Provisional chapter

Fibrosis in Crohn's Disease

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Additional information is available at the end of the chapter

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1. Introduction

The objectives of this chapter are to review clinical and pathophysiologic aspects of fibrosis in inflammatory bowel disease, particularly in Crohn's disease. Potential therapeutic strategies and the current status of preclinical animal models to evaluate these therapeutic strategies will also be discussed.

2. Clinical considerations

Crohn's disease (CD) and ulcerative colitis (UC) are chronic, relapsing inflammatory gastrointestinal diseases which often onset in young adulthood. Unlike in UC, where inflammation is limited to the mucosa, CD patients frequently develop transmural disease which can extend to involve the muscularis and serosa. While inflammatory disease accounts for much of the symptomatology associated with CD, significant morbidity results from fibrotic lesions and their resulting complications.

CD can be broadly categorized as having three major clinical subtypes: stricturing, penetrating or inflammatory (nonstricturing nonpenetrating) disease [1]. This categorization is reflected by the inclusion of disease behavior (stricturing, penetrating or inflammatory) as one of three variables elected for inclusion in the Vienna classification in 1998 [2]. Review of natural history data from shows that the majority of patients undergo progression from inflammatory disease to development of complications including stricture [3]. Even though most patients present with inflammatory disease only and later progress to a more complicated disease phenotype, a subset of patients will present with stricturing or penetrating disease. In population-based studies, 19-36% of patients newly diagnosed with CD present with disease complications such as strictures, fistulas or abscesses [4-6].

Intestinal stricture is a common and serious complication of long term CD. Critical intestinal stricture formation will occur in at least one-third of CD patients within 10 years of onset [7-9]. By contrast, fibrosis associated with UC is generally limited to the mucosa and stricture formation is rare although rectal strictures, when they occur, can be problematic to manage [10]. Advances in CD treatment have yet to make significant impact on the incidence of strictures and the associated morbidity [11].

While surgical resection is a highly effective short term treatment, remission after surgical resection in CD is only temporary. The disease course in CD patients postsurgery is relatively consistent and has allowed development of a postoperative recurrence model [12, 13]. In this model, a focal inflammatory infiltrate forms in the ileum above the anastomosis followed by aphthous ulcers which are endoscopically visible in two-thirds of patients within 3 months after surgery. These patients go on to develop extensive superficial and deep ulcers that precede the development of a new stricture. Postoperative reoccurrence is very common with fewer than 5% of patients having normal endoscopy results 10 years after surgery. Symptoms occur on average 2-3 years after lesions are observed.

In patients with a fibrostenotic disease behavior, symptomatic strictures tend to return despite medical therapy and this leads to repeated bowel resections and eventually to short bowel syndrome [14-16]. Endoscopic balloon dilation can provide a treatment option for patients with fibrostenotic strictures 7 cm or less in length although there is some risk of perforation [17, 18]. Strictureplasty can be a useful bowel-preserving surgical option for stenosing small bowel CD in patients with multiple obstructions and in those vulnerable to short bowel syndrome [19]. The incidence of postoperative recurrence is very similar between strictureplasty and resection [11].

Strictures can arise due to either inflammatory or fibrotic processes. There is evidence that direct steroid injection can provide symptom relief in some CD patients with anastomotic strictures, presumably in patients with active inflammation and relatively little fibromuscular proliferation at the anastomotic site [18]. Given the risk of multiple bowel resection surgeries and the possibility of short bowel syndrome, the ability to differentiate between inflammatory or fibrotic strictures could be used to drive treatment decisions. Endoscopy-based techniques such as colonoscopy, small intestinal endoscopy, and capsule endoscopy have high clinical utility but only visualize the mucosal surface. Cross-sectional imaging techniques can be used to visualize deep layers of the intestinal wall and assess for strictures. Computed tomography enterography (CTE) has become the most widely used cross-sectional imaging technology for CD although concern about cumulative radiation exposure, particularly in young patients, has led to interest in alternative imaging modalities [20, 21].

Imaging methods such as magnetic resonance (MR) enterography or ultrasonography are effective in determining the anatomic location and length of affected intestinal segments and these techniques, as well as CTE, have shown a good correlation with endoscopy [22-24]. However, distinguishing stricture composition remains a challenge. In 2006, the European Crohn's and Colitis Organization (ECCO) stated in their consensus report on treatment of CD that current techniques are insufficiently accurate to differentiate between inflammatory

and fibrostenotic strictures [25]. A recent study confirms that, while the combination of MR-enterography and ultrasound as well as the combination of ^{18}FDG-PET/CT and ultrasound are highly efficient in detecting CD strictures, no current imaging techniques shows sufficient sensitivity or specificity to reliably differentiate inflamed from fibrotic strictures [26]. The clinical need for this for such differentiation remains high and may require both technologic advancement and establishment of criteria for grading or distinguishing strictures which contain both fibrotic and inflammatory components [27]. [25]. In clinical practice, a variety of diagnostic tools including imaging techniques and inflammatory biomarkers are often applied in an effort to obtain sufficient evidence to inform treatment decisions [28].

Therapeutic strategies have evolved over the past decade and having the ability to predict disease outcomes could guide the clinician's choice of therapy. The goals of CD treatment should include 1.) steroid-free sustained clinical remission; 2.) mucosal healing; 3.) potential induction and maintenance of radiological healing; 4.) prevention of surgery; 5.) maintenance of normal gastrointestinal function; and 6.) prevention of disability [6]. There are a number of therapeutic options available for the treatment of inflammation in CD patients, however none of these have been demonstrated to be effective in preventing preventing or treating fibrostenosis thereby failing to achieve at least 2 of the 6 major treatment goals in patients with fibrostenotic disease. There have been some reports of regression of strictures and of overall benefit following infliximab treatment in a subset of patients with small bowel stricturing disease [18, 29, 30]. This data is largely anecdotal and remains controversial as large controlled trials have not yet been performed.

In CD, disease location (ileal vs. colonic) remains relatively stable but clinical behavior can alter significantly over time [8, 31, 32]. During the first few years of disease, inflammatory forms predominate, whereas, after 40 years, most patients have experienced complications and are classified as having penetrating or stricturing disease[33]. However, the rate at which disease behavior evolves can vary widely between CD patients and those differences would determine therapeutic strategies if rapidly progressing patients could be identified. For example, initiation of more aggressive treatment early in the course of disease has the potential to result in better outcomes, however, these therapies can also lead to greater risk of toxicity and adverse effects [34, 35]. Decisions on whether to use a conservative or an aggressive treatment strategy in newly diagnosed patients could be informed by the ability to identify patients at higher risk for developing disabling or complicated disease.

3. Fibrosis risk factors

A number of retrospective studies have identified specific disease characteristics that may be of use in predicting risk for individual CD patients. These include an initial CD diagnosis under age 40, need for steroid therapy at diagnosis, and perianal fistulizing disease [31, 36]. Localization of inflammation to the small bowel has also been identified as predictive of progression to more complicated disease and higher rate of surgery [37]. However, all of these clinical features tend to correlate with the presence of small intestinal disease and do

not necessarily identify which patients with small bowel disease are at greatest risk of developing fibrostenosing disease.

Genetic polymorphisms play an important role in susceptibility to CD so the use of genetic markers to provide risk stratification and drive clinical decisions is very attractive. The observation that some CD patients are susceptible to stricture development early and often in their clinical course while others never develop a stenosing phenotype argues for the existence of a genetic background which predisposes to stricturing behavior in CD.

One of the genes linked with susceptibility to CD is the CARD15/NOD2 gene which encodes a protein involved in bacterial recognition and activation of nuclear factor kB. Carriers of two mutant alleles have 17 to 42 times the risk while carriers of one mutation have 1.5 to 3 times the risk of developing CD [38, 39]. There is a significant body of evidence suggesting that the main NOD2/CARD15 variants (Arg702Trp, Gly908Arg, and Leu1007insC) are associated with risk for developing stenotic disease and increased need for surgery [40-44]. However, this finding is not reproducible in every CD cohort evaluated [45, 46]. One large meta-analysis of the NOD2/CARD15 literature indicated that carrying at least one NOD2/CARD15 variant increased the risk of both small intestinal disease and of the stenosing phenotype [47]. The discrepancy between studies may be due to differences in definitions of disease behavior between studies and, perhaps, to the particular genetic epidemiological analyses used. At present, understanding whether the relationship between the NOD2/CARD15 variants and a stenosing phenotype is a true association or whether it instead reflects aspects of disease duration and ileal localization remains a matter of controversy.

Other gene polymorphisms have been described as associated with a fibrostenotic phenotype although the existing data is much less extensive than that available for NOD2/CARD15. The ATG16L1 gene encodes for a protein involved in autophagy and mutations in this gene have been associated with stricturing disease as well as perianal involvement in CD [48]. CX3CR1, the receptor of CX3CL1 (fractalkine), is involved in regulation of inflammatory response and the V249I polymorphism has been reported to be associated with intestinal strictures [49]. A recent study linked the receptor for advanced glycation endproducts (RAGE) -374T/A polymorphism to protection from stricturing phenotype in CD. The polymorphism increases RAGE gene transcription which may provide protection by increasing levels of soluble RAGE leading to neutralization of proinflammatory mediators [50]. Some fibrostenosis-related polymorphisms have been observed in combination with NOD2/CARD15. CXCL16 is a chemokine involved in bacterial defense mechanisms. CD patients with at least one CXCL16 p.Ala181Val allele and one CARD15/NOD2 variant had a higher incidence of stricturing and penetrating phenotype as well as stenosis as patients with the NOD2 variant alone [51].

Despite their potential, genetic markers may never fully be able to predict the clinical course of CD. The low frequency, incomplete penetrance, and interplay with other genetic polymorphisms greatly complicate interpretation of genetic markers. In addition, environmental factors can modulate disease history and impact phenotypic features. It is probable that genetic markers will need to be integrated with other clinical and serologic information in order to be useful predictors of disease course and to inform treatment decisions.

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4. Serologic biomarkers

Serologic markers may identify CD patients at higher risk of developing disease-related complications. Some of the best characterized serologic markers associated with CD are directed against microbial peptides. Most of the available data is from cross sectional studies in which the patient samples analyzed have been collected at various times in the disease course allowing for comparison of serum from before, concomitant with, and after diagnosis or treatment of bowel stricture.

Disease progression from non-complicated CD to stricturing and/or penetrating phenotypes has been significantly associated with the presence and magnitude of serologic response to microbial antigens. This concept was initially triggered by the observation that high anti-Saccharomyces cerevisiae antibody (ASCA) levels were found to be associated with fibrostenosing and penetrating disease and with the need for surgery [52]. This observation was repeated in another cross sectional study where ASCA positive patients were more likely to undergo surgery within 3 years of diagnosis than ASCA negative patients [53]. Time to first complication was shown to be shorter in ASCA positive pediatric CD patients than in ASCA negative patients [54]. In addition, anti-I2 (an antibody directed against *Pseudomonas* fluorescens), anti-OmpC (the outer membrane porin protein of *Escherichia* coli) and anti-CBir1 (anti-flagellin) levels have also been shown to associated with fibrostenotic disease [55-60]. A multi-center study evaluated the association of ASCA, anti-I2, anti-OmpC, and anti-CBir1 reactivity with disease course in a large cohort of pediatric CD patients and found that the frequency of fibrostenotic or penetrating disease increased in parallel with the number of antigens recognized [61]. Combining anti-microbial antibody titers and evaluation of NOD2 variants or other gene polymorphisms may improve detection of patients at higher risk of developing fibrostenotic disease [62].

The predictive value of other serologic markers for fibrostenotic disease has been evaluated on a much more limited basis than the anti-microbial antibodies. C-reactive protein (CRP) is widely used to monitor inflammatory disease activity and one prospective study found a significant association between CRP levels and subsequent risk of intestinal resection in patients with ileal disease [63]. Despite the prominent place of extracellular matrix proteins in composition of fibrostenotic lesions, little association has been found with levels of these molecules in the circulation [64]. However, one study did find that higher levels of plasma fibronectin were associated with stricture formation in CD patients [65]. Growth factors have also been evaluated in a limited fashion. Serum levels of YKL-40, a mammalian glycoprotein member of the chitinase family, has been reported as increased in CD patients with stricturing disease compared to those without strictures [66]. Another study found serum levels of basic fibroblast growth factor, a cytokine promoting fibroblast activation and proliferation, were higher in CD patients with intestinal strictures compared to patients with fistulizing or inflammatory phenotypes [67]. Prospective studies will be required to determine which, if any, of these serologic tests may have potential as a clinically useful biomarker of fibrostenotic disease.

5. Histologic features

Stricture due to fibrostenotic change resulting in chronic obstruction is a major pathologic event in chronic CD. Histologically, CD strictures are characterized by hyperplasia of the intestinal muscle layers which is typically manifest as islands of smooth muscle cells in the submucosa surrounded by dense collagen deposits. These regions of smooth muscle proliferation may become so extensive that they obliterate the submucosa [68]. Transmission electron microscopy studies show alteration of muscle cells of the muscularis propria, especially the inner muscle layer, including hypertrophy, synthesis and deposition of collagen, and focal cellular necrosis [69].

Despite the general categorization of strictures as inflammatory, fibrostenotic or both, fibrosis is often well correlated with inflammation and the majority of strictures contain some degree of both processes [70]. When histologic tissue inflammation and fibrosis were compared in a relatively small cohort of patients undergoing surgical resection, the authors found that all specimens which were significantly fibrotic were also significantly inflamed [71]. This may accurately reflect the reality of stricturing disease in many patients, however, this has not yet been confirmed in studies of larger patient cohorts and the results may be skewed because histologic evaluation is only possible in patients undergoing surgical resection. This excludes stricture patients who either respond to aggressive medical therapy or who undergo bowel-sparing procedures which may inadvertently exclude many patients who fall on either end of the inflammation/fibrosis spectrum.

Further consideration of the relationship between histologic categorization and disease behavior is needed and new histologic scoring systems may be required which consider cellular composition or other features in order to more effectively categorize strictures. One retrospective study has been published where biopsies were evaluated to determine if certain histologic characteristics correspond to eventual development of complicated CD [72]. The authors report that severe lymphoid infiltration of the lamina propria with crypt atrophy and absence of intraepithelial lymphocytes correlates with non-stricturing/non-penetrating disease while these features were absent in 80% of CD patients with stricturing disease. Once again, these findings were based on a small cohort size and need further evaluation but do accord with the larger concept of inflammatory vs. fibrostenotic stricture processes.

Other studies comparing histologic features with biologic behavior are limited. At least two studies have noted an association of mast cells in the submucosa and especially in the muscularis propria with stricture formation in CD patients [73, 74]. When compared to normal bowel or non-strictured CD bowel, mast cell numbers were significantly higher in the thickened muscularis propria of CD strictures. No increase in mast cells was associated with ulcerative colitis or other intestinal inflammatory conditions. Also, epitheliold granulomas have been implicated as a risk factor for progression to complicated disease behavior. Epitheliod granulomas are one of the most characteristic histologic features in biopsies or resected tissue from patients with CD although only about 15-25% of patients present with

this lesion. Several studies have shown an association between the occurrence of epitheliod granulomas, especially at presentation, and a more aggressive disease course [75].

6. Pathophysiology of fibrosis

Tissue injury or inflammation triggers a cascade of wound healing activities in the surrounding cell populations. Normal wound healing is a tightly regulated and coordinated series of events triggered by secretion of mediators from activated immune and mesenchymal cells which induce cell proliferation, migration, and extracelluar matrix (ECM) production. Wound healing activity is followed by resolution of inflammation and tissue remodeling. A balance must be achieved between processes involved in ECM production and degradation and those involved in cellular hyperplasia (proliferation and cell death). In the intestinal tract, tissue repair and regeneration are of great importance in mucosal homeostasis and intestinal barrier function. Rapid wound healing and restitution of an intact mucosal barrier is crucial for controlling mucosal inflammation. However, excessive wound healing response can result in fibrosis and stricture formation while insufficient tissue repair can result in fistula formation.

The classic model of wound healing has 4 phases: hemostasis, inflammation, proliferation, and remodeling [76, 77]. In the hemostasis phase, platelet degranulation and fibrin formation provide both hemostasis and a provisional matrix for subsequent healing events to take place. Cytokine and chemokine expression, initially by the innate immune system and later including the adaptive immune system, drives the inflammatory phase. During the proliferative phase, activated fibroblasts and myofibroblasts secrete collagen and other matrix molecules which provide a granulation tissue scaffold on which tissue structure repair can commence. During the proliferative phase, cytokines and growth factors regulate reconstitution of the mucosal epithelium allowing closing of the epithelial defect. Angiogenesis and lymphangiogenesis also take place during this phase and there is expansion of the fibroblast/myofibroblast population with concomitant ECM production. Finally, in the remodeling phase, myofibroblasts produce matrix-modifying molecules which assist in the restoring anatomic structural integrity and completing the transition from wound to normal or near normal intestine architecture.

When severe mucosal tissue damage occurs, myofibroblasts migrate to the edges of the tissue defect. The ability of myofibroblasts to migrate to the wound area and synthesize ECM proteins is critical in proliferative phase of intestinal wound healing [78]. Migration of subepithelial myofibroblasts can be mediated by a variety of soluble factors such as transforming growth factor –β (TGFβ), insulin-like growth factor (IGF-1), platelet-derived growth factor-AB (PDGF-AB), and epidermal growth factor (EGF) [79]. Fibronectin, synthesized by myofibroblasts, is essential and is largely responsible for autocrine induction of intestinal myfibroblast migration [80].

Wound healing and myofibroblast migration can be affected by chronic inflammation [79]. Subepithelial myofibroblasts isolated from CD patients show a significant reduction in mi-

gration response when compared to cells from control patients [81]. Similar reduction in fibroblast migration can be induced by treatment with tumor necrosis factor (TNF) or gamma interferon (IFN-g) suggesting that an inflammatory environment can induce changes in myofibroblast function [82]. However, environmental impact on fibroblast migration is complex. For example, fibroblasts from lung tissue with dense fibrosis show higher PDGF-driven migratory potential than do fibroblasts from tissues at an early stage of fibrosis [83]. A recent paper compared migratory potential in colonic fibroblasts isolated from CD patients with either fistulizing (penetrating) or fibrotic (stricturing) disease [81]. These authors showed that, while migratory potential is reduced in CD patients with fistulizing disease, there is in an increase in fibroblast migratory potential in patients with fibrotic disease.

Fibrosis in CD is thought to result from an excessive wound healing response. For reasons that are not wholly understood, the wound repair process in a subset of CD patients continues to progress rather than reaching a termination and allowing for tissue remodeling. Ultimately, the fibrotic process leads to thickening of the intestinal wall and luminal narrowing which can result in bowel obstruction. There are three hallmark pathological features which characterize intestinal stricutres in CD: proliferation of mesenchymal cells including myofibroblasts, smooth muscle cells and fibroblasts; hypertrophy of smooth muscle cells and myofibroblasts ; and accumulation of excess extracellular matrix proteins [84].

Mesenchymal cells in the intestine can be broadly classed as fibroblasts, smooth muscle cells or myofibroblasts on the basis of immunostaining properties with antibodies to vimentin (V) and smooth muscle actin (A) [85, 86]. Fibroblasts are typically V+/A- and are present in the intestinal submucosal and serosa. Subepithelial myofibroblasts (SEMF) are found adjacent to intestinal epithelial cells and are V+/A+. Intestinal smooth muscle cells of the muscularis mucosa and muscularis propria are normally V-/A+. All of these mesenchymal cell types have been implicated in collagen production in CD patients [69, 87, 88].

Activated fibroblasts or myofibroblasts in tissues undergoing a fibrotic process may be derived from a variety of sources. There are three general mechanisms which allow for tissue accumulation of these cells: proliferation of existing tissue fibroblasts, recruitment of fibroblast precursor cells from bone marrow, and transformation either of epithelial cells via epithelial to mesenchymal transistion (EMT), or of endothelial cells by endothelial to mesenchymal transition (EndoMT) [89, 90]. Proliferation and activation of tissue fibroblasts occurs in response to profibrotic signals from infiltrating inflammatory cells or from colonic epithelial cells exposed to proinflammatory cytokines [91]. Soluble inflammatory mediators also drive recruitment of fibroblast precursor cells (fibrocytes) from bone marrow. These fibrocytes migrate from the bloodstream into tissues undergoing pathologic fibrosis in response to specific chemokine gradients [89]. EMT and EndoMT are induced by TGFβ [92]. The relative significance of each of mechanisms discussed above to activated fibroblast/myofibroblast accumulation at the site of injury in CD is not yet fully understood

Smooth muscle hyperplasia surrounded by collagen deposits is the major histologic feature of fibrostenotic CD. This smooth muscle proliferation expands and disrupts the muscularis mucosa. Thickening of the muscular layer is associated with an increase in the number of vimentin-positive cells [93, 94]. In severely affected tissue, even histologically normal mus-

cularis mucosa is populated largely by V+/A- and V+/A+ cells rather than the V-/A+ smooth muscle cells seen in normal muscularis mucosa from non-CD patients. This suggests a transition from an enteric smooth muscle cell phenotype toward a fibroblast or myofibroblast phenotype.

Mesenchymal cells including myofibroblasts as well as smooth muscle cells of the muscularis mucosa and muscularis propria are the main producers of ECM proteins in the intestine. These ECM proteins include structural proteins such as collagen, matricellular proteins such as osteopontin and thrombospondin, and other specialized proteins such as vitronectin and fibronectin. Collagen is the major ECM component associated with intestinal fibrosis. The most common collagen subtypes in normal intestine are type I, type III, and type V in order of abundance. In intestinal fibrosis, there is an increase in total collagen as well as specific and relative increases in collagen types III and V [95-97].

Fibronectin and vitronectin are ligands for the αVβ3 integrin and, in the presence of fibronectin, smooth muscle IGF-1-stimulated IGF-1 receptor activation is augmented [98]. This suggests that increased production of these proteins by smooth muscle cells at sites of intestinal stricture could activate αVβ3 integrin and further increase secretion of collagen as well as promote cellular proliferation creating a positive feedback loop which could further subvert the normal healing process. Fibronectin is also an important mediator in focal adhesion kinase (FAK) signaling pathways involved in cell migration [99]. Myofibroblasts synthesize abundant fibronectin which is largely responsible for the autocrine induction of intestinal myofibroblast migration [100].

The balance between formation and breakdown of ECM proteins determines the net deposition in tissues. In intestinal fibrosis, mechanisms to degrade ECM fail to keep pace with deposition. Matrix metalloproteinases (MMPs), a large family of proteolytic enzymes, are responsible for the breakdown of ECM components. The proteolytic activity of MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPs) and an imbalance between MMP and TIMP activity can result in excessive deposition of ECM proteins with subsequent fibrosis [101]. Higher levels of constitutive TIMP-1 expression have been shown in intestinal myofibroblast culture derived from fibrotic CD patients than those from normal individuals [102].

Transforming growth factor –β (TGFβ) is a pleotrophic cytokine and one of the most influential factors in fibrotic processes. It is a component of Th17 as well as regulatory T cell type immune responses as well as a profibrotic mediator. TGFβ exerts profibrotic effects through its ability to regulate collagen expression and extracellular matrix dynamics. There are three isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3. TGFβ1 activates the canonical Smad signaling cascade leading to translocation of the Smad receptor complex into the nucleus and regulation of gene transcription including ECM genes such as collagen I, collagen III, and fibronectin [103]. TGFβ also induces EMT in organ-fibrosis inducing diseases [104] and can to induce EndoMT in vitro via a "noncanonical" signaling pathway [105]. In CD patients, TGFβ1 and TGFβ3 are increased in in smooth muscle, fibroblasts and myofibroblasts from the strictured region when compared to normal intestine [102, 106].

TGFβ family proteins are important in regulating the synthesis and breakdown of ECM proteins [107]. TGFβ1 downregulates MMP expression and enhances the expression of TIMP-1 [101]. Characterization of the role of TGFβ expression in disease has been done using myofibroblast cultures. Myofibroblasts from normal intestine predominantly express TGFβ3 while those patients with fibrotic CD had significantly lower expression of TGFβ3 and higher levels of TGFβ1 and TGFβ2 [102, 108].

Insulin-like growth factor has also been implicated in the pathogenesis of stricture formation [109]. Intestinal smooth muscle cells express IGF-1 which activates the IGF-1 receptor thereby regulating smooth muscle cell hyperplasia by simultaneously stimulating proliferation and inhibiting apoptosis [110, 111]. Studies using CD tissue from patients undergoing intestinal resection show increased expression of both IGF-1 as well as synergistic IGF binding protein 5 (IGFBP-5) in lesional tissue [112]. Localization studies show IGF-1 is upregulated in smooth muscle cells in regions of stricture when compared to tissue from surgical margins.

7. Preclinical models

A major challenge facing scientists interested in developing treatments for CD-associated fibrotic disease is the need for a robust animal model which develops morphologic features and utilizes pathogenic processes similar to those characterized for the human disease. Preclinical models should provide a consistent environment for testing intervention strategies and quantifying outcomes. At present, no animal model exists which reproduces the unique histologic features associated with CD intestinal strictures. There are several models which address some aspects of CD stricture pathogenesis and those are reviewed below.

Intestinal inflammation and fibrosis can be induced in rats by injection of peptidoglycan-polysaccharide (PG-PS) into the intestinal wall or by repeated rounds of trinitrobenzene sulfonic acid (TNBS) treatment [113, 114]. PG-PS injection causes acute inflammation which peaks by 2 days followed by remission. Spontaneous reactivation of inflammation occurs in genetically susceptible rat strains by 12-17 days and is characterized by progressive transmural granulomatous enterocolitis [115]. Multiple cycles of intrarectal injection of TNBS in ethanol also induces a granulomatous transmural inflammatory response which becomes dominated by chronic inflammation and fibrosis after cycle 4[113, 116]. This model features transmural collagen deposition which is most prominent in the submucosa. Smooth muscle proliferation and expansion into the submucosal space is not a feature of these models.

Given that no existing preclinical model completely mimics changes found in human CD fibrostenosis, assessment of the value of models should be based on the presence of pathways of interest as well as tractability in testing potential therapeutic entities. These rat models show transmural inflammation associated with transmural fibrosis as well as overexpression of TGFβ and/or IGF-1 in a manner consistent with human disease [116-119]. However, given the availability of reagents and other research tools, mouse models of intestinal fibrosis are more desirable to the research community.

While many murine models of inflammatory colitis or enteritis exist, these models are generally not suitable for study the pathogenesis of stricture formation or for testing intervention strategies because they generate very little intestinal fibrosis. Fibrotic models have been challenging to develop given the inherent resistance of mice when compared to other species in development of fibrotic disease [94]. However, progress is being made in this area.

Ileocecal resection is a common surgical intervention in CD and is associated with high rates of disease recurrence[120]. After surgery, recurrence of inflammation and/or fibrosis typically occurs at the anastomosis and in the small intestine immediately upstream of the anastomosis. A model of ileocecal resection in IL-10 gene knockout mice has been described which develops inflammation and fibrosis both at the anastomosis site and in other regions of the small intestine [121]. This approach is attractive because it models a major clinical feature of CD fibrostenotic disease and is highly relevant to future clinical trials where therapeutics targeting CD fibrosis will likely be evaluated for prevention of postsurgical recurrence. However, IL-10 null mice do not spontaneously develop small intestinal inflammation and this surgical approach may need to be combined with one of the existing murine ileitis models to achieve the most relevant preclinical model.

Chronic TNBS treatment has been used to induce colitis in mice as well as rats. In the mouse, TNBS with concomitant administration of ethanol as an epithelial barrier disrupter induces intestinal ulceration and inflammation. This model is widely used to investigate acute inflammation in the gut. Chronic TNBS treatment has been tested in an effort to develop a more robust intestinal fibrosis model in mouse [122]. This model has been reported to have some common features with CD including transmural inflammation and stricturing with proximal dilation and fibrosis. Affected animals have increased expression of MMP-1 and collagen type 1. Fibrosis in this model can be enhanced by treatment with indomethacin, a cyclooxygenase (COX) inhibitor which can block the antifibrotic effects of COX-2 [123].

Dextran sulfate sodium added to drinking water is frequently used to induce epithelial injury and acute colitis in mice. Fibrosis with associated increase in collagen, TGFβ, and matrix metalloproteinase expression has been described in C57BL6 mice following a single 5 day cycle of DSS exposure [124]. The authors were also able to show an increase in fibroblasts (V+/A-) and myofibroblasts (V+/A+) cells in the mucosa and submucosa. While this likely reflects a primary intestinal wound healing response rather than the chronic fibrotic process suggested by the authors, it is worth considering if this could be a useful pathway model which might allow rapid testing the effect of therapeutic candidates on specific elements of the wound healing/fibrotic response. Other groups have investigated the effect of multiple cycles of DSS exposure on fibrotic response in FVB-N and C57BL6 mice [125]. A single cycle of DSS exposure in C57BL6 mice does result in ECM deposition followed by mucosal repair and normalized mucosal architecture. Multiple cycles of DSS exposure did not result in enhanced fibrosis in FVB-N mice, however, it did result in prolongation of a fibrotic response in C57BL6 mice as measured by procollagen α1(I) promoter-GFP reporter transgene reporter activity. Further characterization will be needed to determine the utility of this model.

Salmonella species are facultative intracellular gram negative bacteria which cause a range of illnesses including, but not limited to, enterocolitis [126]. *Salmonella enterica* serovar Typhimurium is an enteric bacterial pathogen which normally causes little intestinal pathology in mice but instead mimics human typhoid. However, a model which utilizes oral streptomycin pretreatment has been developed which allows study of *S*. Typhimurium-induced cecal inflammation [127]. This work has been extended by utilizing attenuated *S*. Typhimurium strains or by infecting resistant mouse strains which carry a functional nramp1 gene to induce chronic infection which results in intestinal fibrosis characterized by transmural collagen deposition and accumulation of fibroblasts in the intestinal submucosa [128]. While increase in collagen deposition is observed throughout the colon, the most intense lesions are present in the cecum. The *Salmonella* model of intestinal fibrosis is unique in that it is induced by bacterial colitis. It results in a relatively long term fibrotic process where fibrosis can be observed in the cecal submucosa at least to day 40 post infection. Similar to human CD, increased TGFβ and IGF-1 are associated with fibrosis in this model.

8. Prevention or treatment of CD fibrosis

While considerable progress has been made, the pathophysiology of fibrostenotic disease in CD patients is incompletely understood. The drug development challenges this creates are greatly compounded by the absence of a well defined and widely accepted preclinical animal model of intestinal fibrosis. Recognition of the unmet need for medical interventions which can effectively prevent or treat CD fibrostenotic disease drives ongoing research in both areas. Despite the challenges, a number of potential therapeutic agents or pathways have undergone preliminary testing. A few of these results are summarized below. The data available for all of these agents is quite limited.

Prostaglandins (PGE1 and 2) are known to inhibit smooth muscle proliferation as well as fibroblast proliferation induced by proinflammatory cytokines [129, 130]. Reduced PGE2 levels are associated with development of fibrosis in idiopathic pulmonary fibrosis (IPF) [131] and indomethacin treatment, which inhibits PGE2, increases fibrosis in the chronic murine TNBS model of colon fibrosis. However, mice deficient in prostaglandin endoperoxide synthase (Ptgs) 2, an enzyme involved in prostaglandin production, showed deficient wound healing following full-thickness colonic biopsy so the effects of prostaglandins may be complex and, perhaps, dependent on the stage of wound healing [132]. Phosphatidyl choline, a polyunsaturated fatty acid which is a precursor to PGE2, has been shown to decrease stricture formation in the rat TNBS intestinal fibrosis model [133]. These data suggest a role for PGE2 in intestinal wound healing and fibrosis but the potential for a therapeutic role requires further investigation.

The steroid hormone retinoic acid (RA) is another potential agent for modification of fibrosis in CD. RA has been shown to have effects on human fibroblast proliferation in cells isolated from IPF lungs [134] and to protect against bleomycin-induced pulmonary fibrosis in mice [135]. More recently, RA has been shown to reduce intestinal fibrosis in the chronic TNBS

mouse model of intestinal fibrosis [123]. Much more research will be needed to determine if RA has promise as a fibrosis modifying agent in CD.

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a phytoalexin found in a variety of plant products including berries, peanuts, grapes and red wine. It has been shown to reduce inflammation in rat colitis [136]. Resveratrol has also been shown to reduce activation of NF-kB in TNBS colitis [137]. A recent paper reports that resveratrol exposure results in decreased collagen synthesis as well as apoptosis in rat intestinal smooth muscle cells [138]. While the data on resveratrol is quite preliminary, the data is of interest because it targets smooth muscle rather than the fibroblasts or myofibroblasts.

Anti-inflammatory and anti-fibrotic effects of the cholesterol lowering 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) have been reported. Statins may play an anti-fibrotic role through inhibition of the activation and proliferation of fibroblasts and by inducing apoptosis of activated fibroblasts [139]. Angiotensin type 1 receptor blockers [140] and the angiotensin-converting enzyme inhibitor captopril [141] have also been proposed as fibrosis inhibitors.

Fibrosis has traditionally been considered an irreversible process. Further testing of these and other agents which have potential to block initiation or inhibit progression of fibrosis may also reveal if medical treatment has the potential to reverse existing fibrotic lesions. Research to further characterize the underlying pathophysiologic processes involved in fibrostenotic disease and to test potential therapeutic approaches remains important to the goal of fully meeting CD therapeutic needs.

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