S100A4 Expression is Increased in Stricture Fibroblasts From Patients with Fibrostenosing Crohn’s Disease and Promotes Intestinal Fibroblast Migration

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Abstract

Introduction: Fibroblasts represent the key cell type in fibrostenosing Crohn’s Disease (FCD) pathogenesis. S100A4 is an EF-hand calcium binding protein family member, implicated in epithelial-mesenchymal-transition (EMT), and as a marker of activated T-lymphocytes and fibroblasts in chronic tissue remodelling. The aim of this study was to examine the expression profile of S100A4 in the resected ileum of patients with FCD.

Materials and Methods: Mucosa, seromuscular explants and transmural biopsies were harvested from diseased and proximal, macroscopically normal margins of ileocaecal resections for FCD. Samples were processed for histochemistry, immunohistochemistry, real-time RT-PCR, Western blotting and transmission electron microscopy. Primary explant cultures of seromuscular fibroblasts were exposed to TGF-β1 (1ng/ml) and S100A4 expression and scratch wound healing activity assessed at 24 hours. CCD-18 Co fibroblasts were transfected with S100A4 si-RNA, and subsequently treated with TGF-β1 (1ng/ml) for 30 minutes or 24 hours, then assessed for S100A4 and Smad3 expression and scratch wound healing activity. Results: S100A4 expression was increased in stricture mucosa, in the lamina propria and in CD3 positive intra-epithelial CD3 positive T-lymphocytes. Fibroblastic S100A4 staining was observed in seromuscular scar tissue. Stricture fibroblast explant culture showed significant upregulation of S100A4 expression. TGF-β1 increased S100A4 expression in cultured ileal fibroblasts. In CCD-18 Co fibroblasts S100A4 siRNA inhibited scratch wound healing and modestly inhibited Smad3 activation. Conclusions: S100A4 expression is increased in fibroblasts as well as immune cells in CD stricture and induced by TGF-β1. Results from knockdown experiments indicate a potential role for S100A4 in mediating intestinal fibroblast migration. (242 words)

Keywords: Crohn’s Disease, Fibrosis, EMT, S100A4, Migration
Introduction

The most commonly affected site in Crohn’s Disease (CD) is the terminal ileum (26) with progressive stricturing due to fibrostenosing type disease (FCD) a major indication for surgery. The cumulative probability of surgery in patients with CD is approximately 10% annually (30), with 70-90% of patients ultimately requiring surgery (14, 25, 36).

Intestinal stricturing in CD involves gross thickening of the bowel wall, attributable in particular to expansion of the submucosal and seromuscular (reticular) regions. Thickening in the latter is characterised by the presence of an expanded population of activated fibroblasts organised into discrete areas of scar tissue, and embedded within extensively infiltrated and hypertrophied mesenteric fat (27, 32). Elucidation of the key steps operative in fibroblast activation and subsequent scar formation in FCD holds the potential to identify new molecular targets for novel anti-fibrotic therapies (6).

Fibroblasts derived from stricture sites have an altered phenotype which persists during explant culture. Increased constitutive expression of pro-fibrotic cytokines transforming growth factor-beta (TGF-β), isoforms 1 (28) and 2 (29), and connective tissue growth factor (CTGF) (1), collagen subtype III (41), tissue inhibitor of metalloproteinase-1 (TIMP-1) (29), vascular cell adhesion molecule (VCAM) (2) and intercellular adhesion molecule (ICAM) (4) have been described. These transcriptomic findings are complimented by functional alterations in stricture fibroblast phenotype including reduced chemotactic migratory capacity in lamina propria myofibroblasts (22), increased total collagen synthesis (20) and enhanced collagen contractile activity (35).

S100A4 (also known as fibroblast-specific protein-1, Fsp-1), is a TGF-β1 inducible, 11kDa EF hand calcium-binding protein coded from chromosome 1q21. It is found intracellularly in homodimeric, and heterodimeric forms (38, 49). Binding of calcium leads to the exposure of a hydrophic interaction domain which permits S100A4 to interact with a wide range of target molecules, thereby endowing cells with enhanced pro-invasive properties (37). Secreted multimeric forms have also been reported which, via the activation of an as yet undefined receptor, lead to the activation of NFkB and p53 stabilisation and have also been implicated in cell migration (18, 40).
S100A4 is of particular interest as a marker of both malignant potential and chronic tissue remodelling (38). Tumour grade and metastatic potential in lower gastrointestinal tract malignancies correlate positively with S100A4 expression (8, 45), complimenting similar findings in breast, pancreatic and gastric carcinoma. With regard to chronic tissue remodelling, increased S100A4 expression has been shown to coincide with the extent of tubular atrophy and fibrosis during chronic renal allograft nephropathy (48) and joint destruction by synovial fibroblasts in rheumatoid arthritis (34).

S100A4 was originally described as a marker of activated fibroblasts, hence the synonym fibroblast specific protein (Fsp-1) (42). However, the cellular origin of S100A4 expression in vivo is diverse, including inflammatory cells of the myeloid and lymphoid lineages, fibroblasts and epithelial cells undergoing EMT (38). S100A4 was found to stain positively in the renal tubulointerstitium from biopsies of chronic fibrotic kidney disease and is capable of inducing EMT when overexpressed in cultured renal tubular epithelial cell lines(42). Consequently, S100A4 has increasingly been adopted as a marker of EMT in studies that attempting to define the contribution of this process to chronic tissue remodelling. Significant disagreement exists in the literature as to whether S100A4 overexpression during chronic tissue remodelling reflects local EMT and fibroblast activation (17), or simply reflects increased inflammatory cell recruitment (21). Studies in rheumatoid arthritis support the hypothesis that high levels of S100A4 expression on various subpopulations of cells, including fibroblasts, have the potential to drive remodelling responses in chronically inflamed tissue (18, 34).

The aim of the present study was to characterise and quantify the expression of S100A4 in the strictured ileum of patients undergoing ileocaecal resection for FCD. Specific emphasis was placed on the presence of S100A4 in lineages likely to directly contribute to stricture formation, namely the mucosal epithelial cells (via EMT) and intestinal fibroblasts. The effect of si-RNA mediated S100A4 depletion on the migratory potential of intestinal fibroblasts in vitro was subsequently examined.

**Materials and Methods**

**Patient cohort**

Samples from 8 patients with FCD were identified for study (Table 1). All gave written, informed consent and the study was approved by the local ethics committees of the Mater
Misericordiae University Hospital and St. Vincent’s University Hospital, Dublin, Ireland.

Biopsies were taken from patients undergoing elective stricture resection. Biopsies were taken from the anti-mesenteric border of the terminal ileum in areas of disease and proximal, macroscopically normal margins. The diagnosis was based on clinical, radiological, and endoscopic examination and histological findings, confirmed on post-operative histology.

Up-to-date clinical information on age at resection, sex, disease duration, current and prior use of corticosteroids, anti-TNF α and/or 5-ASA therapies, as well as patient smoking status were obtained from in-patient charts and confirmed by pre-operative researcher interview. Sample assessment and processing were carried out within four hours of collection.

**Histological Studies**

Transmural biopsies taken at resection were fixed overnight in 10% non-buffered formalin, embedded in paraffin, and 4μm sections cut and mounted. Rehydrated sections were incubated with haematoxylin and eosin or for assessment of fibrosis, incubated sequentially in Bouin's Fixative, Wiegert's iron haematoxylin, and Gomorri’s trichrome stain (all Polysciences Europe GmbH, Eppelheim, Germany), and 0.5% acetic acid. Sections were then dehydrated, mounted, and cover-slipped.

**Immunohistochemical Studies**

Prior to primary antibody incubation, 4μm sections were deparaffinised, endogenous peroxidase quenching was carried out in 3% hydrogen peroxide in methanol and antigen retrieval performed in 0.5M citric acid buffer. Sections were probed for S100A4 (polyclonal rabbit anti-human, A5114, Dako, Ireland) , CD3 (polyclonal goat anti-human CD3δ chain, sc-26431, Santa Cruz Biotechnology, U.S.A.), pan cytokeratin (mouse monoclonal anti-human cytokeratin C2562, Sigma-Aldrich, Ireland) and α-smooth muscle actin (α-SMA), (alkaline phosphatase (AP) conjugated monoclonal mouse anti-human α-SMA, A-5691, Sigma-Aldrich, Ireland) A one hour, room temperature incubation of 1:200 dilutions of all primary antibodies in 0.2% bovine serum albumin in phosphate-buffered saline was used. Visualisation of non-conjugated primary antibody binding proceeded via the use of biotinylated secondary antibody and horseradish peroxidase (HRP)/di-amino-benzidine based staining according to the Vectastain Universal ABC kit (Vector Laboratories, UK) protocol. Sections were counterstained with Harris haematoxylin, dehydrated and coverslipped. For double staining of S100A4 and α-SMA, subsequent to development of the S100A4 HRP
signal, sections were washed in Tris-buffered saline and then incubated with the anti-α-SMA AP conjugate for 1 hour followed by development of staining using the Fast Red substrate system (Dako, Ireland). For S100A4/CD-3 double staining, S100A4 HRP stained sections were incubated for 1 hour with anti-CD3 antibody and signal detected using an anti-goat glucose oxidase staining system (Vector Laboratories, UK). All double stained sections were counterstained with Gill’s haematoxylin, mounted in glycerol, cover-slipped and sealed. Histological and immunohistochemical images were captured using the Aperio ScanScope XT Digital Slide Scanner with image processing in Aperio Image Scope (Aperio, CA).

Transmission Electron Microscopy

Fresh paired biopsy samples from resections (n=3) were immediately placed in fresh 2.5% glyceraldehyde and then post-fixed in 1% osmium tetraoxide for 1 hour followed by dehydration in an ascending ethanol series. Sections were then embedded in Epon for overnight polymerisation at 60°C. Semi-thin sections were stained with toluidine blue for selection of mucosal areas for ultramicrotomy. Ultrathin sections (80nm) were subsequently cut and placed on 100-mesh copper grids, stained with 0.4% lead citrate and 2% uranyl acetate and examined by transmission electron microscopy (Tecnai Twin, FEI). Tight junction (TJ) and adherens junction (AJ) patency was evaluated in mid-villous mucosal epithelial cells by measuring the width (nm) of junctions at 5 cell-cell adhesion sites. Quantification was repeated over 3 discrete locations in the mucosa. All images were calibrated and recorded using a MegaView 3 digital recording system (Olympus).

Fibroblast Isolation and Culture

Intestinal fibroblast cultures were established using a primary explant technique as previously described (1). Briefly, seromuscular biopsies were taken from macroscopically normal and diseased terminal ileum in patients undergoing resection for fibrostenosing CD. Biopsies were taken prior to bowel devascularisation and seeded onto a petri dish for sterile culture in 10% foetal bovine serum (FBS). Cells obtained by this technique adopt an elongated morphology in culture and stain positively for vimentin and negatively for desmin. For all experiments fibroblasts were used between the third and sixth passage.


*Real-time RT-PCR*

CD tissue was placed in RNAlater at the time of resection (Sigma-Aldrich Ireland). Mucosal and seromuscular layers were identified, dissected, snap-frozen in liquid nitrogen, homogenized, placed in an acid-guanidinium-phenol-chloroform (Trizol Reagent, Invitrogen, Dublin, Ireland) and stored at -80°C until processed. Fibroblast cultures were harvested from culture flasks using a cell scraper and placed in Trizol Reagent. Total RNA was precipitated in isopropanol, washed in ethanol and resuspended in DEPC water. RNA concentration and quality were assessed by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, DE, U.S.A.) at a 260:280 ratio. Total RNA (1μg) was treated with DNase I then used to synthesize cDNA using the random primer method.

Gene target expression was analysed using real-time PCR TaqMan assay on an ABI PRISM 7900HT Sequence Detection system (Applied Biosystems, U.S.A.). The primers and probes for these targets were supplied as pre-optimised Gene Expression Assays (Applied Biosystems, U.K.). The probes for the target genes were labelled with the fluorescent dye FAM on the 5’ end and a nonfluorescent quencher on the 3’ end. The endogenous control 18S RNA was purchased from Applied Biosystems as a predeveloped assay reagent (PDAR) with the probe labelled with VIC at the 5’ end and TAMRA on the 3’ end to facilitate dual reporter assay. Default thermal cycling conditions were: 2 minutes at 50°C, 10 minutes at 95°C for enzyme activation, and then 40 cycles of 15 seconds at 95°C for denaturation and 1 minute at 60°C for annealing and extension. Results were analysed using the relative delta ct method of analysis with candidate gene expression normalized to the housekeeping gene, 18S rRNA (annotated as RQ mRNA on figure axes).

*Protein Extraction and Western Blot Analysis*

Total protein was isolated from snap-frozen CD mucosa by homogenisation in tissue lysis buffer as previously described (12). Total protein from fibroblast culture was isolated using RIPA buffer also as previously described (1, 12). For Western blotting studies, protein (30μg) was resolved on a 12% Bisacrylamide-acrylamide-SDS gel (75 minutes at 140V) prior to transfer to 0.2μm pore PVDF Immobilon membranes (Millipore Corp., Ireland) at 100V for 80 minutes in Tris-Glycine-Methanol transfer buffer. Blots were incubated with rabbit monoclonal anti-human S100A4 (antibody as listed for immunohistochemistry) at a dilution of 1:500. Active serine 423/425 phosphorylated Smad3 (p-Smad3) and total smad3 were
detected using 1:1000 dilutions of the rabbit anti-human antibodies AB52903 and AB28379 respectively (both Abcam, U.S.A.) Anti-rabbit secondary antibody was incubated at a concentration of 1:5000. Incubation of membranes with 1:5000 dilutions of mouse monoclonal anti-human β-actin (F3022, Sigma-Aldrich, Ireland) was used to develop loading control signals. Protein density on scanned Western blots was determined using the Image J Software v1.26t (National Institutes of Health U.S.A.).

siRNA transfection

Studies of siRNA knockdown of S100A4 expression were carried out in the normal human colonic fibroblast line CCD-18Co (ATCC, CRL 1459). S100A4 siRNA was purchased from Qiagen (Valencia, CA, U.S.A.) and scrambled siRNA (Ambion Inc., Cambridge, UK) was used as a control for off-target effects. The sequence of selected regions to be targeted by the siRNAs were 5′-AACGAGGTGGACTTCCAAGAG-3′ for S100A4, and 5′-AATTCTCCGAACGTGTCTCGT-3′ for the scrambled non-silencing siRNA. Initial dose-response experiments (data not included) determined the optimum dose of S100A4 siRNA to be 5nM. Transfection of siRNA was achieved using Lipofectamine 2000 (Invitrogen, Ireland) in GIBCO Opti-MEM I Reduced-Serum 1x Medium (Invitrogen, Ireland). After 6 hours the medium was changed to antibiotic-free 1% FBS RPMI 1640 medium. Cells were further treated or harvested for analysis 24 hours from initial transfection.

Cell Migration

Wells of a six well plate with a horizontal 0.6mm pre-etched undersurface were seeded with 5 x 10⁴ fibroblasts. A perpendicular horizontal “scratch wound” was made in the cell layer at 80% confluence and high-resolution images of wound closure obtained using a microscope-mounted, A70 Powershot Camera (Cannon Inc, Ireland). Images were taken at the point of intersection of scratch wound and etch, using set distances and magnification. Initial images at experimentation (time zero) were assessed to determine equal area for migration. Intersection images were obtained at 12-hour time-periods. Image J Software v1.26t (National Institute of Health, U.S.A.) was used to assess the area uncovered by cells, and the migratory capacity of the cells calculated by comparing the rate at which cells migrated towards each other in multiple sets. The rate of closure was expressed as percentage recovered per unit time.
Statistics

Data were assessed for normality using the Kolmogorov-Smirnov test and analysed parametrically using unpaired Students t-test and one-way ANOVA with Bonferroni correction. p< 0.05 was considered statistically significant. For all data for which significance beyond the 99% confidence interval was obtained, results are quoted as p< 0.01.

Results

Patient characteristics

Biobanked material from 15 CD resections included tissue from 8 patients with FCD (6 male and 2 female) (Table 1). The mean age of these patients was 33.2 years (+/- 5.6) and median disease duration was 8.5 years (IQR=5). A single patient had never been treated with corticosteroids, while of the remaining corticosteroid experienced patients there were 2 currently in treatment, 4 within one year of surgery and 1 having been treated 1 to 5 years previously). Three patients had been treated with infliximab while 5 had received 5-ASA. Of the cohort, only 1 patient had undergone previous resection for CD. Only 2 of the cohort identified themselves as current smokers with the remainder stating never to have smoked.

Transmural thickening in FCD is due to collagen deposition and fibrofatty proliferation in expanded submucosa and seromuscular layers.

The diseased section (Fig. 1b) demonstrates transmural thickening, fibrofatty proliferation and vascular congestion (arrow) in comparison to its macroscopically normal adjacent terminal ileal counterpart (Fig. 1a). High-power examination of Gomorri’s Trichrome sections demonstrates the formation of mature, fibrillar collagen scar tissue within the submucosa (Fig. 1e) and seromuscle (Fig. 1f) in comparison to their normal adjacent counterparts (Fig. 1c and d).

Junctional adhesion complex disruption in FCD mucosal epithelium

T.E.M. analysis of TJ structure demonstrated that in all samples examined, Diseased tissue was characterised by dilatation and unzipping of the TJ relative to a patent, closed “tight” morphology observed in normal adjacent specimens. Morphometric comparison of adherens junction (AJ) diameter demonstrated a 41% increase in diameter in Diseased tissue versus Normal Adjacent tissue (22.5nm +/- 0.8 vs. 31.8 +/-2.8 p=0.001), (Fig 2).
S100A4 expression is upregulated on mRNA and protein analysis FCD mucosa.

Diseased mucosa demonstrates a 5.4-fold increase in S100A4 mRNA (1 +/- 0.9 vs. 5.4 +/- 0.9, p=0.01) (Fig. 3A) and a 79% increase in protein (24 +/- 18 vs. 126 +/- 17, p=0.02) (Fig. 3B) expression.

S100A4 localisation in FCD mucosa.

Mucosal S100A4 positivity is concentrated in the lamina propria in grossly Normal tissue sections (Fig. 4, a and b). A similar profile is found in diseased sections, in which staining also becomes prominent in cells continuous with the mucosal epithelium (Fig. 4 c and d). Similar patterns of staining are observed to S100A4 when stained with CD3 (Fig. 4e), suggesting that they are T-lymphocytes (arrow in e), Pan-cytokeratin staining (Fig. 4f) of the mucosa of diseased tissue sections shows that cytokeratin is exclusively localized to mucosal epithelial cells residing within the epithelial lining.

S100A4-positive/α-SMA negative fibroblasts populate collagen plaques of stenotic terminal ileal seromuscle.

Low power fields (x8) (Fig. 5a and c) demonstrate sparse S100A4 staining in the reticular connective tissue of Normal Adjacent specimens and intense staining in fibrotic plaques surrounded by fibrofatty proliferation in diseased seromuscle. High power (x40) fields demonstrate S100A4-positive mononuclear cells in normal adjacent seromuscle (Fig. 5b) and elongated, spindle-shaped cells, consistent with activated fibroblasts (Fig. 5d), in the fibrotic plaques of diseased seromuscle. Stricture fibroblasts do not stain for α-SMA (pink Fast Red stain in surrounding muscularis and microvasculature).
Stricture fibroblast explant cultures overexpress S100A4 mRNA and protein.

Primary explant cultures of seromuscular FCD terminal ileal fibroblasts were derived from diseased areas and compared to those from macroscopically normal adjacent sections. Confluent paired sets of fibroblasts show a 4.9-fold increase in S100A4 mRNA levels (1 +/- 1.9 vs. 4.9 +/- 1.6, p< 0.01) from disease-derived explants on real-time RT-PCR analysis (Fig. 6A). Western blotting analysis shows a striking increase in S100A4 protein expression in diseased fibroblast explants (Fig. 6B).

TGF-β1 increases S100A4 mRNA expression in explanted fibroblasts from normal adjacent tissue of FCD resections and in cultured CCD-18Co human colonic fibroblasts.

Primary culture of fibroblasts from proximal normal resection margins were treated with the pro-fibrotic cytokine TGF-β1 (1ng/ml) for 24 hours. This led to a 2.9-fold increase in S100A4 mRNA expression (1 +/- 1.2 vs. 2.9 +/- 0.6, *p< 0.01) (Fig. 7A). Treatment of CCD-18Co fibroblasts for 24 hours with TGF-β1 increased S100A4 expression 3-fold (1.0 +/- 0.2 vs. 3.0 +/- 0.6, p< 0.01). S100A4 siRNA transfection decreased by 0.4-fold baseline S100A4 mRNA expression (1.0 +/- 0.2 vs. 0.5 +/- 0.1, p=0.01) and caused complete inhibition of TGF-β1 driven induction of S100A4 mRNA expression (3 +/- 0.6 vs. 0.3 +/- 0.2, p< 0.01) (Fig. 7B).

siRNA inhibition of S100A4 expression reduces fibroblast migratory capacity

Normal adjacent margin fibroblast explant cultures were treated with TGF-β1 (1ng/ml), causing a significant increase in migratory capacity at 24 hours (p< 0.01) (Fig. 8A). The effect of S100A4 siRNA (si-S100A4) on basal and TGF-β1 induced fibroblast migration was then examined in CCD-18 Co fibroblasts (Fig. 8B). The si-S100A4 alone caused a reduced migratory capacity at 24 hours versus control and scrambled siRNA treatments (p< 0.01) and was equally effective at inhibiting TGF-β1 enhancement of migration (p< 0.01).
S100A4 siRNA treatment and TGF-β1 induced Smad3 activation in CCD-18Co fibroblasts.

CCD-18Co fibroblasts were treated with either scrambled or si-S100A4 siRNA constructs for 24 hours then exposed to TGF-β1 (1ng/ml) for 30 minutes. An average 35% reduction in cellular S100A4 protein was observed in the si-S100A4 groups versus cells treated with a scrambled construct (combined scrambled groups 0.85 +/- 0.11 vs. combined si-S100A4 groups 0.57 +/- 0.11 p< 0.01). Western blotting of p-Smad3 demonstrated that basal p-Smad3/total Smad3 ratios were significantly reduced (26%), in the presence of si-S100A4 (0.85 +/- 0.07 vs. 0.63 +/- 0.13, p=0.05). TGF-β1 incubation caused a 1.8 fold increase in Smad3 phosphorylation in cells treated with scrambled siRNA (0.85 +/- 0.07 vs. 1.53 +/- 0.01 p< 0.01) and a 2 fold increase in si-S100A4 treated cells (0.63 +/- 0.13 vs. 1.31 +/- 0.07, p< 0.01). Absolute levels of TGF-β1 induced p-Smad3 were significantly decreased (15%) in the presence of si-S100A4 (1.53 +/- 0.01 vs. 1.31 +/- 0.07, p=0.02).

Discussion

This study examined the expression profile of S100A4 in the strictured ileum of patients with FCD, in order to establish whether S100A4 might be implicated as a component of EMT and/or fibroblast activation, as previously reported in diverse disease states (17, 18, 34, 42).

S100A4 expression was increased in the mucosa at stricture sites, principally within the lamina propria but also in cells continuous with the mucosal epithelium, that appear to be intra-epithelial T-lymphocytes. S100A4 expression was also observed in α-SMA negative fibroblasts populating discrete areas of scar within the expanded fibrofatty tissue of the strictured ileum. Constitutive increases in S100A4 expression in fibroblast explants obtained from these sites were also found. S100A4 upregulation was observed in intestinal fibroblasts treated with TGF-β1 and upregulation was required for the migratory response to scratch wounding.

The increased levels of S100A4 in immune cells observed in the strictured mucosa are consistent with reports of S100A4 expression in activated T-lymphocytes and macrophages in breast cancer (7), and co-localisation of S100A4 with the common leukocyte antigen CD45 in the ureteric obstruction model of progressive renal fibrosis in rodents (21). These changes
reflect the process of mucosal inflammation characteristic of active CD. While the most apparent focus of this phenomenon is the lamina propria, S100A4 expression in intra-epithelial lymphocytes cells attests to the expansion of the gamma/delta T-cell receptor subset, during intestinal inflammation (10).

Loss of junctional complex proteins such as E-cadherin occurs as part of the EMT paradigm (15) due to a combination of direct transcriptional (e.g. E-box repression (13)), and indirect enzymatic (e.g. via matrix metalloproteinases (9, 46)) effects of pro-EMT cytokines such as TGF-β1. Overexpression of S100A4 in squamous cell carcinoma lines is sufficient to cause a decrease in E-cadherin and β-catenin expression (31).

In CD, disruption of junctions is known to underpin increases in gut permeability and act as a driver of progressive disease (39). Although this study is limited by the small study pool, the present results demonstrate that the loss of apical junctional patency is evident and quantifiable in the FCD mucosal epithelium. It does not occur in conjunction with de novo epithelial expression of S100A4. It could be argued that S100A4 expressing epithelial cells might not be detectable within the mucosa due to migration to the lamina propria, where they may be traced through retained expression of epithelial markers such as cytokeratin.

The appearance of cytokeratin positive cells in the fibrofatty layer of the bowel wall of diseased segments of FCD resections. The possibility that S100A4 staining fibroblasts might also be found in the lamina propria and submucosa cannot be excluded, however the density of S100A4-positive cells, and the difficulty with which myofibroblasts and apical projections of the muscularis mucosae can be differentiated in the lamina propria, led the authors to focus on the more delineated areas of scar in the fibrofatty tissue. These
S100A4 positive fibroblasts were α-SMA negative, indicating that they did not have myofibroblast or smooth muscle phenotype and were highly unlikely to be derived from muscle hyperplasia. This suggests that they represent a different subtype from the lamina propria myofibroblast, in which several functional studies have been carried out (20, 22, 28, 29, 41). The existence of an S100A4 positive, α-SMA negative subpopulation of activated fibroblasts has been described both in the tumour associated stroma of murine models of pancreatic and breast cancer (44) and in areas of tubulointerstitial fibrosis in the DBA/2-pcy murine model of polycystic kidney disease (33). An increase in S100A4 expression in response to TGF-β1 was observed in ileal fibroblast cultures established from the proximal, normal adjacent tissue of resections. This is in agreement with previous reports that show S100A4 as a TGF-β1 responsive gene in type II alveolar epithelial cells (47).

The principal cellular behaviour associated with S100A4 is migration. The major identified intracellular pathway mediating this effect centres on alterations in cytoskeletal dynamics caused by the binding of S100A4 to the heavy chain of non-muscle myosin, thereby preventing the phosphorylation of myosin by protein kinase C2, and its subsequent association with actin (19). A number of reports also attest to a receptor for advanced glycation products (RAGE) mediated role for extracellular S100A4 in migration (reviewed in (3)).

The scratch wound healing assay provides a convenient method of assessing migration in cultured fibroblasts (24). The migratory capacity of intestinal fibroblasts in this assay was enhanced by TGF-β1 and was dependent upon S100A4 expression. This finding implies a role for increased expression of S100A4 in the migratory potential of fibroblasts in FCD. Interestingly, Leeb et al. previously described a reduction in the chemotactic migratory potential of lamina propria fibroblasts isolated from endoscopic biopsies of inflamed tissue from patients with CD (22) and that reduced migratory potential coincided with hypophosphorylation of focal adhesion kinase (FAK). Conversely, short term TGF-β1 incubation of lamina propria fibroblasts from non-inflamed areas of the bowel increases FAK phosphorylation and enhances migration (5). Prolonged treatment causes an attenuation of migratory potential and coincides with de novo expression of α-SMA and the development of a myofibroblast phenotype (5).

Sustained S100A4 expression in fibroblasts populating fibrofatty scar tissue, in the absence of myofibroblastic maturation may parallel findings in fibroblasts derived from idiopathic
pulmonary fibrosis, which show increased migratory potential, the magnitude of which is 
positively influenced by the density and maturation of scar tissue from which they were 
derived (43).

A small but significant decrease in activatory Smad3 phosphorylation in fibroblasts was 
observed in the presence of siRNA mediated inhibition of S100A4, under both basal and 
TGF-β1 treated conditions. That the relative magnitude of TGF-β1 induction of Smad3 
phosphorylation was not reduced in siRNA treated cells points to a non-TGF-β1 dependent 
mechanism.

Ligation of the proposed extracellular receptor for S100A4, the RAGE receptor, has been 
shown to effect Smad3 activation in a TGF-β receptor independent manner (23); therefore, 
differences in Smad3 phosphorylation per se may reflect a deficit in the extracellular actions 
of S100A4. However, the magnitude of reduction of Smad3 activation observed is not 
coherent with central Smad signalling representing the major mechanism behind the pro-
migratory effect of S100A4 observed in the present study. However, a deficit in extracellular 
S100A4 could influence migration in addition to its effects on the formation of intracellular 
S100A4 dimers in cytoskeletal dynamics. RAGE expression has been observed on both 
dermal fibroblasts obtained from patients with systemic sclerosis (11) and in human synovial 
fibroblast culture (16) while studies in human pulmonary artery smooth muscle cells show 
that extracellular S100A4 increases migratory capacity in a RAGE dependent manner (40).

The present data indicate that S100A4 may play a role during stricture formation in FCD as a 
mediator of the migratory effects of TGF-β1 on intestinal fibroblasts. The relative roles of the 
intracellular and extracellular forms of S100A4 in fibroblast activation represent a future 
direction in research with the potential to identify therapeutic entry points in the treatment or 
prevention of fibrostenosis in Crohn’s Disease.

**Conflicts of Interest**

The authors hold no conflicts of interest.

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Table and Figure Legends

Table 1: Patient characteristics
From a biobank of a total of 13 FCD patient samples, samples from 8 patients were used in the presented analyses. The table outlines the patient demographic, disease history, treatment regimens and tobacco usage for these patients. All mRNA, protein and explants culture analysis was performed with paired Normal Adjacent and Diseased tissues from the first 5 patients listed while T.E.M. studies were carried out using paired samples from the remaining 3 patients.

Figure 1: Intestinal wall thickening and collagen deposition in FCD.
Representative haematoxylin and eosin stained 4μm sections of (a) proximal, macroscopically normal adjacent terminal ileum and (b) a stricture site from a patient with FCD (diseased) (x10). Gomorri’s Trichrome stain of normal adjacent (c) and diseased (e) terminal ileal submucosa; and normal adjacent (d) and diseased (f) terminal ileal seromuscle (x150).

Figure 2: Apical junctional complex ultrastructure in FCD mucosal epithelium
Ultrathin sections of FCD mucosa (n=3 paired sets of Normal Adjacent and Diseased tissue) were examined by transmission electron microscopy (T.E.M., x75,000) and tight junction (TJ) and adherens junction (AJ) patency evaluated. Increases in AJ diameter were quantified and compared between normal and diseased tissue,*p=0.001 versus Normal Adjacent

Figure 3: S100A4 expression in FCD mucosa.
S100A4 expression was compared between the dissected mucosa of terminal ileal strictures (Diseased) and adjacent, case-matched, macroscopically normal resection margin mucosa (Normal Adjacent) from patients with FCD. (A) Real-time RT-PCR analysis of S100A4 mRNA expression. *p=0.011 versus normal adjacent mucosa n=5. (B) Western blotting and densitometric quantification of S100A4 protein expression. β-Actin was used as a loading control. #p=0.018 versus Normal Adjacent mucosa. n=5.

Figure 4: Immunohistochemical localisation of S100A4 in FCD mucosa
Localization of S100A4 in 4μm sections of Normal Adjacent and Diseased sections of terminal ileal tissue from FCD. S100A4 staining in Normal Adjacent (x200 (a) and x400 (b)) and Diseased terminal ileal mucosa (x80 (c) and x400 (d)). Double staining of Diseased tissue mucosal epithelium for S100A4 (brown) and CD3 (black), (x400 (e)). Pan-cytokeratin immunohistochemistry showing discrete mucosal epithelial staining in a blunted villous unit from Diseased tissue (x200 (f)). All data are representative of 3 paired sets per group.

**Figure 5:** S100A4 expression in seromuscular fibrofatty plaques in FCD.
Double-staining of 4μm sections of the terminal ileal muscularis and seromuscle for S100A4 (brown) and α-SMA (red) in Normal Adjacent margins and Diseased areas in FCD. Positive staining of mononuclear cells is found in Normal Adjacent specimens (a x8, b x40). Positive fibroblastic (arrow in d) and mononuclear cells are found in the FCD seromuscle (c x8, d x40). Representative of 3 paired sets per group.

**Figure 6** S100A4 expression in FCD fibroblast explant culture.
S100A4 expression was compared in primary explant cultures of intestinal fibroblasts established from the seromuscular layer of the Normal Adjacent margins and Diseased sites of terminal ileal resections for FCD (A) Real-time RT-PCR *p< 0.01, n=5 paired sets. (B) Western blotting of the same samples, where β-actin was used as a loading control. n=5 paired sets.

**Figure 7:** TGF-β1 increases S100A4 mRNA expression in primary cultures of ileal fibroblasts and CCD-18Co colonic fibroblasts (A) Fibroblast cultures established from Normal Adjacent tissue from FCD stricture resections was treated TGF-β1 (1ng/ml) for 24 hours and S100A4 mRNA expression assessed by real time RT-PCR, *p< 0.01, n=3. (B) CCD-18 Co fibroblasts were transfected with S100A4 or scrambled siRNA (5nM), then treated with TGF-β1 (1ng/ml) or vehicle (control) for 24 hours and S100A4 mRNA expression assessed by real-time RT-PCR *p=0.015, Control scrambled siRNA versus S100A4 siRNA; #p< 0.01, Control scrambled siRNA versus Control scrambled siRNA + TGF-β1 (1ng/ml); ψp< 0.01, Control scrambled siRNA + TGF-β1 (1ng/ml) versus S100A4 siRNA + TGF-β1 (1ng/ml).

**Figure 8:** S100A4 siRNA inhibits scratch wound healing in CCD-18Co fibroblasts. (A) Primary fibroblast explant cultures derived from the proximal macroscopically normal
adjacent seromuscle in ileal FCD resections were treated with TGF-β1 (1ng/ml) or vehicle (Control) in a scratch wound assay (*p< 0.01, versus Control). (B) CCD-18 Co fibroblasts were either left untreated (Control), or pre-treated with a scrambled si-RNA construct (Scrambled) or an S100A4 siRNA construct. Following transfection, a scratch wound assay was performed to compare migratory capacity over 24 hours with and without 1ng/ml TGF-β1 treatment, (#p and *p< 0.01, versus control), n=9.

**Figure 9**: S100A4 siRNA does not prevent TGF-β1 induced smad3 activation in CCD-18Co fibroblasts.

CCD-18Co fibroblasts were treated with either scrambled or S100A4 targeted siRNA constructs exposed to TGF-β1 (1ng/ml) for 30 minutes and then examined by Western blotting (A) for S100A4, p-Smad3, total smad3 and β-actin. (B) Densitometric analysis of the effect of 5nM si-S100A4 on S100A4 protein expression (*p< 0.01 vs. scrambled, n=3 per group) (C). Densitometric analysis of the effect of si-S100A4 on p-Smad3/total Smad3 ratios and total Smad3/β-actin ratios, &p< 0.05 scrambled control versus si-S100A4 control, *p< 0.01 TGF-β1 groups versus cognate controls, #p< 0.05 scrambled TGF-β1 versus si-S100A4 TGF-β1 treated groups (n=3 per group).
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FIGURE 2

(T.E.M. x76,000)

Normal Adjacent

Diseased

T.J.

A.J.

*
FIGURE 3

A

R Q S100A4 mRNA expression

Groups

Normal Adjacent Diseased

B

Densitometric Units

Groups

Normal Adjacent Diseased

S100A4 (11 kDa)

β-actin (42kDa)

Groups

Normal Adjacent Diseased
FIGURE 4
FIGURE 6

A

RQ S100A4 mRNA expression

Groups

Normal Adjacent  Disease

B

S100A4 (11 kDa)  β-actin (42kDa)

Groups

Normal Adjacent  Diseased
FIGURE 7

A

B

A

B

*  

#  

Ψ

Control  TGF-β1

Control  TGF-β1

scrambled siRNA
S100A4 siRNA
FIGURE 8

A

B

Groups

Groups

Area (sq. pixels)

Area (sq. pixels)