Characterization of myofibroblasts isolated from the intestine of patients with inflammatory bowel disease [version 1; peer review: 1 approved, 1 approved with reservations]

Serge Dionne¹, Sophie Restellini¹, Jamie Koenekoop¹, Pedro Salvador Escribano¹², Ciriaco A. Piccirillo³, Patrick Charlebois⁴, A. Sender Liberman⁴, Barry Stein⁴, Carl Frederic Duchatellier¹, Ernest Gerald Seidman¹

¹Division of Gastroenterology, Research Institute, McGill University Health Center, Faculty of Medicine, McGill University, Montréal, QC, H3G 1A4, Canada
²Department of Pharmacology, Faculty of Medicine, University of Valencia, Valencia, 46010, Spain
³Department of Microbiology and Immunology, Research Institute, McGill University Health Center, Faculty of Medicine, McGill University, Montréal, QC, H4A 3J1, Canada
⁴Colorectal Surgery, Research Institute, McGill University Health Center, Faculty of Medicine, McGill University, Montréal, QC, H4A 3J1, Canada

Abstract

Background: Intestinal fibrosis represents a serious complication of inflammatory bowel diseases (IBD), often necessitating surgical resections. Myofibroblasts are primarily responsible for interstitial matrix accumulation in fibrotic diseases. However, intestinal myofibroblasts (IMF) remain inadequately characterized. The aim was to examine fibroblast markers and fibrosis-associated gene expression in IMF isolated from resected intestine from IBD and control patients. As well as determining the effect of the fibrogenic cytokine TGFβ.

Methods: Intestinal resections were obtained (n=35) from consenting patients undergoing elective surgery (2014-16). Primary cultures of IMF were isolated using DTT and EDTA and cultured. Viability and phenotypic characterization of IMF was carried out by flow cytometry and fluorescence microscopy. IMF (passages 3-8) were treated for 24 hours. Cytokines were quantified in IMF by real time PCR and in supernatants using the human pro-inflammatory cytokine panel.

Results: All markers and most fibrosis mediators studied were preferentially expressed by IMF compared to mucosal tissue. Metalloproteinases (MMP) 2 and 3, as well as their inhibitor TIMP1, are highly expressed by IMF. They also highly expressed inflammatory mediators, including IL-6, IL-8, CCL2 and PTGS2. Whereas mucosal expression of pro-inflammatory cytokines such as TNFα and IL-17 is
increased in IBD, that of fibrosis mediators was not different. Fibrosis-related gene expression in IMF from IBD patients and controls was similar, but IMF from IBD expressed higher levels of several inflammatory genes. IMF from CD and UC had mostly similar expression profiles. TGFβ induced expression of fibrogenic genes αSMA, COL1A1, CTGF, FN1 and LOX. TGFβ-stimulated IMF released increased levels of IL-6, whereas IL-6, IL-8, as well as small amounts of IFN-γ and IL12p70 were produced following stimulation with IL-1β+IL-23.

**Conclusions:** This study extends knowledge about the pathogenesis of fibrosis in IBD. Further research in the identification of mechanisms involved in IMF activation and fibrogenesis are required.

**Keywords**
intestinal myofibroblasts, inflammatory bowel disease, fibrosis, crohn's disease, ulcerative colitis, inflammation, cytokines gene expression
**Introduction**

The two major forms of inflammatory bowel disease (IBD), Crohn’s disease (CD) and ulcerative colitis (UC), are characterized by chronic and relapsing intestinal inflammation that develop as the result of an abnormal regulation of immune responses, likely directed at least in part, against the host commensal gut microbiota\(^1\)–\(^3\). The prevalence of IBD is estimated to be over 3.6 million persons in North America and Europe, and the incidence is increasing in Asia and Africa\(^5\)–\(^8\). Genetic studies have identified over 200 susceptibility loci for IBD, mostly shared between CD and UC\(^9\)–\(^12\). These loci are enriched for pathways that interact with environmental factors to modulate intestinal homeostasis, including IL23R, ATG16L1, IRGM and NOD2\(^13\).

The natural history and clinical course of IBD is extremely heterogeneous. Up to 16% of UC patients require a colectomy within 10 years, whereas about 50% of patients with CD develop complications such as strictures, fistulas, and abscesses that frequently require surgery within the same period\(^14\)–\(^16\). Intestinal fibrosis is a common and potentially serious complication of CD, but not UC, that results from the reaction of intestinal tissue to the damage inflicted by chronic inflammation\(^12\). NOD2 has been long considered the most important genetic predictor of the evolution toward a fibrostenosing phenotype\(^14\)–\(^16\). Fibrosis in CD is also associated with polymorphisms of the JAK2, ATG16L1, CX3CR1 and MMP3 genes\(^17\).

An improved understanding of the cellular and molecular mechanisms that underlie the pathogenesis of intestinal fibrosis is needed. The mechanisms are complex, dynamic, and likely involve multiple cell types and soluble factors\(^18\). Intestinal myofibroblasts (IMF) play important roles in inflammation and tissue remodeling\(^19\). The development of fibrosis results from an imbalance in extracellular matrix (ECM) deposition and degradation. Alpha-smooth muscle actin (\(\alpha\)-SMA) positive myofibroblasts were identified as the primary cell type responsible for interstitial matrix accumulation in fibrotic diseases\(^20\)–\(^22\). A hallmark of mesenchymal cell activation is the acquisition of a myofibroblast phenotype, whereby fibroblasts transform into myofibroblasts acquiring smooth muscle features, most notably the expression of \(\alpha\)-SMA and synthesis of mesenchymal cell related matrix proteins. TGF-\(\beta\), a prototype of the TGF-\(\beta\) superfamily, is widely considered to be the major profibrogenic cytokine that is responsible for the myofibroblast differentiation and subsequent matrix synthesis\(^23\)–\(^25\). Although the TGF-\(\beta\)/Smad pathway is considered a driving force of fibrosis, myofibroblasts are activated by numerous paracrine mediators in their environment that promote their production of ECM and proliferation including, TGF-\(\beta\), PDGF, CTGF, IGFI/II, bFGF and various interleukins: IL-1\(\beta\), IL-6 and IL-13\(^26\)–\(^28\).

Although inflammation is necessary for fibrosis, recent evidence indicates that once initiated, fibrosis in CD can progress independently of inflammation\(^1\). Consequently, current anti-inflammatory treatments may not prevent fibrosis once excessive ECM deposition has commenced\(^29\)–\(^31\). Matrix stiffness is capable of further activation of intestinal fibroblasts and can contribute to progression of fibrosis independently of inflammation\(^32\)–\(^34\).

There is currently little information about the identity, abundance and characteristics of intestinal mesenchymal cells such as fibroblasts and IMF under normal and pathological conditions. In this study, we examined the expression of fibroblast and IMF molecular markers in the intestine from patients with CD, UC and from non-IBD control patients.

**Methods**

**Human intestinal tissue acquisition and consent**

The McGill University Health Center’s research ethics board approved the study design and consent forms. Intestinal resections were obtained (2014-2016) from patients undergoing elective intestinal surgery who voluntarily gave written informed consent to participate. Written informed consent for publication of the participants/patients’ details and/or their images was obtained from the participants/patients/parents/guardian/relative of the participant/patient. Resected tissue was obtained from 15 CD and 6 UC patients, as well as from uninvolved surgical specimens (>5 cm from the tumor margin) of 14 control patients undergoing colectomy for carcinoma or polyps. The characteristics of the patient groups are shown in Table 1.

**Isolation and culture of human intestinal subepithelial fibroblasts and myofibroblasts**

Primary cultures of IMF were isolated and cultured according to the method reported by Mahida et al.\(^35\). Briefly, tissue specimens were trimmed of fat and thoroughly washed. The mucosa was cut into 0.5 cm pieces and incubated in 1.5 mM DTT (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) in HBSS for 15 min at 37°C to remove mucus. Tissue was washed and then incubated in HBSS containing 2 mM EDTA for 2 × 30 min in a shaker at 37°C.

The resulting mucosal samples denuded of epithelial cells were cultured at 37°C, in RPMI-1640 supplemented with 10% fetal calf serum (FCS) to allow myofibroblasts to migrate out, in order to establish primary cultures. Established colonies of IMF were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 1% non-essential amino acids (Gibco, Carlsbad, CA, USA), and 200 mM glutamine (Sigma). Experiments were performed on passages 3–8.

**Viability and Phenotypic Characterization of Primary Myofibroblast Cultures**

Cells obtained as above were prepared for flow cytometry as follows. After trypsinization, cells were counted and distributed in 1×10⁶ per sample tubes. Cells were washed twice in PBS and blocked with Human BD Fc Block (BD Biosciences, Mississauga, ON) according to the manufacturer’s instructions. Samples were then incubated with eBioscience™ Fixable Viability Dye eFluor 450 (Life Technology Inc., Burlington, ON) for 30 min in ice, washed again in PBS and incubated in PBS containing the surface marker antibodies for 20 min in ice (Table 2). Samples were then permeabilized with BD Cytofix/Cytoperm Buffer (BD Biosciences) for 20 min on ice and then washed with BD Perm/Wash (BD Biosciences) and incubated with BD Perm/Wash Buffer containing intracellular marker antibodies for 20 min on ice (Table 2). Samples were washed with BD Perm/Wash and
Table 1. Description of the IBD patients and controls studied.

|                           | CD     | UC     | Controls |
|---------------------------|--------|--------|----------|
| **Number**                | 15     | 6      | 14       |
| **Females, n (%)**        | 8 (53.3) | 3 (50) | 6 (42.9) |
| **Age at resection, median (range)** | 36.9 (18-68) | 41.9 (19-56) | 60.4 (44-78) |
| **Age at diagnosis**      |        |        |          |
| • A1 (<16)                | 6      | 2      |          |
| • A2 (17-40)              | 7      | 4      |          |
| • A3 (>40)                | 2      | 0      |          |
| **Treatment**             |        |        |          |
| • None, n (%)             | 6 (40) | 3 (50) | 14 (100) |
| • 5-ASA alone             | 0 (0)  | 0 (0)  |          |
| • Thiopurine or methotrexate | 4 (26.7) | 0 (0)  |          |
| • TNF inhibitor           | 5 (33) | 3 (50) |          |
| • Oral corticosteroids    | 3 (20) | 1 (16.7)|          |
| **Disease location: CD**  |        |        |          |
| • L1-terminal ileum      | 7      |        |          |
| • L2-colon               | 4      |        |          |
| • L3-ileocolonic         | 4      |        |          |
| • L4-upper GI tract      | 0      |        |          |
| **Disease location: UC**  |        |        |          |
| • E1-proctitis           |        | 0      |          |
| • E2-left side colitis   |        | 0      |          |
| • E3-pancolitis          |        | 6      |          |
| • E4-proximal colitis    |        | 0      |          |
| **Disease behavior: CD**  |        |        |          |
| • B1- Inflammatory       | 4      |        |          |
| • B2-Stricturing         | 7      |        |          |
| • B3-Penetrating         | 4      |        |          |
| **Perianal disease, n (%)** | 2 (13.3) |        |          |

CD: Crohn’s disease, IBD: inflammatory bowel disease, UC: ulcerative colitis

Table 2. Specific antibodies used for immunofluorescence and flow cytometry.

| Marker                          | Intracellular/Extracellular | Sample ul (1:100) | Manufacturer/Catalogue # |
|---------------------------------|-----------------------------|-------------------|--------------------------|
| Alpha Smooth Muscle Actin       | Intracellular               | 5                 | Abcam/ab197240           |
| FSP-1 (S100A4)                  | Intracellular               | 5                 | Biolegend/370005         |
| CD90                            | Extracellular               | 2.5               | BD Biosciences/563804    |
| EpCAM                           | Extracellular               | 5                 | BD Biosciences/347199    |
| CD45                            | Extracellular               | 2.5               | BD Biosciences/560566    |
| FAP PE                          | Extracellular/Intracellular | 10                | R & D Systems/ FAB3715P-025 |
| CD271 PE-Cy7 (LNGFR)            | Extracellular/Intracellular | 5                 | BD Biosciences/ 562122   |

In some experiments, cells were incubated with SMAD (5uM, x 2hr) and/or TGFα (3 ng/ml, overnight).

**Multiplex cytokine array**

IMF were plated on 6 well-plates and treated for 24 hours. The medium was collected and stored at -80°. Supernatants of the cultures were tested for IFNγ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-13, using the human pro-inflammatory cytokine panel (Meso Scale Diagnostics, Rockville, MD).
Real time polymerase chain reaction (qPCR)
mRNA from IMF was isolated with RNeasy Mini Plus kit (Qiagen,
Toronto, ON, Canada) according to the manufacturer's instruc-
tions. cDNA was generated from 1 µg of total RNA using Tran-
scriptor First Strand cDNA Synthesis kit (Sigma-Aldrich Canada
Ltd). Real-time RT-PCR was performed by a “StepOnePlus”
RT-PCR (Life Technologies, Burlington, ON) using PerfeCTa
SYBR green Fast Mix (Quanta Biosciences, Beverly, MA). Primers
were as described in Table 3.

Primers were ordered from Integrated DNA Technologies
(Coralville, IA). Gene expression was standardized using
GAPDH expression. Results were quantified using the ΔCt or
2ΔCt method.

Table 3. Sequence of primers used.

| GENE NAME   | PRIMER SEQUENCES       |
|-------------|------------------------|
| FAP         | forward: 5'-TAT TCA GAG TAA CAC ACG ATT CA-3' 
             | reverse: 5'-ACT TCT TGC TTG GAG GAT AG-3'   |
| FSP-1/S100A4| forward: 5'-CTT CTT CTT TCT TGG TTT GGT-3' 
             | reverse: 5'-AGC AGT CAG GAT GAA CAC-3'      |
| CD90        | forward: 5'-AGA GAC TTG GTG GAG GAG-3'    
             | reverse: 5'-CTG AGA ATG CTG AGG ATG-3'      |
| ACTA2/αSMA  | forward: 5'-CTG TTC CAG CCA TCC TTC AT-3'  
             | reverse: 5'-CCG TGA TCT TCT GCA TT-3'       |
| COL1A1      | forward: 5'-GAG AGC ATG ACC GAT GGA TT-3'  
             | reverse: 5'-CCT TCT TGA GGT TGC CAG TC-3'   |
| CTGF        | forward: 5'-GCT CTT CAG CCA TCT TCC TCT TT-3' 
             | reverse: 5'-GAT GCA CTT TTT GCC CCT TCT-3'   |
| CCN1        | forward: 5'-AAT GGA CCT CGC ATC CTA TA-3'  
             | reverse: 5'-TTT TTC CAG AAC GCC GCA-3'       |
| TIMP1       | forward: 5'-TTG ACT TCT GGT GTC CCC AC-3'  
             | reverse: 5'-CTG TTG TTT CTG TGG CTG AT-3'   |
| MMP9        | forward: 5'-TTG GTC CAC CTG GTTCAA AT-3'   
             | reverse: 5'-ACG ACG TCT TCC AGT ACC GA-3'   |
| MMP2        | forward: 5'-GCC CCA AGG AGG TGA TCT TG-3'  
             | reverse: 5'-GCT TGC GAG GGA AGA AGT TGT-3'  |
| MMP3        | forward: 5'-CAA TTT CAT GAG CAG CAA CG-3'  
             | reverse: 5'-AGG GAT TAA TGG AGA TG C-3'     |
| IL-6        | forward: 5'-CAA AGA TGG CTG AAA AAG ATG GA-3' 
             | reverse: 5'-CTG TTC TGG AGG TAC TCT AGG T-3'|
| IL-8        | forward: 5'-CTC TCC TGA TTT CTG CAG CTC-3' 
             | reverse: 5'-GTC CAC TCT CAA TCA TCC TCA C-3'|
| IL-17       | forward: 5'-ACT ACA ACC GAT CCA CCT CAC-3' 
             | reverse: 5'-ACT TTG CCT CCC AGA TCA CAG-3'  |
| TNFA        | forward: 5'-AGG CCG TGC TCG TCT CTC AG-3'  
             | reverse: 5'-GGC TAC AGG TCT GTC ACT CG-3'   |
| IL23A       | forward: 5'-GGG ACA CAT GGA TCT AAG AG-3'  
             | reverse: 5'-GCA AGG AGA ACT GAC TGT TG-3'    |
| PTGS2       | forward: 5'-ATA TGT TCT CCT GCG TAC TGG AA-3' 
             | reverse: 5'-GCC CTT CAC GTT ATT GCA GAT G-3'|
| FN1         | forward: 5'-CCC CTT GGG TCA CCT ATT AC-3'  
             | reverse: 5'-CGG TCA GTC GGT ATC CTG TT-3'   |
| NOX4        | forward: 5'-GGT AGG AGA CCA AGA GTG TCC GG-3' 
             | reverse: 5'-GCT GTA TAA CCA AGG GCC AG-3'   |
| LOX         | forward: 5'-TGG CAG TCT ATG TCT GCA CC-3'  
             | reverse: 5'-CTA TGG CTA CCA CAG GCG AT-3'   |
Statistical analysis
Data were analyzed using GraphPad and presented as median. Statistical analysis of the results between groups was determined by the Mann-Whitney test. Significance was set as p<0.05.

Results and discussion
Characterization of primary IMF cultures
The viability of IMF cells as determined by flow cytometry was 97.8% (n= 3/group; passages 3-8). No difference was observed between UC, CD and control groups. Immunofluorescence microscopy revealed that the IMF were αSMA+, CD90+, FSP1 +, and negative for hematopoietic and epithelial markers CD45 and EpCAM. Representative images are illustrated in Figure 1.

Expression of fibroblast markers by primary IMF cultures
We examined the gene expression of fibroblast markers in IMF cultures and compared their levels with those in the mucosa. The expression of fibroblast activation protein (FAP) was more than 200 times higher in IMF cultures than in mucosal tissue. Levels of Fibroblast specific protein 1 (FSP1, also known as S100A4) and those of the stromal marker CD90/Thy-1 were 5–8 times greater in IMF than in the mucosa. The myofibroblast marker αSMA in primary IMF cultures was 2-fold the mucosal level (Figure 2A). We then examined the expression of different mediators implicated in the fibrotic process. As illustrated in Figure 2A, IMF expressed high levels of type 1 collagen (COL1A1) and fibronectin 1 (FN1). Connective tissue growth factor (CTGF/CCN2), a matricellular protein recognized to promote matrix protein deposition and fibrogenesis, was similarly expressed by IMF and mucosa. CCN1, another matricellular protein, was preferentially expressed by IMF.

Fibrosis is a pathological condition characterized by the deposition of excessive or abnormal ECM components, including collagen type I. Metalloproteinases (MMP) are responsible for the degradation of ECM components and are thus play a central role in ECM remodeling. Their activity is controlled by tissue inhibitors of metalloproteinase (TIMP). MMP-2 and MMP-3 are highly expressed in IMF (over 100 times the mucosal levels). However, MMP-9 transcript levels were higher in the mucosa (p=0.05). IMF were found to be an important source of TIMP1, with expression largely exceeded that of the mucosa (Figure 2B). Accumulating evidence points toward the NAD(P)H oxidase of the Nox family and particularly Nox4 as the predominant enzyme source for ROS generation in fibrotic disease. As shown in Figure 2B, IMF expressed significant levels of NOX4 transcripts.

The inflammatory potential of IMF was then investigated. IMF expressed high levels of pro-inflammatory cytokines IL-6 and IL-8, more than 40 and 30 times the mucosal levels, respectively (Figure 3). The chemokine CCL2 was also more expressed by IMF cultures than mucosal tissue. Transcripts of cytokines such as TNFα, IFNγ, IL-17A and IL-23A were undetectable in most IMF samples analyzed. PTGS2, also known as cyclooxygenase-2 or COX-2, is highly expressed by IMF, approximately 200 times the mucosal levels.

Mucosal gene expression of fibrosis mediators
We then examined mRNA level of fibrosis related genes in the mucosa of IBD patients and of controls. No significant differences were found in fibroblast markers and fibrosis gene expression in mucosa from IBD patients compared to controls. MMP3, MMP9 and TIMP1 expression tended to be higher in IBD tissue, but the differences were not statistically different due to the large variation in IBD mucosa (data not shown). There was no clear expression profile related to the pathology except for TIMP1 which was more expressed in UC than in CD (Figure 4). Pro-inflammatory cytokine expression was greater in mucosa isolated from IBD patients. Expression of IL-17 and TNFα, but not IL-23A, were significantly greater in the intestine from IBD patients (Figure 4). Values for IL-6 and IL-8 were too dispersed to achieve significance.

**Figure 1. Immunostaining of IMF primary cultures.** A) Representative photomicrograph of colonic myofibroblasts isolated from a resection of a control patient. Cells have a fibroblast shape and express expressed a marker of myofibroblast, a-SMA (in green). B) IMF cultured from a colonic resection in a patient with ulcerative colitis. Cells also express CD 90 (in red).
Figure 2. Comparative expression of fibrosis-related mediators and fibroblast markers by intestinal myofibroblasts (IMF) compared to intestinal mucosa levels in human bowel resections. Gene expression was determined by real-time qPCR, and data normalized compared to GAPDH expression. Data are presented as medians. * $p<0.05$, ** $p<0.005$, *** $p<0.000$
Figure 3. Inflammation-related gene expression in intestinal myofibroblasts (IMF) and intestinal mucosa resections. Gene expression was determined by real-time qPCR, and data normalized compared to GAPDH expression. Data are presented as medians. * p< 0.05, ** p< 0.005, *** p < 0.001
Figure 4. Mucosal gene expression from control and inflammatory bowel disease (IBD) patients. Gene expression was determined by real-time qPCR, and data normalized compared to GAPDH expression. Data are presented as medians. * p< 0.05, ** p< 0.005
Fibrosis-related gene expression in IMF from control and IBD patients.
Expression of fibroblast markers and fibrosis-related genes was not different between IMF generated from IBD and control patients (Figure 5). Increased CTGF expression by IBD IMF was marginally (significant p=0.053). FSP-1 expression was not different between IBD and control groups, but IMF from UC patients expressed lower transcript levels compared to those from controls (p=0.0357).

Inflammatory gene expression in IMF from control and IBD patients
IMF from IBD resections expressed higher levels of IL-6 than those from controls (p=0.0357, Figure 6). IL-8 was nearly 100 times more expressed by IMF from IBD but due to the small number of samples the difference did not reach statistical significance. CCL2 expression was also 30 times more in IMFs from IBD patients. There was a trend towards higher PTGS2 expression in IMF from CD patients compared to those from controls and UC patients.

Effect of TGF-β on fibrosis-related gene expression in IMF from control & IBD patients
TGFβ stimulation of IMF resulted in an increased expression of profibrotic genes COL1A1 and CTGF. CCN1 as well as FN1 expression were also upregulated following stimulation with TGFβ (Figure 7). Lysyl oxidase (LOX), a collagen modifying enzyme required for the cross-linking of collagen, was also induced. There was marked upregulation of αSMA of IMF. On the other hand, TGFβ down-regulated expression of fibroblast markers FSP1 and CD90. TGFβ slightly induced MMP9 and TIMP1 expression, although not significantly. No significant effect of TGFβ was observed on NOX4, IL-6, IL-8 and PTGS2 expression, although IL-6 expression was increased in 4 of 6 IMF cultures.

Modulation of inflammation-related gene expression in IMF by fibrogenic and inflammatory signals
To assess the inflammatory properties of IMF, we determined their cytokine release following fibrogenic and inflammatory stimulation. IMF spontaneously released IL-6 and IL-8 (Figure 8a). TGFβ induced IL-6 release (Figure 8b). Stimulation with IL-1β+IL-23 increased IL-6 and IL-8 production. In addition, small amounts of IFNγ and IL-12p70 were released (Figure 8c).

Fibrosis is a chronic, progressive process characterised by an excessive deposition of collagen and other ECM components. Intestinal fibrosis is a common complication of CD, an intractable disorder, forcing patients to undergo bowel resections over their lifetime. More than 40% of CD patients with ileal involvement will require one or more resections of strictures 28. Although less common in UC, longstanding disease is believed to cause fibrosis resulting in altered bowel function 29. In this study, isolation techniques used yielded multiple cell populations present in the intestinal lamina propria including: immune cells, epithelial cells, and MF. This permitted the establishment of primary IMF cultures with a high rate of viability. After the first passage, only MF remained, as identified by their unique phenotype (Figure 1).

A variety of signals promote fibroblast differentiation into IMF and augment ECM expression. TGFβ represents the prototype of the profibrogenic mediators, with its unique ability to drive myofibroblast activation through both canonical and non-canonical signaling pathways leading to expression and deposition of ECM 30.

There is little information about the identity, localization, and abundance of the different intestinal mesenchymal cell types. Various studies show that fibroblasts isolated from different tissues are morphologically and functionally heterogeneous subpopulations 31. Several fibroblast markers have been described, but none of them is unique to fibroblasts, and not all fibroblasts express the proposed markers. This lack of specific markers has impeded the characterization of IMF and their putative precursors. Moreover, characterization of these markers under normal and pathological conditions is still lacking. This study aimed to determine the expression of several markers as well as fibrosis related mediators in IMF derived from the CD, UC and control patients.

Among the markers available to identify fibroblasts, Thy-1 (CD90), FAP and FSP1 (S100A4) are the most extensively studied 32. Our results show that all three markers are highly expressed by IMF compared to the intestinal mucosa. IMF also express higher levels of the myofibroblast marker αSMA.

Heterogenous expression of surface receptor Thy-1 in fibroblasts from several tissues is well established. Normal lung fibroblasts express Thy-1, whereas myofibroblasts in the fibroelastic foci in idiopathic pulmonary fibrosis lack Thy-1 expression 33. It has been shown that loss of Thy-1 in human lung fibroblasts induces a fibrogenic phenotype 34. In contrast to lung fibroblasts, TGFβ up-regulated αSMA expression only in Thy-1+ myometrial and orbital fibroblasts 35. These results show that the presence rather than the absence of CD90 apparently favors the appearance of a myofibroblast phenotype in response to TGFβ. In this study, we did not observe any difference in CD90 expression between IMF from IBD patients and controls.

Our results reveal that IMF are also enriched in several mediators. COL1A1 and FN1 were highly expressed in IMF. However, expression of the fibrogenic gene CTGF, also known as CCN2, a key mediator of ECM production in pathological fibrotic conditions, was similar to that in the mucosa. This is likely because the epithelium is an important source of CTGF 36. CCN1/CYR61 expression was augmented in IMF compared to the intestinal mucosa. To our knowledge, this is the first time that CCN1 expression is reported in IMF. CCN1 levels in parenchymal liver cells were relatively low compared to that in hepatic stellate cells and portal myofibroblasts 37. The same study found that overexpressed CCN1 significantly inhibited production of collagen type I, attenuated TGFβ signaling and induced production of reactive oxygen species (ROS), leading to dose-dependent cellular senescence and apoptosis. On the contrary, CCN1 has been shown to augment TGF-β signaling and contribute to fibrogenic responses to lung injury 38. Its role in intestinal fibrosis is still unknown.
Figure 5. Fibrosis-related gene expression in intestinal myofibroblasts (IMF) cultures obtained from resected bowel in control and inflammatory bowel disease (IBD) patients. Gene expression was determined by real-time qPCR, and data normalized compared to GAPDH expression. Data are presented as medians.
Figure 6. Inflammatory gene expression in intestinal myofibroblasts (IMF) obtained from control and inflammatory bowel disease (IBD) patients. Gene expression was determined by real-time qPCR, and data normalized compared to GAPDH expression. Data are presented as medians. * p< 0.05

Figure 7. Effect of TGFβ on fibrosis-related gene expression intestinal myofibroblasts (IMF) obtained from control and inflammatory bowel disease (IBD) patients. * p< 0.05, ** p< 0.005, *** p < 0.001.
Figure 8. Effect of TGFβ and IL-1β+IL-23 on cytokine production by intestinal myofibroblasts (IMF) obtained from control and inflammatory bowel disease (IBD) patients. Data are mean ± SEM from 3–5 different IMF cultures from IBD and control, respectively (A and B) and 2 IMF cultures from both IBD and control IMF cultures (C).
It was recently demonstrated that CCN1 promotes mucosal healing in murine colitis. Mechanistically, CCN1 induced IL-6 in macrophages and fibroblasts and promoted intestinal epithelial healing\(^3\). IL-6 is produced by several cell types in the lamina propria. Our data indicates that IMF cultures spontaneously released IL-6 and IL-8. In addition, we found that both fibrogenic and inflammatory stimuli can up-regulate IL-6 production. IL-6 has been shown to induce production of collagen I and fibronectin in fibroblasts from normal lungs and in idiopathic pulmonary fibrosis. In vivo neutralization of IL-6 trans-signaling resulted in a reduction in pulmonary inflammation and fibrosis, associated with improvement in respiratory function\(^4\). Neutralization of autocrine IL-6 reversed STAT3 phosphorylation and normalized expression of TGF\(\beta\)1 in structured intestinal muscle\(^5\).

Conclusions

We demonstrated that TGF\(\beta\) induced several pro-fibrotic genes in IMF. Although IMF are reported to be activated and to express \(\alpha\)SMA\(^6,18,22,28\), stimulation with TGF\(\beta\) resulted in a 20-fold increase of \(\alpha\)SMA expression. This is accompanied by upregulation of COL1A1, FN1 and CTGF. Interestingly, TGF\(\beta\) increased expression of LOX, an enzyme required to modify collagen, a pre-requisite for the cross-linking of collagen. Inhibition of LOX has recently been reported to alleviate lung fibrosis by modulating the inflammatory response preceding myofibroblast accumulation\(^6\). NOX4 expression was also induced by TGF\(\beta\). NOX4 modulates TGF\(\beta\)/SMAD-signaling via intracellular ROS production. Increased expression of NOX4 has been reported in idiopathic pulmonary fibrosis, suggesting its role in pathogenesis\(^7\). FSP1 and CD90 expression were decreased by TGF\(\beta\). \(\alpha\)SMA expression by IMF and down-regulated by IFN\(\gamma\). IL-1 and TNF\(\alpha\) induced loss of fibroblast Thy-1 surface expression \textit{in vitro}\(^8\). IFN\(\gamma\), in combination with TNF\(\alpha\), has been associated with the loss of pericryptal intestinal myofibroblasts\(^9\). Whether decreased FSP1 and CD90 expression by TGF\(\beta\) has a functional consequence on IMF is currently unknown. A recent study provided evidence of HIF-1 dependent induction of Notch ligands associated with M1 macrophages\(^10\). In contrast to M2 macrophages, M1 cells activate Notch signaling pathway in epithelial cells. It was suggested that the prevalence of M2 over M1 macrophages in the mucosa of CD patients may mediate the diminished enterocyte differentiation and impaired mucosal regeneration observed in these patients\(^11\). The current study extends our knowledge about the pathogenesis of fibrosis in IBD. Further research in the identification of mechanisms involved in IMF activation and fibrogenesis are required. A better understanding of the reciprocal regulation of macrophage phenotype and mucosal repair following intestinal damage will help to establish new approaches to CD therapy.

Data availability

Underlying data

F1000Research: Dataset 1. The following raw data sets are provided as comma separated values (.csv) files: https://doi.org/10.5256/f1000research.13906.d23172

- Figure 1 dataset on immunostaining of IMF primary cultures.
- Figure 2 dataset on the expression of fibrosis-related mediators and fibroblast markers by intestinal myofibroblasts (IMF) compared to intestinal mucosa levels in human bowel resections.
- Figure 3 dataset on the inflammation-related gene expression in intestinal myofibroblasts (IMF) and intestinal mucosa resections.
- Figure 4 dataset on the mucosal gene expression from control and inflammatory bowel disease patients.
- Figure 5 dataset on the fibrosis-related gene expression in intestinal myofibroblasts cultures obtained from resected bowel in control and inflammatory bowel disease patients.
- Figure 6 dataset on the inflammatory gene expression in intestinal myofibroblasts obtained from control and inflammatory bowel disease patients.
- Figure 7 dataset on the effect of TGF\(\beta\) on fibrosis-related gene expression intestinal myofibroblasts obtained from control and inflammatory bowel disease patients.
- Figure 8 dataset on the effect of TGF\(\beta\) and IL-1\(\beta\)+IL-23 on cytokine production by intestinal myofibroblasts obtained from control and inflammatory bowel disease patients.

Grant information

Support for this research was in the form of funds provided to EG Seidman as a Tier 1 Canada Research Chair in immune mediated gastrointestinal disorders. PS Escobar was provided salary support through the Spanish government with a predoctoral training grant Resolución de la Presidencia de la Agencia Estatal de Investigación, por la que se conceden ayudas a la movilidad predoctoral para la realización de estancias breves en Centros de I+D, convocatoria 2016. S Restillini was provided salary support by the Government of Switzerland through her home institute Geneva University Hospital.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
References

1. Abraham C, Medzhitov R: Interactions between the host innate immune system and microbes in inflammatory bowel disease. Gastroenterology 2011; 140(6): 1729–37. PubMed Abstract | Publisher Full Text | Free Full Text

2. Imhof M, Vich Vila A, Boderer MJ, et al.: Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. Gut 2016; 67(1): 108–119. PubMed Abstract | Publisher Full Text | Free Full Text

3. Fava F, Danese S: Intestinal microbiota in inflammatory bowel disease: friend or foe? World J Gastroenterol 2017; 17(5): 557–566. PubMed Abstract | Publisher Full Text | Free Full Text

4. Molodecky NA, Soon IS, Rabi DM, et al.: TGF-beta and fibrosis in different organs - molecular pathway imprints. Nat Med 2005; 11(9): 979–986. PubMed Abstract | Publisher Full Text | Free Full Text

5. McGovern DP, Kugathasan S, Cho JH: Increasing incidence and prevalence of the inflammatory bowel diseases has decreased over time: a systematic review and meta-analysis of population-based cohorts. Gastroenterology. 2016; 150(6): 1147–1165. PubMed Abstract | Publisher Full Text | Free Full Text

6. Liu JZ, van Sommeren S, Huang H, et al.: Genotype Influences on the Development of Fibrosis in Crohn’s Disease. Inflamm Bowel Dis. 2014; 20(11): 2198–2206. PubMed Abstract | Publisher Full Text | Free Full Text

7. Liu JZ, van Sommeren S, Huang H, et al.: Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet. 2015; 47(9): 979–986. PubMed Abstract | Publisher Full Text | Free Full Text

8. Huang C, Hartlun T, Oku DT, et al.: Characterization of genetic loci that affect susceptibility to inflammatory bowel diseases in African Americans. Gastroenterology. 2015; 149(5): 1575–1586. PubMed Abstract | Publisher Full Text | Free Full Text

9. Khor B, Gardet A, Xavier RJ: TGF-beta and fibrosis in different organs - molecular pathway imprints. Nat Med 2005; 11(9): 979–986. PubMed Abstract | Publisher Full Text | Free Full Text

10. Frikha AD, Dykeman J, Negron ME: Risk of surgery for inflammatory bowel disease has decreased over time: a systematic review and meta-analysis of population-based studies. Gastroenterology. 2015; 149(5): 1575–1586. PubMed Abstract | Publisher Full Text | Free Full Text

11. Verstockt B, Cleynen I: Genetic Influences on the Development of Fibrosis in Crohn’s Disease. Front Med (Lausanne). 2016; 3: 24. PubMed Abstract | Publisher Full Text | Free Full Text

12. Powel DW, Pinchuk IV, Saada JI, et al.: TGF-beta and fibrosis in different organs - molecular pathway imprints. Nat Med 2005; 11(9): 979–986. PubMed Abstract | Publisher Full Text | Free Full Text

13. Wynn TA, Ransingh-da TR: Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012; 18(7): 1028–1040. PubMed Abstract | Publisher Full Text | Free Full Text

14. Latela G, Rogler G, Bamias G, et al.: Results of the 4th scientific workshop of the ECCO (I): pathophysiology of intestinal fibrosis in IBD. J Crohns Colitis. 2014; 8(10): 1147–1165. PubMed Abstract | Publisher Full Text | Free Full Text

15. Porters D, Brenmoehl J, Löffler I, et al.: TGF-beta and fibrosis in different organs - molecular pathway imprints. Biochim Biophys Acta. 2009; 1792(8): 746–55. PubMed Abstract | Publisher Full Text | Free Full Text

16. Li C, Kuenmerle JP: Mechanisms that mediate the development of fibrosis in patients with Crohn’s disease. Inflamm Bowel Dis. 2014; 20(7): 1250–8. PubMed Abstract | Publisher Full Text | Free Full Text

17. Scharf M, Bruckner RS, Rogler G: The two sides of the coin: Similarities and differences in the pathomechanisms of fistulas and strictures formations in inflammatory bowel disease. United European Gastroenterol J. 2016; 4(4): 506–51. PubMed Abstract | Publisher Full Text | Free Full Text

18. Balestrini JL, Chaudtry S, Sarrazy V, et al.: The mechanical memory of lung fibroblasts. Integr Biol (Camb). 2012; 4(4): 410–421. PubMed Abstract | Publisher Full Text | Free Full Text

19. Johnson LA, Rodansky ES, Sauder KL, et al.: Matrix stiffness corresponding to stricture bowel induces a fibrogenic response in human colonic fibroblasts. Inflamm Bowel Dis. 2013; 19(5): 901–903. PubMed Abstract | Publisher Full Text | Free Full Text

20. Mahda YR, Bellinger J, Mack S, et al.: Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. Am J Physiol. 1997; 273(6 Pt 1): G1341–1348. PubMed Abstract | Publisher Full Text

21. Barnes JL, Gorin Y: Myofibroblast differentiation during fibrosis: role of NADPH oxidases. Kidney Int. 2011; 79(9): 934–956. PubMed Abstract | Publisher Full Text | Free Full Text

22. Cosnes J, Gower-Rousseau C, Seksik P, et al.: Epidemiology and natural history of inflammatory bowel disease. Gastroenterology. 2011; 140(6): 1785–94. PubMed Abstract | Publisher Full Text | Free Full Text

23. Gordon IO, Agrawal N, Goldblum JR, et al.: Fibrosis in ulcerative colitis: mechanisms, features, and consequences of a neglected problem. Inflamm Bowel Dis. 2014; 20(11): 2135–2150. PubMed Abstract | Publisher Full Text | Free Full Text

24. Ippolito C, Colucci R, Segnani C, et al.: Fibrotic and Vascular Remodelling of Colonic Wall in Patients with Active Ulcerative Colitis. J Crohns Colitis. 2016; 10(10): 1194–204. PubMed Abstract | Publisher Full Text | Free Full Text

25. Siiram G, Biglardi PL, Biglardi-Qi M: Fibroblast heterogeneity and its implications for engineering organotypic skin models in vitro. Eur J Cell Biol. 2015; 94(11): 483–512. PubMed Abstract | Publisher Full Text | Free Full Text

26. Ramírez G, Siiram GS, Sanders Y, et al.: Absence of Thy-1 results in TGF-β induced MMP-9 expression and confers a fibrotic phenotype to human lung fibroblasts. Lab Invest. 2011; 91(6): 1206–1218. PubMed Abstract | Publisher Full Text | Free Full Text

27. Kornhams P, Schaffrath C, Van de Leur E, et al.: The anti-fibrotic effects of CCN1/VCN1 in primary portal myofibroblasts are mediated through induction of reactive oxygen species resulting in cellular senescence, apoptosis and attenuated TGF-β signaling. Biochim Biophys Acta. 2014; 1843(5): 902–914. PubMed Abstract | Publisher Full Text | Free Full Text

28. Carvalho J, Medrano-Rodriguez N, et al.: The matricellular protein CCN1 enhances TGF-β/SMAD3-dependent profibrotic signaling in fibroblasts and contributes to fibrogenic responses to lung injury. FASEB J. 2016; 30(6): 2135–2150. PubMed Abstract | Publisher Full Text | Free Full Text

29. Choi JS, Kim KH, Lau LF: The matricellular protein CCN1 promotes mucosal healing in murine colitis through IL-4. Mucosal Immunol. 2015; 8(6): 1285–96. PubMed Abstract | Publisher Full Text | Free Full Text

30. Le TT, Karmouty-Quintana H, Melicow E, et al.: Blockade of IL-6 Trans signaling attenuates pulmonary fibrosis. J Immunol. 2014; 193(7): 3755–3768. PubMed Abstract | Publisher Full Text | Free Full Text

31. Li C, Iness A, Yoon J, et al.: Noncanonical STAT3 activation regulates excess TGF-β1 and collagen I expression in muscle of strictureing Crohn’s disease. J Immunol. 2015; 194(7): 3462–3471. PubMed Abstract | Publisher Full Text | Free Full Text

32. Cheng T, Liu Q, Zhang R, et al.: Lysyl oxidase promotes bleomycin-induced lung fibrosis through modulating inflammation. J Mol Cell Biol. 2014; 6(6): 506–515. PubMed Abstract | Publisher Full Text | Free Full Text

33. Hecker L, Vittal R, Jones T, et al.: NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med. 2009; 15(9): 1077–1081. PubMed Abstract | Publisher Full Text | Free Full Text

34. Desmoulière A, Rubbia-Brandt L, Abdou A, et al.: Alpha-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by gamma-interferon. Exp Cell Res. 1992; 199(1): 64–73. PubMed Abstract | Publisher Full Text | Free Full Text

35. Francoeur C, Boudroussy Y, Sellanta A, et al.: Degeneration of the pericryptal myofibroblast sheath by proinflammatory cytokines in inflammatory bowel diseases. Gastroenterology. 2009; 136(1): 268–277.e3. PubMed Abstract | Publisher Full Text | Free Full Text

36. Ortiz-Masiá D, Cosín-Roger J, Calatayud S, et al.: Macrophages Activate Notch Signalling in Epithelial Cells: Relevance in Crohn’s Disease. J Crohns Colitis. 2016; 10(6): 582–692. PubMed Abstract | Publisher Full Text | Free Full Text

37. Dionne S, Restiliini-Kherad S, Koenekoop J, et al.: Dataset 1 in: Characterization of myofibroblasts isolated from the intestine of patients with inflammatory bowel disease, F1000Research. 2019. http://www.doi.org/10.2526/f1000research.13906.d231722
Andrew Silver
Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London (QMUL), London, UK

The authors have presented an account of their study on characterising human myofibroblasts from IBD patients, both Crohn's disease (CD) and ulcerative colitis. The authors are to be commended for obtaining human tissue from a good number of patients - the effort involved is significant. However, I do have some important comments on the manuscript, which I feel should be addressed so that the reader is better informed.

Comments:

1. There is insufficient detail on and phenotyping of the resection specimens. CD is a very diverse disease. There are a number of factors, e.g. site, active/inactive disease, presence of stricture etc., that can have a major impact on gene expression. The reader is unable to assess pathological features with respect to the expression data.

2. Figure 1 is incomplete. Why only a control patient for panel A? Why CD90 for panel B and only one specimen from a UC patient? Why are so few cells in A showing a-SMA expression? Negative controls? Magnification? Use of overly? Make sure the legends fully explain the figure - see also comments below.

3. I am unclear as to why the numbers of data points varies so often within (mucosa versus IMF) and between graphs (e.g., CCN1 Versus FN1) within a given Figure? The numbers appear very different to those indicated in the text. There could be a number of reasons why this has happened. The authors should explain this anomaly clearly along with any potential influence loss of data points has had on their analyses.

4. There are insufficient mRNA expression data points to do a sub-group (UC and CD) analysis. Given the nature and diversity of these diseases I would have focused on one or the other, especially given that fibrosis and subsequent stricture formation predominately affect CD patients. From the clinical table, it appears that seven Crohn's patients had a structuring phenotype, how do markers of fibrosis in IMFs isolated from these patients compare with
inflammatory IMFs for UC/CD controls? The authors could also compare their mRNA data to what is already published in the literature.

5. mRNA levels of some inflammatory cytokines are enriched in IMFs over mucosa, how do the authors explain this, given IMFs are not the primary cytokine producing cells?

6. Data and text to justify the use of GADPH as the normaliser would be useful.

7. Figures 7 and 8 are very difficult to follow because of a lack of information in the text and the legend. For example, the title for Figure 7 states, “gene expression intestinal myobroblasts (IMF) obtained from control and inflammatory bowel disease (IBD) patients” yet there is only a single bar for each gene? This should be explained in the legend and then numbers used should be given in the legend. The use of black plus, I think, two shades of grey which do not match the key complicate matter further. Once again it would be more clinically impactful to have compared response between different diseases and phenotypes. Currently numbers are too small to do this.

8. In Figure 8 have the cultures been pooled? Why are the numbers different between panels A/B and C? What were the criteria for selecting these particular cultures over others?

9. There is no clear explanation as to how the data presented in Figures 7 and 8 relate to the mRNA data given in earlier Figures. There is a lack of explanation as to why select the various cytokine combinations for stimulation experiments.

10. Overall there is an absence of protein validation.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular genetic colorectal
I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 24 May 2019

https://doi.org/10.5256/f1000research.15118.r48693

© 2019 Gopalakrishnan A. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Archana Gopalakrishnan
University of Maryland, Baltimore, Baltimore, MD, USA

Since fibrosis is a major complication in IBD patients and the published literature indicates a fibrogenic role of myofibroblasts, the authors have an important rationale for understanding the characteristics of myofibroblasts in the intestines (IMF). However, this study has introduced a number of variables, such as mucosal tissues versus IMF and IBD patient versus control groups that are confusing to the overall aim of the study. Some of the areas that can be better explained include:

1. It is unclear in the Results pertaining to Figures 2 and 3 from which of the four groups of patients the IMF cultures were characterized.

2. When examining the inflammatory potential of IMF, an elevated level of IL-6 and IL-8 is reported, but not TNF and IL-17. It would be interesting to hear a speculation on why this is so.

3. IMFs from control groups and IBD groups did not vary in their expression of fibrosis markers - doesn't this undermine the role of IMFs in IBDs, where fibrosis is a major complication?

4. Several references have been made to the lung mucosal surface that differs significantly from the intestinal mucosa.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Innate immunity, tuberculosis induced immune response, macrophages, inflammation, flow cytometry, qRT-PCR, cytokines and chemokines.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com