolysis.

Immunostaining of collagen types I and III showed differential distribution of these subtypes in the gut wall. Type I was seen mainly in the SM and around the myenteric plexus at later time points. In contrast, type III was seen in the MP, MM, and SM. Collagen III staining in the SM was only seen at early time points whereas that in the MP became intense at later time points. Immunostaining based semi-quantification showed that collagen I decreased progressively while type III showed no change. However, in the MP, type III staining increased progressively and the ratio of type III and I showed a significant increase from ~0.3 at day 3 to ~0.8 at day 21. It has been reported that collagen type III is increased in CD strictures,[35 36] such that the ratio of type III:I is ~1, while the normal ratio is ~0.3.[37] Taken together, these findings demonstrate a pattern of fibrosis reminiscent of that found in CD.

Smooth muscle cells are considered to be the main source of collagen in the fibrotic intestine.[38] In the present study, collagen III immunostaining was seen around smooth muscle cells in the MM and MP, suggesting that smooth muscle cells produced the collagen. On the other hand, vimentin immunopositive (V^+) cells were scattered throughout the mucosa, SM, and muscle layers consistent with the inflammatory infiltrate. However, A^+ cells were barely seen in the SM, suggesting that the $V^+A^-D^-$ phenotype of fibroblasts plays an important role in fibrogenesis in SM. Although many of the V^+ cells were $V^+A^-D^-$ in the muscle layer, $V^+A^+D^+$ cells were also seen, indicating that both the $V^+A^-D^-$ phenotype of fibroblasts and the $V^+A^+D^+$ phenotype of myofibroblasts, together with muscle cells, were contributing to fibrosis formation in the muscle layer. These results are similar to findings in CD.[34] The myenteric plexus was also V^+ at day 21 and was surrounded by collagen, suggesting that the transformation of cells such as the interstitial cells of Cajal towards a fibroblast phenotype might have occurred.[34]

The inflammatory cell infiltrate included CD3+ lymphocytes and F4/80+ macrophages. These cells were significantly increased in the AdMCP-1 treated group, suggesting that exogenous MCP-1 chemoattracts these cells. It has been reported that MCP-1 recruits monocytes and lymphocytes but does not activate them.[38] In this study, monocytes and lymphocytes, which had been recruited by MCP-1, could have been activated by the adenovirus itself. Coexistence of lymphocytes and fibroblasts raises the possibility that they interact with each other towards the development of fibrosis, as it has been reported that fibroblasts, which express CD40, and T lymphocytes, which express CD40 ligand (CD40L), interact and activate each other and promote fibrogenesis.[39] CD40L is thought to promote fibrosis by activating fibroblasts.[40] The absence of collagen deposition in T and B cell deficient $Rag2^{-/-}$ mice in this study provides evidence for the critical role of lymphocytes in the generation of fibrosis in this model. In contrast, the increase in F4/80 positive macrophages in $RAG2^{-/-}$ mice at the same level as in wild-type mice indicates that macrophages play a lesser role in fibrogenesis in this model.

It is well known that TGF-β is a major fibrogenic cytokine and has been shown to be induced by MCP-1 in vitro.[41] In this study, the local TGF-β level was significantly increased at days 3 and 7 after AdMCP-1 injection. At the later time points, declining levels of MCP-1 were probably insufficient to maintain high levels of TGF-β. We speculate that the increased level of TGF-β contributes to the fibrosis seen in this model.

MMPs and TIMPs also play a major role in tissue remodelling and are regulated by several cytokines or chemokines. For example, it is known that TGF-β increases TIMP-1 expression and decreases MMP-1 expression in human fibroblasts,[42] leading to a fibrogenic imbalance of MMP/TIMP. On the other hand, MCP-1 increases both MMP-1 and TIMP-1 expression in human fibroblasts.[43] In this study, we observed local upregulation of TIMP-1 and MMP-3 expression in AdMCP-1 treated mice. The ratio of TIMP-1/MMP-3 was significantly higher in this group compared with AdDL70 treated mice. These result suggest that MCP-1 increased TIMP-1 expression both directly and indirectly through TGF-β at least in the early stage of this model. Thus an imbalance of MMP/TIMP in the early stage might be a contributing factor in collagen deposition in this model.

In conclusion, we have demonstrated for the first time that MCP-1 is capable of inducing fibrosis in the intestine. The mechanisms regulating MCP-1-induced fibrosis include enhanced infiltration of lymphocytes and interaction between lymphocytes and fibroblasts/myofibroblasts and subsequent upregulation of TGF-β and TIMP-1 production. Enhanced production of TGF-β and TIMP/MMP imbalance might be key initial factors in MCP-1 mediated fibrosis. MCP-1 could therefore to be a novel target in developing novel therapeutic strategies for intestinal fibrosis that might include direct intramural injection of antagonists or anti-sense into the resection margins during surgery for strictures.

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