Smad7 Sustains Inflammation in the Gut: From Bench to Bedside

Irene Marafini, Silvia Sedda, Davide Di Fusco, Michele M Figliuzzi, Francesco Pallone and Giovanni Monteleone

Department of Systems Medicine, University of Rome Tor Vergata, Italy

Corresponding author: Giovanni Monteleone, Department of Systems Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy, Tel: +39.06.72596158; Fax: +39.06.72596391; E-mail: Gi.Monteleone@Med.uniroma2.it

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Abstract

In Crohn’s disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel disease (IBD) in human beings, the pathological process is driven by an excessive immune response that is directed against components of the luminal flora and inappropriately controlled by immunosuppressive mechanisms. One such a mechanism involves TGF-β1, a pleiotropic cytokine that targets both immune and non-immune cells in the gut. TGF-β1 is highly expressed in inflamed mucosa of IBD patients but paradoxically it is unable to activate Smad-associated intracellular signalling and suppress inflammatory cytokine responses. This is because IBD-related inflammation is marked by elevated levels of Smad7, an inhibitor of TGF-β1 signalling. Consistently, knockdown of Smad7 with a specific antisense oligonucleotide restores TGF-β1 function, inhibits inflammatory cytokine production, and ameliorates colitis in mice. In this article we review the available data supporting the pathogenic role of Smad7 in gut as well as the results of a recent phase 1 trial assessing the safety and tolerability of a Smad7 antisense oligonucleotide in CD patients.

Keywords: Smad7; Gut; Inflammation

Introduction

Crohn’s disease (CD) and Ulcerative Colitis (UC) are the major chronic inflammatory bowel diseases (IBD) in humans. CD generally involves the terminal ileum and colon, but it can present anywhere in the alimentary tract, from the mouth to the anus, with inflammatory lesions that are often transmural and discontinuous. UC involves the rectum, and the inflammation typically confined to the mucosa or submucosal layers may extend proximally in a continuous pattern thus affecting part of the colon or the entire colon [1].

The aetiology of both CD and UC is unknown, but the rapid advancement of molecular techniques and the possibility to use several models of colitis have led to a better knowledge of mechanisms that orchestrate the tissue-destructive inflammatory response in these disorders. The most accepted hypothesis is that IBD result from the interaction between genetic and environmental factors that eventually leads to an exaggerated mucosal immune response directed against luminal bacteria [1-6]. This process seems to be facilitated, or at least perpetuated, by defects in counter-regulatory mechanisms. Indeed, mutations in genes encoding for the suppressive cytokine IL-10 or IL-10 receptor (R) are associated with paediatric forms of IBD characterized by early and aggressive course [7,8] and lack of IL-10 in mice favours the development of bacteria-driven colitis [9-11]. Along the same line is the evidence that mice deficient in transforming growth factor (TGF)-β1, a pleiotropic cytokine with potent immunoregulatory properties, develop a multifocal immune-inflammatory disease, also involving the colon [12]. Gut inflammation can occur in transgenic mice that express a functionally inactive form of TGF-βR II on T cells and therefore are unable to respond to TGF-β1 [13]. Moreover, in murine models of colitis, neutralization of TGF-β1 leads to severe colitis while the presence of functional TGF-β1 is associated with either complete protection from the development of colitis or reduced severity of colitis [14,15].

Initial studies aimed at characterizing the expression of TGF-β1 in IBD showed elevated levels of RNA transcripts in inflamed mucosa of patients with CD and patients with UC as compared to uninfamed mucosal areas of the same patients and normal controls [16], thus excluding the possibility that defects in TGF-β1 production could contribute to sustain the IBD-related detrimental immune response. In contrast, a large body of evidence indicates that IBD are marked by disruption of TGF-β1 signalling due to elevated levels of the intracellular inhibitor, Smad7 [17].

In this article we review the data supporting the pathogenic role of Smad7 in gut and discuss the benefits and risks of the use of Smad7 inhibitors in CD patients.

TGF-β1-Associated Smad Signalling in the Gut

Within the gut mucosa of healthy individuals, several cell types [i.e. epithelial cells, macrophages, regulatory T cells (Tregs), myofibroblasts, and mast cells] can both produce and respond to TGF-β1 [12,18]. TGF-β1 is secreted as part of a latent complex, which includes latent TGF-β binding protein and latency-associated peptide (LAP), and in this form it cannot bind to its receptor [19]. TGF-β1 can be activated upon being released from the complex through the proteolytic action of a number of proteinases or upon the interaction between the tripeptide integrin-binding motif on LAP and the corresponding binding sequence on αvβ3, αvβ5, αvβ6 or αvβ8 integrins, expressed on the surface of epithelial cells, dendritic cells and myofibroblasts [20,21]. Active TGF-β1 binds the subunit II of its trans-membrane heterodimeric serine/threonine kinase receptor, thus promoting activation of subunit I, which in turns allows the phosphorylation and activation of the intra-cellular proteins Smad2 and Smad3 [22]. Once activated, Smad2 and Smad3 form a complex with Smad4 that eventually translocates to the nucleus where it regulates transcription of a wide spectrum of target genes (Figure 1) [22,23]. Several observations support the role of TGF-β1-associated Smad3 signalling in the maintenance of intestinal immune
homeostasis. First, elevated levels of phosphorylated form of Smad3 (p-Smad3) are seen in intestinal biopsy samples and lamina propria mononuclear cells (LPMCs) of normal individuals [24]. Blockade of TGF-β1 in normal colonic LPMC and biopsies with a specific anti-TGF-β1 antibody also up-regulates expression of inflammatory markers (i.e., T-bet, IFN-γ) while treatment of the same samples with exogenous TGF-β1 is accompanied by enhanced expression of p-Smad3 and suppression of inflammatory pathways (e.g., TNF-driven NF-kB activation) [25]. Mice lacking Smad3 die early in life and Smad3 mutation associates with the development of inflammatory lesions in colon and many other organs [26]. Finally, in inflamed gut of patients with CD and patients with UC, p-Smad3 expression is markedly reduced [24] and treatment of IBD LPMC with TGF-β1 up-regulates expression of inflammatory cytokine inhibition [25].

TGF-β1 also activates the small GTPase Ras, phosphoinositide 3-kinase, and mitogen-activated protein kinases such as ERKs, p38, and c-Jun N-terminal kinases, which may interact with Smad proteins and ultimately modulate the effects of the cytokine [22,27,28]. However, the contribution of such molecules in the control of gut inflammation is not yet known.

**IBD-Related Inflammation is Associated with High Smad7**

The fact that TGF-β1-associated Smad signalling is impaired in IBD led us to investigate the factors/mechanisms involved in such a defect. We focused our attention on Smad6 and Smad7, two inhibitors of TGF-β1-induced Smad3 phosphorylation [22,23]. By Western blotting of total proteins extracted from IBD and control samples, we showed that Smad7 but not Smad6 was up-regulated in involved mucosa of patients with CD and patients with UC [24]. Smad7 competes with Smad2 and Smad3 for the binding to TGF-βRI, thereby blocking their phosphorylation [23]. Smad7 inhibits TGF-β1 signalling also by recruiting Smurf-containing E3 ubiquitin ligase, which in turn degrades TGF-βRI, and by interacting with growth arrest and DNA damage protein, a regulatory subunit of protein phosphatase 1, resulting in TGF-βRI dephosphorylation [29,30].

In IBD mucosa, both T cells and non-T cells express elevated levels of Smad7 [24]. Recent data from our laboratory indicate also that non-immune cells, such as epithelial cells, are an additional source of this protein in inflamed mucosal areas of IBD patients (personal unpublished observations). The exact mechanism that regulates Smad7 expression in the single cell types remains to be clarified. Initial studies with cell lines showed that Smad7 can be positively regulated by inflammatory cytokines (e.g., IFN-γ, TNF-α, IL-7) or TGF-β1 itself at transcriptional level [24]. However, blockade of each of such cytokines in cultures of IBD LPMC with neutralizing antibodies did not affect Smad7 expression [24]. Moreover, no significant change in Smad7 RNA transcripts was seen between IBD and controls [31]. Smad7 also undergoes dynamic post-translational modifications, which make the protein resistant to proteasome-mediated degradation in the cytoplasm. In particular, the stability of Smad7 is controlled by competition between acetylation and ubiquitination, which compete for the same lysine residue. Because such residues are targeted by Smurf-mediated ubiquitination, their acetylation prevents the ubiquitination and protects Smad7 protein against proteasomal degradation. Interestingly, in IBD mucosa, Smad7 is acetylated and not ubiquitinated, and this phenomenon appears to be mainly related to the activity of the transcriptional coactivator p300, which interacts with Smad7 and leads to its acetylation [31]. P300 is over-expressed in IBD samples and its knockdown associates with a marked down-regulation but not normalization of Smad7 levels [31]. This later finding raises the possibility that additional factors can contribute to stabilize Smad7 in IBD. In this context, for example, it is known that Smad7 is regulated by SIRT1, a component of the mammalian Sirtuin family proteins. SIRT1 deacetylates the lysine residues of Smad7 and additional proteins involved in the progression of immune-inflammatory processes, such as the transcription factors Stat3, NF-kB p65, and c-Jun [32,33]. Interestingly, SIRT1 expression is markedly reduced in IBD tissue, particularly in cells which express high Smad7 [34].

![Figure 1: Schematic illustration showing the TGF-β1-associated Smad pathway in normal (A) and inflamed (B) intestine.](image-url)
Up-regulation of Smad7 also occurs in Helicobacter Pylori-related gastritis but not in celiac disease mucosa [35,36].

The Pathogenic Role of Smad7 in the Gut

As Smad7 is a negative regulator of TGF-β1 and this cytokine has immune-regulatory properties in the gut, we planned to clarify the role of Smad7 in the control of intestinal inflammation by using two different approaches. Initially, Smad7 was inhibited with a specific antisense oligonucleotide in culture of IBD LPMC and mucosal explants. Knock-down of Smad7 increased p-Smad3 and markedly reduced TNF-α and IFN-γ production [24]. All these effects were suppressed by a neutralizing antibody against TGF-β1, indicating that the anti-inflammatory effects seen in cultures treated with Smad7 antisense oligonucleotide were mediated by TGF-β1. TGF-β1 signalling is also required for the function of Foxp3-expressing Tregs, a subset of T cells that plays a key role in the maintenance of the gut immune homeostasis [37,38]. Notably, effector CD4+ T cells isolated from the gut mucosa of IBD patients, but not those isolated from controls, were resistant to Treg-mediated suppression. This phenomenon was, at least in part, because of high Smad7, since knockdown of Smad7 restored the responsiveness of colitogenic CD4+ T cells to Foxp3-expressing Tregs [39]. TGF-β1 is also over-produced in the inflamed colons of mice with both trinitrobenzene sulfonic acid (TNBS) and oxazolone-mediated colitis, two experimental models that exhibit immunological similarities with CD and UC, respectively [40,41]. In both these models, p-Smad3 was reduced in the colon, while Smad7 was increased. Oral administration of Smad7 antisense oligonucleotide reduced Smad7, enhanced p-Smad3 expression in the colon of mice with either TNBS- or oxazolone colitis, thereby leading to inhibition of inflammatory cytokine production and attenuation of colitis [42].

More recently we generated a T cell-specific Smad7 transgenic (tg) mouse on C57B6 genetic background. Smad7 tg mice do not develop spontaneously any sign of colitis over a period of 8 weeks. By using the T cell-transfer model of colitis, we showed that the adoptive transfer of Smad7 tg naïve CD4 T cells, in the absence of Tregs, produced a more pronounced synthesis of IFN-γ and severe colitis than those seen in mice reconstituted with wild-type cells [39]. The tendency of Smad7 tg mice to develop a Th1-mediated colitis was evident even when mice were co-transferred with Tregs, which are known to suppress T cell-transfer colitis [39].

The susceptibility of Smad7 tg mice to colitis was also investigated by treating mice with 3 cycles of dextran sodium sulphate (DSS) to mimic human chronic-relapsing colitis. Smad7 tg mice developed a more severe colitis as shown by endoscopic view of the colons, histologic analysis of colonic specimens and evaluation of inflammatory cytokine production [43]. Altogether these results suggest that selective overexpression of Smad7 in T cells renders these cells resistant to Treg-mediated suppression with the downstream effect of enhancing the synthesis of pathogenetic cytokines.

From Bench to Bedside: A Phase I Clinical Trial of Smad7 Antisense Oligonucleotide in Patients with Active Crohn’s Disease

A pharmaceutical compound containing the specific Smad7 antisense oligonucleotide has been recently developed. The antisense oligonucleotide is formulated as a solid oral dosage form protected by an external coating that determines gastro-resistance and allows the compound to reach the terminal ileum and right colon. To determine whether this compound was safe, a phase 1 clinical, open-label, dose escalating study was conducted in patients with active, steroid dependent/resistant CD [44]. Fifteen patients were enrolled and divided in three cohorts receiving once a day for 7 days the compound at doses of 40 mg, 80 mg and 160 mg. The treatment was well-tolerated and no serious adverse events (AE) occurred. Twenty-five AE were documented in 11 patients, but most of them were mild and not related to treatment. Treatment was associated with a marked reduction of IFN-γ and IL-17A-producing CCR9-positive T cells in the blood, a phenomenon that could be therapeutically relevant as CCR9-positive T cells represent a subset of inflammatory T cells with gut homing properties, which are increased in the active phases of CD [44,45]. Moreover, a rapid clinical improvement was seen in all the patients.

Since TGF-β1 is pro-fibrogenic in many organs [46,47] all patients receiving Smad7 antisense oligonucleotide were closely monitored for the development of small bowel strictures through Small Bowel Intestine Ultrasonography over a period of 6 months [48]. No patient developed small bowel strictures or experienced obstructive symptoms during the study. Moreover, no significant change in serum markers of fibrosis, like basic fibroblast growth factor (bFGF), human chitinase 3-like1 (YKL-40), was seen following treatment [48].

Conclusions

The pre-clinical data discussed in this article suggest that high Smad7 in intestinal LPMC contributes to sustain detrimental signals in the gut and raise the exciting possibility that resolution of IBD-related inflammation might be accomplished by enabling endogenous immunosuppressive mechanisms to function. Indeed, knockdown of Smad7 allows the abundant TGF-β1 in inflamed tissues to become functional and suppress effector cytokine production [24]. This hypothesis is supported by the preliminary results emerging from the phase 1 clinical trial showing that treatment of CD patients with oral compound containing Smad7 antisense oligonucleotide is safe and associates with clinical improvement [44]. Phase 2 and 3 clinical trials will be necessary to confirm such data. Meanwhile, some key issues remain to be addressed. For example, we do not know whether, in IBD, there is a cell-specific regulation of Smad7 and which factors/mechanisms account for high Smad7 in specific cell types. While there is no doubt that over-expression of Smad7 in immune cells sustains inflammatory signals, it is conceivable that the pathogenic role of Smad7 in the gut may also rely on its ability to suppress TGF-β1 activity in non-immune cells. In particular, elevated levels of Smad7 in IBD epithelium could inhibit the ability of TGF-β1 to promote epithelial cell margination and hence mucosal healing [49]. Further experimentation needs to clarify the exact role of Smad7 in colitis-associated colorectal cancer (CRC). In this context, we have recently shown that Smad7 tg mice are less susceptible than wild-type mice to develop colitis-associated CRC [43], and this phenomenon seems to be related to enhanced production of TGF-β1 in the colon of Smad7 tg mice and ability of this cytokine to kill colonic epithelial cells [50]. However, Smad7 expression is up-regulated in both human sporadic and colitis-associated CRC tissue and inhibition of Smad7 reduces, rather than enhances, human CRC cell growth and survival [50,51]. Future clinical trials will also be useful to determine whether inhibition of inflammatory pathways by Smad7 antisense oligonucleotide associates with enhanced risk of opportunistic...
infections given the central role of effector cytokines in controlling the host response against bacteria and viruses [52].

Declarations of Personal Interests

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