Journal of Crohn's and Colitis, 2016, 377–386
doi:10.1093/ecco-jcc/jjv228
Advance Access publication December 17, 2015
ECCO Scientific Workshop Paper

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Results of the Fifth Scientific Workshop of the ECCO (II): Pathophysiology of Perianal Fistulizing Disease

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Abstract

The fifth scientific workshop of the European Crohn's and Colitis Organization (ECCO) focused on the relevance of fistulas to the disease course of patients with Crohn's disease (CD). The objectives were to reach a better understanding of the pathophysiological mechanisms underlying the formation of CD fistulas; to identify future topics in fistula research that could provide insights into pathogenesis; to develop novel therapeutic approaches; and to review current therapeutic strategies (with clarification of existing approaches to prevention, diagnosis and treatment). The results of the workshop are presented in two separate manuscripts. This manuscript describes current state-of-the-art knowledge about fistula pathogenesis, including the roles of epithelial-to-mesenchymal transition and cytokine matrix remodelling enzymes, and highlights the common association between fistulas and stenosis in CD. The review also considers the possible roles that genetic predisposition and intestinal microbiota play in fistula development. Finally, it proposes future directions and needs for fistula research that might substantially increase our understanding of this complex condition and help unravel novel therapeutic strategies and specific targets for treatment. Overall, it aims to highlight unanswered questions in fistula research and to provide a framework for future research work.

Key Words: Inflammatory bowel disease; Crohn's disease; fistula; epithelial-to-mesenchymal transition; mouse models; cytokines; genetic predisposition; intestinal microbiota; fibrosis

1. Introduction

Most patients with Crohn's disease (CD) are initially diagnosed on the basis of inflammatory pathological changes. At diagnosis, only up to one-third of patients have evidence of a stricturing or penetrating intestinal complication.[1,2] However, in the setting of longstanding and chronically relapsing disease the inflammatory disease phenotype often shifts towards a stricturing and/or penetrating phenotype that is characterized by severe complications such as stenosis or fistulas. About 70% of CD patients suffer from fistula or stenosis and associated intestinal obstruction during their lifetime

and at least 60% of CD patients require surgery at least once within 20 years following their initial diagnosis.[3] Fistulas, mainly perianal, affect between 17 and 50% of CD patients.[4] Previous studies have demonstrated that the extent of disease at diagnosis is associated with fistula development.[4] In contrast, patients with ileitis alone and patients undergoing laparotomy and bowel resection have a reduced risk.[5]

The driving force behind the development of CD-associated fistulas may be the phenomenon of epithelial-to-mesenchymal transition (EMT). This is a physiological process involved in embryogenesis, organ development, wound healing and tissue remodelling, but also plays a major role in pathological processes such as tissue fibrosis and cancer progression.[6,7] It is a mechanism by which epithelial cells lose their essential epithelial-defining properties, including apico-basal polarity and epithelial-specific cell contacts, and gain characteristics of mesenchymal cells, e.g. increased motility and cell spreading.[6] EMT is characterized by down-regulation of epithelium-specific proteins such as E-cadherin and claudin-4 and by up-regulation of mesenchymal proteins such as vimentin.[6]

This review aims to provide a comprehensive overview of current knowledge about fistula pathogenesis, highlighting available data about the molecular pathogenesis of CD fistulas and novel factors that might also play a role. Ideas that might contribute to the identification of future needs and directions in fistula research are explored. These, in turn, might help the development of novel therapeutic strategies.

2. Histopathological assessment and pathophysiology of CD-associated fistulas

2.1. Definition

A fistula (literally a 'pipe') is a tract between two epithelium-lined surfaces. In CD, fistulas affect up to 50% of patients[8,9] and are most often perianal (54% of the total), entero-enteric (24%) or recto-vaginal (9%). Perianal fistulas are not specific for CD. Other causes include infection, hidradenitis suppurativa and malignancy. Tuberculosis can mimic CD, but has a much lower prevalence than CD with regard to fistulas and perianal disease.[10,11] Usually, the cause of fistula development remains unknown.[12]

2.2. Histology

Diagnosis of perianal fistulas depends on clinical assessment.[13] There are classification systems, but these do not require histology.[13] Biopsy, excision of associated skin tags or excision of the fistula may be done to confirm a diagnosis of CD and exclude other aetiologies.[13] The histological features of fistulas are largely non-specific. A fistula tract may be identifiable microscopically, lined by granulation tissue and/or squamous epithelium and typically filled with debris, erythrocytes and acute inflammatory cells.[8] Chronic inflammation and fibrosis are common.

Granulomas may occur in and around perianal fistulas. In a patient with no established cause, they raise the possibility of CD. However, a multinucleate foreign body-type giant cell reaction can occur in any type of fistula and is not specific. Furthermore, a granulomatous reaction could reflect other aetiologies, such as mycobacterial infection, fungal infection, sarcoid or even nearby neoplasia.[12] The granulomas of CD are typically well circumscribed and discrete with relatively few giant cells and no necrosis, but usually cannot be distinguished reliably from non-CD granulomas. Most perianal samples from CD patients do not contain granulomas, even when the disease is established.[13]

Fistulas probably arise as a chronic consequence of an acute inflammatory process with infection and suppuration.[12] For example, a deep penetrating ulcer in the rectum or anus might fill with faecal material that is forced into the underlying tissue by luminal pressure. Anal gland or anal duct abscesses could also serve as a point of origin. The process of tissue destruction may be maintained by luminal antigens and bacteria.

Recently, CD fistulas have been investigated in more detail. In a study of intestinal and perianal fistulas from CD and non-CD patients, 27–31% had a lining of flattened intestinal or narrow squamous epithelium and all were surrounded by granulation tissue. In non-epithelialized areas there was a lining of myofibroblast-like cells (termed 'transitional cells' in later studies), which could form a new basal membrane. Fistulas in CD, but not control fistulas, had areas with disordered myofibroblasts and fragmented basal membrane, raising the possibility that mechanisms of fistula formation in CD differ from those in other settings.[8] However, CD medications might also influence the histology.[8]

Inflammatory cell populations in and around fistulas have been studied. In one report, CD fistulas typically had central infiltration by CD45R0$^+$ T cells, an underlying band of CD68$^+$ macrophages and a dense CD20$^+$ B-cell infiltrate in the outer wall. In contrast, control fistulas typically had a dense macrophage infiltrate and sparse CD20$^+$ B cells or CD45R T cells.[8] In another study, CD4$^+$ CD161$^+$ T cells with a Th17, Th17/Th1 and Th1 phenotype accumulated in CD perianal fistulas.[14]

2.3. Epithelial-to-mesenchymal transition

A process that is characteristic of fistula pathogenesis in CD is EMT, which is a process of transformation from a differentiated epithelial cell to a mesenchymal-type cell.[9] This process is associated with embryogenesis, organ development and wound repair and also with pathological fibrosis, tumour growth and metastasis.[9] It favours the acquisition of the ability to migrate and to penetrate into adjacent layers. In experimental models it confers invasive and metastatic characteristics and a stem cell phenotype on cancer cells.[15] Histologically, tumour budding in colorectal carcinoma [CRC] may reflect EMT and often has negative prognostic value.[16]

In EMT, cells may express epithelial markers, such as cytokeratin 8 and cytokeratin 20, together with mesenchymal markers, e.g. vimentin and smooth muscle actin. They have reduced expression of adhesion molecules, such as E-cadherin, and up-regulation of transcription factors, including SNAIL1 and SLUG.[9] Known inducers of EMT include transforming growth factor (TGF)-β and tumour necrosis factor α (TNF-α). Aspects of the process of EMT may be facilitated by specific factors; e.g. SLUG and β6-integrin facilitate cell invasiveness.

The process of EMT may be involved in the pathogenesis of CD fistulas.[9] In CD fistulas are lined wholly or partly by myofibroblast-like cells/transitional cells (TCs) that express cytokeratins 8 and 20 and probably represent intestinal epithelial cells that have undergone EMT.[17] Features in and around CD fistulas that suggest EMT onset include: lower levels of E-cadherin in TCs than in adjacent epithelial cells; SLUG expression in TCs and in underlying mesenchymal cells; overexpression and nuclear localization of SNAIL1 in TCs; high levels of TGF-β and β6-integrin in TCs and in the zone between TCs and intestinal epithelial cells; and strong expression of TNF-α and its receptor in TCs.[9,17,18]

Other molecules relevant to EMT have also been studied. Interleukin (IL)-13 favours fibrosis, is induced by TGF-β and induces the expression of SLUG and β6-integrin in EMT models and in intestinal epithelium. Ets1 is a transcription factor and proto-oncogene

that mediates the activation of β6-integrin during EMT in an experimental model of CRC. Dickkopf homologue 1 (DKK1) influences cell migration, is a regulator of EMT and has been associated with progression of carcinomas. IL-13, IL-13 receptor-α, Ets1 and DKK1 are highly expressed in TCs along CD fistula tracts, favour EMT and may play a role in CD fistula pathogenesis.[9,19,20]

Nucleotide oligomerization domain (NOD)2 is a receptor for the bacterial cell wall muramyl dipeptide (MDP). NOD2 mutations have been associated with a fistulizing course in CD.[19] Also, MDP stimulates expression of TNF-α, TGF-β, SNAIL1, IL-13 and Ets1 in intestinal epithelial cells and lamina propria fibroblasts from fistulas. These observations support the suggestion that bacteria have a role in CD fistula formation, possibly as a result of EMT.[19]

2.4. Matrix remodelling enzymes

The intercellular matrix is constantly remodelled by a number of enzymes that degrade all components of the extracellular matrix [ECM], namely the matrix metalloproteinases (MMPs). Increased MMP activity eventually results in immune-mediated tissue injury and has been associated with a number of pathologies, such as cancer growth and CD.[21–23] The importance of MMPs for the development of CD is highlighted by the fact that in the murine dextran sulphate sodium-induced colitis model, targeted deletion of MMP-9 has a protective effect,[24,25] while mice overexpressing MMP-9 in the intestinal epithelium develop more severe colitis when compared with wild-type animals.[26] Furthermore, addition of MMP-3 caused extensive tissue injury in an *ex vivo* human foetal model of intestinal inflammation and tissue injury was effectively blocked by inhibiting MMP activity.[27] The physiological inhibitors of MMPs are the tissue inhibitors of MMPs (TIMPs) which are also secreted by the MMP-producing cells.[21]

In CD fistulas, strong MMP-3 expression is observed independently of the stage of inflammation. Expression of MMP-3 mRNA and protein is detected in mononuclear cells and fibroblasts.[28] Furthermore, inactive and active MMP-9 is expressed around CD fistulas and raised mRNA and protein levels are found in granulocytes and fibroblasts.[28,29] The activated isoform of MMP-13 is present in the supernatant of untreated CD fistula colonic lamina propria fibroblasts (CLPFs), but is almost absent in the supernatant of non-fistula CLPFs. Expression of MMP-13 protein is clearly detectable in mononuclear cells around CD fistulas.[9,29] In contrast, expression of MMP-1 and MMP-7 is only weak around CD fistulas, MMP-10 is not detectable and MMP-2 protein is equally expressed in fistula and control tissue. Activated MMP-2 can only be found in CD fistulas.[28] Protein levels of TIMP-1, TIMP-2 and TIMP-3 are low around CD fistulas.[28] These observations suggest a critical role for matrix remodelling enzymes in fistula pathogenesis.

In summary, a possible scenario for CD fistula formation is a genetically triggered, aberrant immune response to bacterial stimuli, including MDP, leading to a severe inflammatory reaction with secretion of TNF-α, IL-13 and TGF-β. TGF-β could then induce factors associated with EMT and cell invasiveness, such as Ets1, SNAIL1 and SLUG, leading to EMT and the production of further molecules associated with cell invasiveness, such as β6-integrin. IL-13 (induced by TGF-β) and its receptor may act in an autocrine manner to favour EMT cell penetration of deeper layers.[30] Other regulatory proteins, such as DKK1, may also participate[9] (Figure 1).

2.5. Fistula-associated neoplasia

Crohn's disease carries an increased risk of gastrointestinal cancer, with a 2- to 3-fold increase in CRC and an increase of up to 30-fold in small bowel cancer.[31] Along with disease extent, disease duration, inflammatory activity and age of onset, the presence of persistent chronic fistulas has been cited as a risk factor for gastrointestinal malignancy.[8] Adenocarcinoma associated with CD fistula, in comparison, is rare.[32] Not infrequently the tumour is a mucinous carcinoma.[33–35] The risk is associated with the duration of perianal fistulizing disease.[31] The tumour may arise directly from the fistula, and occasionally there is dysplasia of the adjacent fistula lining.[36] It has been suggested that adenocarcinomas associated with CD fistulas probably represent anal gland carcinoma.[8] EMT may also be important. In a detailed study of a fistula-associated adenocarcinoma in CD there was aberrant SLUG transcription factor expression in the transitional cells of the fistula, suggesting a role for EMT in carcinogenesis.[31]

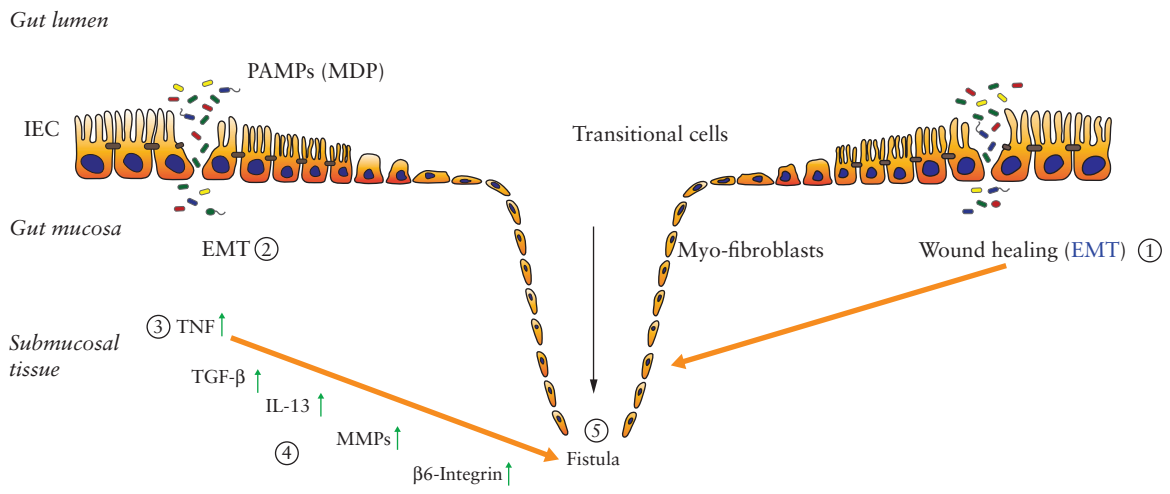

Figure 1. Pathogenesis of Crohn's disease-associated fistulae. Due to an epithelial barrier defect, several pathogen-associated molecular patterns (PAMPs), such as muramyl dipeptide (MDP), are able to enter the gut mucosa. Both the process of wound repair (1) and the inflammatory response caused by PAMPs (2) induce the event of epithelial-to-mesenchymal transition (EMT). First, increased expression of TNF is initiated (3), resulting in up-regulation of TGF-β production. This triggers a signalling cascade of molecules associated with cell invasiveness, such as β6-integrin (4). The enhanced activity of matrix metalloproteinases (MMPs) and the up-regulation of protein expression favour the transformation of intestinal epithelial cells (IECs) towards invasive myofibroblast forms, which results in fistula formation (5).

2.6. Relationship between CD-associated fistulas and hidradenitis suppurativa

Hidradenitis suppurativa (HS) is a chronic disease that most often affects the axilla but may be perianal. Abscesses, sinuses, fistulas and scarring may occur. Histologically, apocrine gland openings contain keratin and debris. CD and HS may occur in the same patient,[37,38] in which case CD usually, but not always, precedes HS.[39] In a study of HS, 10/101 patients had discrete epithelioid granulomas away from the site of inflammation. Of these, one had known CD and one sarcoidosis, and investigations revealed CD in a further two.[40] The remaining seven had no cause other than HS. Foreign body-type granulomas were found in 25%, typically related to ruptured hair follicles, sinus tracts or sweat glands[40].

The anatomical distribution of HS raises the possibility of an apocrine gland disorder, but follicular occlusion is now the favoured cause.[41,42] Infection may play a role, though this is disputed.[43] Occluded, dilated follicles may rupture, with extrusion of keratin and bacteria, an inflammatory response and destruction of pilosebaceous units and other adnexae. Fragments of follicular epithelium might be the origin of sinus tracts.[43] Hormonal imbalance may play a role, but the evidence conflicts; other contributory factors could include free radical production by neutrophils.[44]

There are a few possible similarities between the pathogenesis of HS- and CD-related fistulas. IL-17, the caspase-1 associated cytokines IL-1β and IL-18 and the IL-23/T-helper cell type 17 pathway have been implicated, and the latter may also play a role in CD fistula formation[37,44,45]. In CD patients, a relatively high frequency of CD4+CD161+ T lymphocytes was detected in fistulas and in HS lesions in one report.[46] Release of pro-inflammatory cytokines has been demonstrated in HS, with increased expression of TNF-α (a recognized contributor to the pathogenesis of CD).[44]

3. Genetic predispositions and specific immunological profiles in fistula development

3.1. Role of genetic predisposition to fistula development

In recent years, more than 200 genetic polymorphisms that confer increased susceptibility to (or protect from) the development of CD have been reported.[47,48] Such genetic predispositions influence the likelihood that a single individual will manifest CD and may also help determine the phenotype of each case. Accordingly, several associations between specific genotypes and fistulizing (including perianal) CD have been reported. Variation exists between different genetic studies, as results are commonly dependent upon the characteristics of the population, including ethnic background and age. In addition, the definition of perianal disease is not uniform: in some studies it comprises the full spectrum of anal lesions, whereas in others it is restricted to those with fistulas.

Results from the IBDchip European Project, which included 1528 Caucasian patients from eight major inflammatory bowel disease (IBD) clinics, provided useful insight into the genetic background of patients with fistulizing CD.[49] The risk of developing internal fistulas was significantly associated with carriage of the PRDM1 (PR domain containing 1, with ZNF domain) rs7746082 variant or any NOD2 variant (increased incidence) as well as of the IL-23R rs11465804 variant (decreased incidence). Carriage of variant alleles for ATG16L1 (autophagy-related 16-like 1) and PRDM1 were associated with earlier appearance of internal penetrating disease, whereas the variant allele for IL-23R was associated with delayed appearance. In all, the authors reported that a 'high' genetic risk score, which was computed from the total number of risk alleles for IL-23R, LOC441108, PRDM1 and NOD2 polymorphisms, was associated with a hazard ratio of 1.43 (95% confidence interval 1.16–1.79, $p = 9.64 \times 10^{-04}$ vs 'low' score) for developing a fistulizing phenotype. In this study, the genetic factor PUS10 (pseudouridylate synthase 10) had a significant protective effect against the development of perianal disease. The authors of the study analysed the genetic characteristics of a West European CD cohort and found predictors for both perianal and non-perianal fistulizing disease.[50] A C allele at the CDKAL1 (CDK5 regulatory subunit associated protein 1-like 1) rs6908425 variant and the absence of NOD2 variants were independently associated with perianal fistulas. Non-perianal fistulas were significantly affected by the presence of a T allele at rs12704036, the presence of any NOD2 variant or the IRGM (immunity-related GTPase family M member) rs4958847 G allele. These studies demonstrate that genetic factors that affect the risk of perianal disease are clearly different from those that are associated with internal fistulas.

Several other genetic associations with fistulizing disease have been reported. Schnitzler et al.[51] found a significant association between the NOD2 variant rs72796353 and the development of perianal fistulas, in the absence of the three major NOD2 polymorphisms. In Korean patients the *TNFSF15* (tumour-necrosis factor superfamily of proteins member 15) rs4574921 CC genotype was significantly associated with the development of perianal fistula.[52] Finally, there are a number of reported associations between genetic polymorphisms and perianal disease without separating patients with and without fistula formation. Such polymorphisms have been described for the genes that encode the carnitine/organic cation transporter (OCTN, *IBD5* locus on 5q31), the autophagy-related protein IRGM, the epithelial barrier-associated protein *Drosophila* discs large homologue 5 (DLG5, in paediatric CD) and the neutrophil cytosolic factor 4 (NCF4).[53–56] Despite the differences between individual studies, it should be noted that genetic susceptibility to fistulizing disease appears to follow the general pattern of CD. Indeed, the majority of reported associations involve genes that encode proteins that participate in the interaction between the immune system and microbial factors or regulate the integrity of the intestinal epithelial barrier.

3.2. Cytokine profile in fistula patients

Patients with fistulizing complications constitute a large and well-defined subpopulation of CD. Nevertheless, it remains largely unknown whether this subgroup carries a distinctive immunological phenotype that underlies the formation of fistulas, although the few studies that have addressed this question have raised the possibility that this may be the case. An Italian group published a study of the serum concentrations of pro-inflammatory cytokines in four groups: CD patients with perianal fistulas and inactive luminal disease; patients with active intestinal CD without perianal manifestations; patients with perianal complications after restorative proctocolectomy; and healthy controls.[57] They found that the presence of fistulas correlated with serum elevations of TNF-α and IL-6 but not of IL-12 or IL-1β. The same group also studied the expression of cytokines in the rectal mucosa and reported that mucosal expression of IL-1β and IL-6 was higher in those with perianal CD than in those with small bowel CD and in healthy controls.[57] Local immunostaining for TNF-α and IL-12 has also been performed in resected specimens from patients with CD and non-CD controls. They found up-regulated expression of both proteins in patients with CD, which was

related to the presence of fistulizing complications. These findings demonstrate up-regulated expression of TNF-α in patients with fistulizing disease and are in line with the significant clinical benefit of anti-TNF-α treatment in perianal CD.

3.3. Cytokine profile in fistula tissue

In an effort to determine the local immunological microenvironment, cytokine expression patterns were also studied in the epithelial lining of the fistula tract.[30] Once again, TNF-α appears to be a principal component, as it is not only highly expressed by the lining epithelium but also by immune cells that surround the fistula, as well as by epithelial cells of the adjacent crypts.[18] TNF receptor-1 is also expressed by epithelialized fistulas. IL-13 and its α1 receptor (IL-13R1) are also abundantly expressed in the fistula lining and this is in contrast to the absence of IL-13 expression both in the healthy intestine and in non-fistulizing lesions of IBD, irrespective of the inflammatory state.[9] Finally, TGF-β localizes in the epithelial cells that line the fistula.[17] The presence of these cytokines in the luminal site of the fistula implies their participation in the pathogenesis of penetrating complications in CD. These cytokines interact with each other and they all affect critical mechanisms of fistula formation such as EMT and the function of colonic lamina propria fibroblasts.

4. Mechanisms of fistula and fibrosis development: similarities and differences

4.1. Pathogenesis of intestinal fibrosis

Fibrosis is the result of a dynamic process involving the increased synthesis of matrix components and a failure of physiological mechanisms of matrix turnover. When a tissue or organ is injured a complex healing cascade is triggered. This well-orchestrated process of tissue repair is of great importance for organ homeostasis. However, an imbalance between excessive and insufficient tissue repair will impair organ function. Formation of ulcers and fistulas on the one hand and fibrosis and stricture on the other represent two sides of the same coin.[58] Fibrogenesis is a consequence of local chronic inflammation and is characterized by deposition of excess ECM that is produced by activated myofibroblasts. The time taken for intestinal fibrotic lesions to progress is highly variable and may range from weeks to decades. Deep ulcers or transmural fissures are more likely to result in fibrotic strictures. However, it is difficult to predict which patients will develop a fibrostenosing phenotype and how rapidly this will occur. Also, it is still unclear which factors trigger disease chronicity, and it is not yet known which factors promote the development of intestinal fibrosis.[58–61]

4.2. Fibrosis in CD patients

The theory regarding fibrosis in CD is based on observation of an extreme healing response to injury. This model predicts that tissue injury causes an initial activation of normal intestinal mesenchymal cells, with a shift to a fibrogenic phenotype.[58–60] These cells are characterized by an enhanced ability to trigger ECM synthesis. However, following acute injury the normal intestinal architecture is restored, and some mechanisms prevent the accumulation of ECM while fibrogenic cells are eliminated. In contrast, the mechanisms serving to degrade ECM are not operative at appropriate levels during fibrosis, and fibrogenic cells are not only maintained but increase in number. The mechanisms regulating these effects are unknown but may include factors associated with CD, such as altered cytokine levels and transmural inflammation.[59–61]

Population-based studies may help us to understand the natural history of fibrosis in IBD.[62,63] In a large paediatric CD population-based registry, a French group reported that more than 70% of patients had an inflammatory phenotype at diagnosis. Eight years after diagnosis, 40% of the children developed a stricturing phenotype.[64] In adults, 79% of CD patients had an inflammatory phenotype at diagnosis. Ten years later, 43% had developed intestinal fibrosis.[65] Also, there is evidence that intestinal inflammation is the major driving force for fibrosis in CD.[64,65] Fibrosis in ulcerative colitis (UC) patients is not as severe as in CD, but it can be observed. De Bruyn and colleagues[66] showed no significant difference in collagen deposition, presence of ECM or myofibroblast numbers between acute and longstanding UC. It seems that acute UC inflammation leads to activation of ECM-degrading enzymes, such as MMP and elastase. Moreover, this activation process improves collagenase activity and contributes to degradation of ECM components, including fibronectin and collagen. As a consequence, selected patients with longstanding UC may experience a lead-pipe colon, with a shortened, stiff and narrowed organ with loss of haustration as well as contractile and absorptive function.[66–68]

4.3. Fibrosis in the liver and the lung

The fibrogenesis process is a common pathway in different conditions. Beyond the intestine, fibrosis may develop in e.g. liver and lung, though with different genesis and evolution. Liver fibrosis is a common long-term pathological consequence of different causes, such as viral hepatitis, including chronic hepatitis B and chronic hepatitis C. Due to continuous replacement of normal liver tissue with ECM, liver fibrosis results in progressive distortion of the normal hepatic architecture. These changes can evolve into cirrhosis.[69] The activation of hepatic stellate cells by inflammation stimulates their differentiation into myofibroblasts, including the capacity to induce proliferation and fibrogenesis. Similar to what occurs in intestinal fibrosis, imbalance between the degradation of stimulating factors and ECM results in an abundance of this matrix and consequently in the development of hepatic fibrosis.[69,70] In the lung, the activation and proliferation of fibroblasts by local or systemic inflammatory mechanisms have been associated with changes in the alveolar epithelium resulting in the accumulation of ECM and remodelling of lung architecture, characteristic features of fibrotic lung disease.[71]

An important point in the intestinal fibrogenesis process is the direct activation of myofibroblasts by pro-inflammatory mediators and subsequent ECM production.[58] On the other hand, in liver diseases, hepatic stellate cells play a key role in disease genesis by their extraordinary ability to differentiate into myofibroblasts.[69] Differently, in the lung local as well as systemic fibroblasts are being recruited and induce early activation of epithelial mesenchymal cells, hence resulting in fibrosis.[71] Briefly, activation and differentiation of fibroblast cells by different routes associated with the imbalance between matrix factors and cell stabilizers seem to be central to the pathophysiology of fibrosis in different organs.

4.4. Fistula versus fibrosis development

In contrast to fibrosis and the related strictures, a fistula is defined as a chronic tract between two epithelium-lined surfaces.[68] When perianal disease is the first manifestation of CD, severe disease is likely and there may be rapid progress from inflammatory manifestations to stricturing or penetrating complications.

Interestingly, there seem to be pathophysiological mechanisms that contribute to the development of both fibrosis and fistulas in IBD

patients. EMT, which seems to be the driving force for fistula development, also plays a key role in the development of fibrosis in CD.[7,72] Furthermore, aberrant activation of matrix remodelling enzymes such as MMP-3 and MMP-9, as well as of cytokines and growth factors such as TGF-β and IL-13, are important for fibrosis as well as fistula development.[7,9,17,28] However, although those factors contribute to the development of both fibrosis and fistulas, it is not clear why stenosis occurs in some patients and fistulas in others. Notably, fistulas are often surrounded by fibrotic tissue, suggesting that these disease manifestations can exist together and might originate from similar events. However, the additional mechanisms that need to occur in CD patients to favour fistula development over fibrosis require further investigation. In addition, it is important to understand why fibrosis and fistulas are a common complication in CD patients but not in UC.

5. Role of microbiota in fistula and fibrosis development: similarities and differences

5.1. Intestinal microbiota in IBD

The role of microbiota in IBD has gained increasing attention in recent years. Previously, there was clear evidence that the intestinal microbiota play a crucial role in mediating disease, mainly in the form of absent inflammation in germfree mice in models of experimental colitis.[73] Furthermore, early clinical studies indicated that faecal stream diversion can ameliorate human IBD.[74] In recent years, it has been demonstrated that microbial diversity increases in 'intestinal' health but decreases in inflammation.[75,76] There is evidence from controlled clinical studies that the transplantation of microbiota might become a therapeutic strategy to ameliorate inflammation, but many questions need to be answered before this approach is considered in practice.[75,76] While the impact of microbiota on inflammation has been studied, their role in fistula and fibrosis development has scarcely been addressed. Here we aim to summarize the available data for IBD as well as for other diseases.

5.2. Intestinal microbiota in fibrotic disease

When different organs are compared we find that there are substantially more data on fibrosis than on fistula development. Ligands to toll-like receptor (TLR) 4 or TLR2, predominantly Gram-negative and -positive bacteria, activate the NFκB (nuclear factor κ-light-chain-enhancer of activated B cells) pathway leading to the secretion of cytokines and chemokines by intestinal mesenchymal cells, thus contributing to the production of pro-fibrotic factors.[77] In addition, experimental models, including SAMP1/YitFc and IL-10-deficient mice as well as benzene sulphonic acid (TNBS)- and peptidoglycan-polysaccharide (PG-PS)-induced colitis, indicate that not only are the intestinal microbiota required for intestinal inflammation but that they also perpetuate gut fibrosis.[73] Activated mesenchymal cells can be considered as major driving factors for intestinal fibrosis, since these cells produce several collagen types, including types I, III and V,[59–61] as well as ECM products such as fibronectin and tenascin C.[78,79] The deposition and formation of networks ultimately contribute to tissue stiffness, which subsequently activates further mesenchymal cells in a positive feedback loop, hence increasing fibrosis development.[80] TGF-β1 can be considered as a central mediator in this context.[81] The majority of these core mechanisms are shared between the human intestine and other organs, such as the liver, lung, kidney and skin.[82] However, there are also organ-specific differences. For instance, TNF-α is a central pro-inflammatory mediator in IBD and several anti-TNF-α antibodies have been approved for treatment of CD as well as UC.[79–82] In other organs, including liver and lung, and in systemic sclerosis, anti-TNF-α treatment seems to mediate an anti-fibrotic effect.[83–85] Mechanistically, this can be explained by a TNF-α-mediated activation of mesenchymal cells resulting in increased TIMP-1 expression and parallel inhibition of MMP-2 activity and collagen degradation.[86] Strikingly, human intestinal myofibroblasts isolated from CD patients exhibit increased production of TIMP-1 and decreased collagen production upon exposure to infliximab.[87]

5.3. Intestinal microbiota in fistulizing disease

Having outlined the impact of microbiota on fibrosis, the question arises of their potential role in fistula formation. In older studies the contribution of the microbiota to perianal fistulas of cryptoglandular origin was investigated, and pathogenic bacteria were not identified.[88–90] Furthermore, only a limited number of bacteria were found. From this the authors concluded that permanent infection is not a major contributing factor to the persistence of fistulas. From today's point of view these conclusions are questionable since conventional culture techniques were applied. Hence these analyses require repetition and the application of modern molecular techniques that are independent of the viability of the bacteria.[91,92] A recent study addressed this deficit and assessed the distal part of surgically removed fistula tracts, analysing fistula content by culture and 16S rRNA gene sequencing. In addition, the presence of the pro-inflammatory peptidoglycan was investigated using immunohistochemistry.[93] The bacterial species identified were bowel- and/or skin-derived and no mycobacteria were identified. Sequencing of 16S rRNA failed to detect bacteria except in one sample, probably due to a low number of organisms. However, peptidoglycans were detected in 90% of patients. The authors concluded that peptidoglycan contributes to the ongoing inflammation in perianal fistulas. Therefore, additional studies are required, applying novel sequencing strategies that will allow the analysis of even small sample sizes. Additionally, Karban and colleagues[94] studied the microbiology of the fistula tract of CD patients. They aspirated and analysed pus from the tract using standard culture techniques. The authors found a predominance of Gram-positive (staphylococci and streptococci) over Gram-negative bacteria, in contrast to perianal fistulas of cryptoglandular origin (not CD-associated fistulas), which preferentially have bacteria of gastrointestinal origin.

6. Defining future directions in fistula research

6.1. Lack of mouse models in fistula research

To date, defining the pathophysiology and pathogenesis of CD-associated fistulas has been a relatively neglected area of research. Our current knowledge about the development of fistulas derives from descriptive immunohistochemical data and from *in vitro* and *ex vivo* cell model experiments. Additional clinical observations are certainly helpful, but are not sufficient to unravel the mechanistic and/or molecular processes that result in fistulas in CD patients.

To obtain a better understanding of the complex aetiology of fistulas, the efforts of a larger number of research groups may be needed. A major drawback of fistula research is the fact that no reliable and reproducible animal model – the mouse in particular – of intestinal fistulas exists. Current colitis models, such as the dextran sodium sulphate colitis model, the TNBS (2,4,6-trinitrobenzenesulphonic acid) colitis model, the T-cell-transfer colitis model and the IL-10 knockout colitis model, do not develop fistulas. This prevents investigators from obtaining sufficient *in vivo* data for unravelling fistula pathogenesis, and in turn developing novel fistula therapy strategies.

6.2. SAMP1/Yit mice

So far, only a few mouse models that might develop intestinal fistulas have been described. The first model is the SAMP1/Yit mouse. After more than 20 generations of brother–sister mating the incidence of perianal disease within the described colony was 4.8% and involved predominantly young female mice at an age between 3.5 and 24 weeks. The male:female ratio was 1:6.7. However, no phenotypic differences in perianal lesions were observed between the sexes. In this model, inflammation is characterized by mucosal ulceration of the anal canal, with fissures that occasionally extend 1 or 2 cm from the anal orifice. Increased submucosal chronic inflammatory infiltrates (lymphocytes, plasma cells and macrophages) in the anal and rectal canals as well as accumulation of neutrophils in the perianal soft tissue, which occasionally coalesce to form perirectal abscesses, can be observed in the affected animals. Anocutaneous fistulas, documented by insertion of a probe and by histological sections, comprise granulation tissue-lined tracts connecting the anus to the lateral skin. Multiple fistulas are not observed and the progression of perianal inflammation to fistulas is observed in 40% of mice, in which superficial perianal ulceration appears to develop into fistulous tracts.[95] However, this demonstrates that this model can be used neither on a routine basis for studying fistula development nor for testing novel fistula therapies.

6.3. Atg7/Xbp1ΔIEC mice

A further novel model is the *Atg7/Xbp1*$^{\Delta IEC}$ mouse, which feature loss of Atg7 and Xbp1 in intestinal epithelial cells. More than 70% of these animals develop discontinuous submucosal or transmural inflammation, characterized by acute and chronic inflammation extending in an abrupt knife-like fashion to the muscularis propria and serosa, closely resembling the fissuring ulcers and fistula tracts observed in human CD. Interestingly, all animals exhibited submucosal or transmural disease in this study after 18 weeks.[96] However, this model needs further validation with respect to fistula development before it can be used on a routine basis.

6.4. Other mouse models

Several surgically induced fistula models have been developed. Unfortunately, these models, which also included rats and dogs, were mostly not feasible because of severe side effects, infections and perioperative mortality. However, one of the mouse models seemed to be usable. In these animals, a caecostoma was created surgically. Although the operation was easy to perform, and although neither spontaneous closure of the fistula nor premature death occurred, this mouse model of an enterocutaneous fistula was not subsequently followed up.[97] Interestingly, in contrast to intestinal fistula models, there are several mouse models for other types of fistula, such as arterio-venous and trachea-oesophageal fistulas.[98–100] Given the importance of *in vivo* models for the investigation of pathogenetic features and the mechanisms underlying certain diseases, it is obvious that the development of a reliable and reproducible *in vivo* fistula model would be of great value.

6.5. Morphological characterization of fistulas

In the absence of a reliable mouse or animal model, *in vitro* and *ex vivo* experiments gain even more importance. For a better understanding of the pathogenesis of CD fistulas, it is critical to define the histological characteristics of CD fistulas and, in particular, the ways in which they differ from non-CD fistulas. Also, more knowledge about possible differences between actively inflamed CD fistulas, CD fistulas after treatment and chronically persisting CD fistulas without signs of acute inflammation would be useful. In addition, more details about the specific cell types and characteristics of the cells along, as well as surrounding, the fistula tracts are required. Though we have studied certain properties of colonic lamina propria fibroblasts derived from CD fistulas, there is relatively little knowledge about functional properties of intestinal epithelial cells, transitional cells, immune cells, etc. in and around fistulas.

6.6. MicroRNAs

On a molecular level, recent data suggest that microRNAs (miRs) play a pivotal role in IBD pathogenesis. MicroRNAs are small non-coding RNAs with a length of 18–23 nucleotides that act as post-transcriptional regulators of gene expression.[101] Schaefer and colleagues[102] have demonstrated that microRNA signatures can distinguish CD from UC and have suggested that microRNAs could contribute to CD pathogenesis. Furthermore, microRNAs seem able to classify different disease behaviour phenotypes in CD and might even have prognostic impact. Of particular interest, the expression levels of miR-215 in index biopsies of CD patients might predict the likelihood of progression to penetrating/fistulizing CD.[103] MicroRNAs represent an emerging field in IBD research, and accordingly it might be interesting to evaluate their role in fistula pathogenesis.

6.7. Intestinal microbiota

As described above, the intestinal microbiota might contribute to fistula pathogenesis in IBD patients. To date, knowledge about microbiota composition within fistula tracts and about possible differences between fistulized and non-fistulized areas in individual patients, as well as differences between patients with fistulas and those without, is almost completely lacking. Therefore, one of the major goals for future fistula research should be to investigate the composition of such microbiota, their effects on intestinal cells, and their relevance to pathogenesis. This might allow the identification of microbiota-based strategies for fistula treatment.

7. Conclusion

In summary, fistulas represent a severe and still unresolved problem in the management of CD patients. Current knowledge about fistula pathophysiology is still poor. It appears that CD fistulas develop as a result of EMT, probably in areas with chronic ongoing inflammation. Furthermore, fistula-associated cells need to acquire markers associated with cell invasiveness that then contribute to the development of invasive fistula tracts. Emerging evidence suggests that a specific immune cell and cytokine profile can be detected around CD fistulas and that genetic factors as well as intestinal microbiota might also be involved

A drawback in unravelling fistula pathogenesis and the development of novel fistula therapies is the absence of a suitable animal model. Therefore, future research should be directed towards the generation of such an *in vivo* model to allow fistula research in real-life circumstances. Additionally, better characterization of fistulas is needed, with more focus on the effects of microRNAs and intestinal microbiota. The aim would be the identification of the driving forces for fistula development, fistula progression and, perhaps, fistula closure. This might in turn facilitate the development of new and more effective therapeutic strategies for the treatment of patients suffering from CD fistula.

Funding

No study sponsors had any involvement in study design, data collection, interpretation or writing of the manuscript.

Conflict of Interest

BS has served as consultant for Abbvie, Janssen, Hospira, MSD, Mundipharma and Takeda and has received speaker fees from Abbvie, Falk, Ferring, Hospira, MSD and Takeda. RMF has nothing to disclose. GB has received lecture honoraria from Takeda, MSD and Abbvie. JCL has provided consultancy and received payment for lectures from Abbvie, Janssen and Takeda. FVT has provided consultancy and received payment for lectures from Abbvie, Janssen, Ferring, Nestlé and Hospira. GR has provided consultancy for Abbot, Abbvie, Augurix, Boehringer, Calypso, FALK, Ferring, Fisher, Genentech, Essex/MSD, Novartis, Pfizer, Phadia, Roche, UCB, Takeda, Tillots, Vifor, Vital Solutions and Zeller; received speaker's honoraria from Astra Zeneca, Abbott, Abbvie, FALK, MSD, Phadia, Tillots, UCB and Vifor; and received educational grants and research grants from Abbot, Abbvie, Ardeypharm, Augurix, Calypso, Essex/MSD, FALK, Flamentera, Novartis, Roche, Takeda, Tillots, UCB and Zeller. MS has received speaker's honoraria from FALK.

Author Contributions

This article is a result of a joint scientific workshop activity. Hence, all authors participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, data interpretation and writing of the article. The final version of the article was approved by all authors.

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