S100A4 expression is increased in stricture fibroblasts from patients with fibrostenosing Crohn’s disease and promotes intestinal fibroblast migration

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Cunningham MF, Docherty NG, Burke JP, O’Connell PR. S100A4 expression is increased in stricture fibroblasts from patients with fibrostenosing Crohn’s disease and promotes intestinal fibroblast migration. Am J Physiol Gastrointest Liver Physiol 299: G457–G466, 2010. First published May 20, 2010; doi:10.1152/ajpgi.00351.2009.—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 and as a marker of activated T lymphocytes and fibroblasts in chronic tissue remodeling. The aim of this study was to examine the expression profile of S100A4 in the resected ileum of patients with FCD. Mucosa, seromuscular explants, and transmural biopsies were harvested from diseased and proximal, macroscopically normal margins of ileocecal resections from patients with 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 transforming growth factor-β (TGF-β) isoforms 1 (28) and 2 (29) and connective tissue growth factor (1), collagen subtype III (41), tissue inhibitor of metalloproteinase-1 (29), vascular cell adhesion molecule (2), and intercellular adhesion molecule (4) has been described. These transcriptomic findings are complemented 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), TGF-β1-inducible, 11-kDa 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 hydrophilic interaction domain, which permits S100A4 to interact with a wide range of target molecules, thereby endowing cells with enhanced proinvasive properties (37). Secreted multimeric forms, which, via the activation of an as yet undefined receptor, lead to the activation of NF-κB and p53 stabilization and have also been implicated in cell migration, have also been reported (18, 40). S100A4 is of particular interest as a marker of malignant potential and chronic tissue remodeling (38). Tumor grade and metastatic potential in lower gastrointestinal tract malignancies correlate positively with S100A4 expression (8, 45), complementing similar findings in breast, pancreatic, and gastric carcinoma. With regard to chronic tissue remodeling, 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 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 epithelial-mesenchymal transition (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 attempt to define the contribution of this process to chronic tissue remodeling. There is significant disagreement in the literature as to whether...
S100A4 overexpression during chronic tissue remodeling reflects local EMT and fibroblast activation (17) or, simply, 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 remodeling responses in chronically inflamed tissue (18, 34).

The aim of the present study was to characterize and quantify the expression of S100A4 in the strictured ileum of patients undergoing ileocecal 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 small interfering RNA (siRNA)-mediated S100A4 depletion on the migratory potential of intestinal fibroblasts in vitro was subsequently examined.

MATERIALS AND METHODS

Patient cohort. Samples from eight patients with FCD were identified for study (Table 1). All gave written informed consent to participate in the study, which was approved by the local ethics committees of the Mater Misericordiae University Hospital and St. Vincent’s University Hospital. Biopsies were obtained from patients undergoing elective stricture resection. Biopsies were taken from the antimesenteric border of the terminal ileum in areas of disease and from proximal, macroscopically normal margins. The diagnosis was based on clinical, radiological, and endoscopic examination and histological findings and confirmed upon postoperative histology. Up-to-date clinical information on age at resection, sex, disease duration, current and prior use of corticosteroids, and anti-TNF-α and/or 5-aminosalicic acid therapies, as well as patient smoking status, was obtained from in-patient charts and confirmed by preoperative researcher interview. Sample assessment and processing were carried out within 4 h of collection.

Histological studies. Transmural biopsies taken at resection were fixed overnight in 10% nonbuffered formalin and embedded in paraffin, and 4-μm sections were cut and mounted. Rehydrolyzed sections were incubated with hematoxylin and eosin or, for assessment of fibrosis, incubated sequentially in Bouin’s fixative, Wiegert’s iron hematoxylin, Gomori’s trichrome stain, and 0.5% acetic acid (all from Polysciences Europe, Eppelheim, Germany). Sections were then dehydrated and mounted, and coverslips were applied.

Immunohistochemical studies. Prior to primary antibody incubation, 4-μm sections were deparaffinized and subjected to endogenous peroxidase quenching in 3% hydrogen peroxide in methanol and phosphatase-conjugated monoclonal mouse anti-human α-SMA; A-5691, Sigma-Aldrich). A 1-h, room temperature incubation of 1:200 dilutions of all primary antibodies in 0.2% bovine serum albumin in phosphate-buffered saline was used. Visualization of nonconjugated primary antibody binding proceeded via the use of biotinylated secondary antibody and horseradish peroxidase (HRP)/diaminobenzidine-based staining according to the Vectastain Universal ABC kit (Vector Laboratories) protocol. Sections were counterstained with Harris hematoxylin and dehydrated, and coverslips were applied. For double staining of S100A4 and α-SMA, after development of the S100A4 HRP signal, sections were washed in Tris-buffered saline and incubated with the anti-α-SMA–alkaline phosphatase conjugate for 1 h; then staining was developed using the Fast Red substrate system (Dako). For S100A4/CD3 double staining, S100A4 HRP-stained sections were incubated for 1 h with anti-CD3 antibody, and signal was detected using an anti-goat glucose oxidase staining system (Vector Laboratories). All double-stained sections were counterstained with Gill’s hematoxylin and mounted in glycerol, and coverslips were applied and sealed. Histological and immunohistochemical images were captured using the Aperio ScanScope XT digital slide scanner with image processing in Aperio Image Scope.

Transmission electron microscopy. Fresh paired biopsy samples from resections (n = 3) were immediately placed in fresh 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 h, and then dehydrated in an ascending ethanol series. Sections were embedded in Epon for overnight polymerization at 60°C. Semithin sections were stained with toluidine blue for selection of mucosal areas for ultramicrotomy. Ultrathin (80-nm) sections 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 (TEM; Tecnai Twin, FEI). Tight junction (TJ) and adherens junction (AJ) patency was evaluated in midvillous mucosal epithelial cells by measurement of the width (nm) of junctions at five cell-cell adhesion sites. Quantification was repeated over three discrete locations in the mucosa. All images were calibrated and recorded using the 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 FCD. Biopsies were taken prior to bowel devascularization and seeded onto a petri dish for sterile culture in 10% 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 passages 3 and 6.

Real-time RT-PCR. CD tissue was placed in RNAlater (Sigma-Aldrich Ireland) at the time of resection. Mucosal and seromuscular layers were identified, dissected, snap-frozen in liquid nitrogen, ho-
mogenized, placed in acid-guanidinium-phenol-chloroform (TRIZol Reagent, Invitrogen, Dublin, Ireland), and stored at −80°C. 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 diethylpyrocarbonate-water. RNA concentration and quality were assessed by spectrophotometry (ND-1000, NanoDrop Technologies) at an excitation wavelength ratio of 260 nm to 280 nm. Total RNA (1 μg) was treated with DNase I and then used to synthesize cDNA using the random primer method.

Gene target expression was analyzed using real-time PCR TaqMan assay on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The primers and probes for these targets were supplied as preoptimized gene expression assays (Applied Biosystems). The probes for the target genes were labeled 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, with the probe labeled with VIC at the 5′ end and TAMRA on the 3′ end to facilitate dual reporter assay. Default thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C for enzyme activation, and then 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. Results were analyzed using the relative ΔCT method of analysis, with candidate gene expression normalized to the housekeeping gene 18S rRNA.

Protein extraction and Western blot analysis. Total protein was isolated from snap-frozen CD mucosa by homogenization 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 blot studies, protein (30 μg) was resolved on a 12% bisacrylamide-acrylamide-SDS gel (75 min at 140 V) prior to transfer to 0.2-μm-pore polyvinylidene difluoride membranes (Immobilon, Millipore) at 100 V for 80 min 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 1:1,000 dilutions of the rabbit anti-human antibodies Ab52903 and Ab28379, respectively (both from Abcam) Anti-rabbit secondary antibody was incubated at a concentration of 1:5,000. Incubation of membranes with 1:5,000 dilutions of mouse monoclonal anti-human β-actin (F3022, Sigma-Aldrich) was used to develop loading control signals. Protein density on scanned Western blots was determined using ImageJ version 1.26t (National Institutes of Health).

siRNA transfection. siRNA knockdown of S100A4 expression was studied in the normal human colonic fibroblast line CCD-18Co (CRL 1459, American Type Culture Collection). S100A4 siRNA was purchased from Qiagen (Valencia, CA), and scrambled siRNA (Ambion, Cambridge, UK) was used as a control for off-target effects. The sequences of selected regions to be targeted by the siRNAs were as follows: 5′-AACGAGGTGGACTTCCAAGAG-3′ for S100A4 and 5′-AATTCTCCGAACGTGTCTCGT-3′ for the scrambled nonsilencing siRNA. Initial dose-response experiments (data not shown) determined the optimum dose of S100A4 siRNA to be 5 nM. Transfection of siRNA was achieved using Lipofectamine 2000 (Invitrogen) in 1× Opti-MEM I (GIBCO) reduced serum medium (Invitrogen). After 6 h, the medium was changed to antibiotic-free 1% FBS-RPMI 1640.

![Intestinal wall thickening and collagen deposition in fibrostenosing Crohn’s disease (FCD).](http://ajpgi.physiology.org/)

**Fig. 1.** Intestinal wall thickening and collagen deposition in fibrostenosing Crohn’s disease (FCD). A and B: representative hematoxylin-and-eosin-stained 4-μm sections of proximal, macroscopically normal adjacent terminal ileum and a stricture site from a patient with FCD (diseased). Arrow shows vascular congestion. Magnification ×10. C–F: Gomorri’s trichrome-stained sections of normal adjacent (C) and diseased (E) terminal ileal submucosa and normal adjacent (D) and diseased (F) terminal ileal seromuscle. Magnification ×150.
Cells were further treated or harvested for analysis 24 h after initial transfection.

**Cell migration.** Wells of a six-well plate with a horizontal 0.6-mm preetched undersurface were seeded with 5 × 10⁴ fibroblasts. A perpendicular horizontal “scratch wound” was made in the cell layer at 80% confluence, and high-resolution images of wound closure were obtained using a microscope-mounted camera (A70 Powershot, Canon). Set distances and magnification were used to obtain images at the point of intersection of scratch wound and etch. Initial images at experimentation \( (\text{time } 0) \) were assessed to determine equal area for migration. Intersection images were obtained at 12-h time periods. ImageJ version 1.26t was used to assess the area uncovered by cells, and the migratory capacity of the cells was calculated by comparing the rate at which cells migrated toward each other in multiple sets. The rate of closure is expressed as percent recovered per unit time.

**Statistics.** Data were assessed for normality using the Kolmogorov-Smirnov test and analyzed parametrically using unpaired Student’s \( t \)-test and one-way ANOVA with Bonferroni’s 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 eight patients (6 men and 2 women) with FCD (Table 1). The mean age of these patients was 33.2 ± 5.6 yr, and median disease duration was 8.5 yr (interquartile range = 5). A single patient had never been treated with corticosteroids; among the remaining corticosteroid-treated patients, two were currently in treatment, four were within 1 yr of surgery, and one had been treated 1–5 yr previously. Three patients had been treated with infliximab, and five had received 5-aminosalicylic acid. Only one patient...
had undergone previous resection for CD. Only two of the patients identified themselves as current smokers; the remainder stated that they had never 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 compared with its macroscopically normal adjacent terminal ileal counterpart (Fig. 1A). High-power examination of Gomorri’s trichrome-stained sections demonstrates the formation of mature, fibrillar collagen scar tissue within the submucosa (Fig. 1E) and seromuscle (Fig. 1F) compared with their normal adjacent counterparts (Fig. 1, C and D).

Junctional adhesion complex disruption in FCD mucosal epithelium. TEM analysis of TJ structure demonstrated that, in all samples examined, diseased tissue was characterized by dilatation and unzipping of the TJ relative to a patent, closed “tight” morphology observed in normal adjacent specimens. Morphometric comparison of AJ diameter demonstrated a 41% increase in diameter in diseased vs. normal adjacent tissue (22.5 ± 0.8 vs. 31.8 ± 2.8 nm, P = 0.001; Fig. 2).

S100A4 expression is upregulated on mRNA and protein analysis of 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 localization 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). Staining patterns similar to those obtained with S100A4 were obtained with CD3 (Fig. 4E), suggesting that the cells are T lymphocytes. Pancytokeratin 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 (×8) fields (Fig. 5, A 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 (×40) 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 with those from macroscopically normal adjacent sections. Confluent paired sets of fibroblasts show a 4.9-fold increase in S100A4 mRNA levels (1.9 vs. 4.9, *P* < 0.01) from disease-derived explants upon real-time RT-PCR analysis (Fig. 6A). Western blot 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.** Treatment of primary culture of fibroblasts from proximal normal resection margins with the profibrotic cytokine TGF-β1 (1 ng/ml) for 24 h led to a 2.9-fold increase in S100A4 mRNA expression (1.2 vs. 2.9, *P* < 0.01; Fig. 7A). Treatment of CCD-18Co fibroblasts for 24 h with TGF-β1 increased S100A4 expression threefold (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).

**S100A4 siRNA treatment and TGF-β1 induced Smad3 activation in CCD-18Co fibroblasts.** CCD-18Co fibroblasts were treated with scrambled or S100A4 siRNA constructs for 24 h and then exposed to TGF-β1 (1 ng/ml) for 30 min. An average 35% reduction in cellular S100A4 protein was observed in the S100A4 siRNA groups compared with cells treated with a scrambled construct (0.85 ± 0.11 vs. 0.57 ± 0.11, *P* < 0.01; Fig. 9). Western blotting of p-Smad3 demonstrated that basal p-Smad3-to-total Smad3 ratios were significantly reduced (26%) in the presence of S100A4 siRNA (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...
S100A4 expression in strictured fibroblasts 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 intraepithelial 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 colocalization 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 intraepithelial lymphocytes attests to the expansion of the T cell receptor γδ-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., matrix metalloproteinases (9, 46)] effects of pro-EMT cytokines such TGF-β1. Overexpression of S100A4 in squamous cell carcinoma lines is sufficient to cause a decrease in E-cadherin and β-catenin expression (31).

**DISCUSSION**

This study examined the expression profile of S100A4 in the strictured ileum of patients with FCD 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

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Fig. 7. Transforming growth factor (TGF)-β1 increases S100A4 mRNA expression in primary cultures of ileal fibroblasts and CCD-18Co colonic fibroblasts. A: S100A4 mRNA expression assessed by real-time RT-PCR in fibroblast cultures established from normal adjacent tissue from FCD stricture resections treated with TGF-β1 (1 ng/ml) for 24 h. *P < 0.01 (n = 3). B: S100A4 mRNA expression assessed by real-time RT-PCR in CCD-18Co fibroblasts transfected with S100A4 or scrambled siRNA (5 nM) and then treated with TGF-β1 (1 ng/ml) or vehicle (control) for 24 h. *P = 0.015, control scrambled siRNA vs. S100A4 siRNA. #P < 0.01; control scrambled siRNA vs. control scrambled siRNA + TGF-β1 (1 ng/ml). ϑP < 0.01, control scrambled siRNA + TGF-β1 (1 ng/ml) vs. S100A4 siRNA + TGF-β1 (1 ng/ml).

S100A4 siRNA (0.85 ± 0.07 vs. 1.53 ± 0.01, P < 0.01) and a 2-fold increase in cells treated with S100A4 siRNA (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 S100A4 siRNA (1.53 ± 0.01 vs. 1.31 ± 0.07, P = 0.02).

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Fig. 8. S100A4 siRNA inhibits scratch wound healing in CCD-18Co fibroblasts. A: results of scratch wound assay of primary fibroblast explant cultures derived from the proximal macroscopically normal adjacent seromuscle in ileal FCD resections treated with TGF-β1 (1 ng/ml) or vehicle (control). *P < 0.01 vs. control. B: CCD-18Co fibroblasts were left untreated (control) or pretreated with a scrambled siRNA construct (scrambled) or an S100A4 siRNA construct. After transfection, a scratch wound assay was performed to compare migratory capacity over 24 h with and without 1 ng/ml TGF-β1. #P < 0.01; *P < 0.01 vs. control (n = 9).
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 fibrosed tubulointerstitium in chronic renal allograft nephropathy has been reported as evidence of local EMT (48). In the present study, no cytokeratin positivity was observed in the lamina propria at stricture sites, despite obvious villous blunting and morphometric evidence of deterioration of epithelial junctional complexes. It was also noted by TEM (data not shown) that, in the mucosal epithelium at stricture sites, interepithelial desmosomal plaques remained wholly intact. As the desmosomal-cytoskeletal linkage occurs via cytokeratin intermediate filaments, the likelihood of observing cytokeratin-positive cells in the context of intact desmosomal structure would seem to be low.

Fibroblastic S100A4 expression was observed within scar tissue 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 us 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 subtype different 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 in the tumor-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 behavior associated with S100A4 is migration. The major identified intracellular pathway mediating this effect centers on alterations in cytoskeletal dynamics.
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caused by the binding of S100A4 to the heavy chain of nonmuscle 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 product (RAGE)-mediated role for extracellular S100A4 in migration (reviewed in Ref. 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 on S100A4 expression. This finding implies a role for increased expression of S100A4 in the migratory potential of fibroblasts in FCD. Leeb et al. (22) described a reduction in the chemotactic migratory potential of lamina propria fibroblasts isolated from endoscopic biopsies of inflamed tissue from patients with CD and reported that reduced migratory potential coincided with hypophosphorylation of focal adhesion kinase. Conversely, short-term TGF-β1 incubation of lamina propria fibroblasts from noninflamed areas of the bowel increases focal adhesion kinase 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 myofibroblast 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 Smad3 phosphorylation in fibroblasts was observed in the presence of siRNA-mediated inhibition of S100A4 under 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 affect 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 is not coherent with central Smad signaling representing the major mechanism behind the promigratory effect of S100A4 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 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 CD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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