Interaction between sphingosine kinase/sphingosine 1 phosphate and transforming growth factor-β/Smad pathways in experimental intestinal fibrosis. An in vivo immunohistochemical study

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Introduction

Intestinal fibrosis is described as an uncontrolled accumulation of Extracellular Matrix (ECM) proteins and represents a common and progressive response to chronic inflammation, characterized by the rearrangement of normal tissue architecture, leading eventually to strictures, stenosis and intestinal obstruction, particularly in Crohn Disease (CD). Crucial effector cells of this process are activated myofibroblasts, derived not only from resident mesenchymal cells (fibroblasts, sub-epithelial myofibroblasts and smooth muscle cells) but also from epithelial and endothelial cells via a process of Epithelial-Mesenchymal Transition (EMT)/Endothelial-Mesenchymal Transition (EndoMT).1,2 Several lines of evidence suggest that EMT is a crucial mechanism in the fibro-proliferative processes.3 Several biological mediators have been implicated in the activation of ECM producing cells, including cytokines, chemokines, growth factors, angiogenic factors and products of oxidative stress. Among these, Transforming Growth Factor-β (TGF-β) plays a central role in sustaining and amplifying tissue remodeling and fibrosis. Previous studies have demonstrated that several anti-fibrotic molecules directly interact with the TGF-β/Smad pathway to modulate intestinal fibrosis.4,5 Although TGF-β signaling is dependent on Smad family protein activity, other molecules, including those that regulate cytoskeletal function, have been implicated in fibrosis. Members of the Rho-GTPase family, in particular RhoA, play a fundamental role in TGF-β-induced α-Smooth Muscle Actin (α-SMA) expression and EMT.1,2 TGF-β also activates the Phosphoinositide 3-Kinase (PI3K)/protein kinase B (AKT)/mammalian Target of Rapamycin (m-TOR)-dependent pathway, deregulated of which contributes to chronic fibrosis in several fibro-proliferative diseases.6

In addition, recent reports also demonstrate a connection between the TGF-β/Smad and sphingosine 1 Phosphate (S1P) pathways in stimulating excessive ECM production in many organs.10 S1P is a ceramide-derived lipid messenger of the sphingolipid family, the synthesis and stabilization of which involves structural membrane components. Sphingosines are bioactive cytosolic lipids, deregulation in the synthesis and degradation of which is toxic and characterized by membrane damage or modification.11

Under normal conditions, cells control cytosolic sphingosine accumulation through the balanced action of S1P-lyase (SPL)-mediated degradation and specific S1P phosphorylate.12 SPL is a crucial regulator of intracellular S1P levels and S1P phosphorylation is catalyzed by Sphingosine Kinase 1 (SPHK1) and Sphingosine Kinase 2 (SPHK2) that exhibit ubiquitous expression but differences in subcellular localization and biochemical properties.13 S1P exhibits both intracellular and extracellular functions: it acts as a second messenger, interfering with intracellular pathways involved in variety of cellular processes but also binds specific S1P1, S1P2, S1P3, S1P4, S1P5 cell surface receptors, involved in cell growth, differentiation, survival, motility, inflammation and fibrosis,12,14 each inducing a specific cellular response to S1P binding.14
through which crosstalk between S1P and SPHK1 and TGF-β signaling results in organ fibrosis.10 In idiopathic pulmonary fibrosis (IPF), S1P directly stimulates SPHK1, Akt and AMPK, resulting S1P production.17 Interaction between S1P/SPHK1 and TGFβ-1 signaling pathways induces the transition of alveolar epithelial cells into myofibroblasts resulting in the expression of fibronectin and α-SMA and progression of lung fibrosis.11 A link between S1P and TGFβ-β-associated fibrosis has also been demonstrated in the cardiac muscle, in which TGF-β stimulates SPHK1, inducing S1P production and activation of cardiac fibrosis via the S1P2 receptor.18 Furthermore, the paracrine/autocrine action of S1P, produced by SPHK1 in the intracellular compartment and released into the extracellular compartment, promotes TGFβ-induced cardiac fibroblasts to myofibroblasts transformation.20 Previous reports have also associated ECM accumulation and renal fibrosis with activation of the SPHK1/SIP and TGFβ pathways.21 Furthermore, diabetic kidney and mesangial cells grown under high glucose conditions exhibit augmented expression of the S1P2 receptor, implicating the S1P-SIP2-MAPK axis in fibronectin overexpression.13 SIP receptors are also expressed by liver and stimulate hepatic stellate cells proliferation and contractility in vitro.22 A potential role for SIP and SIP receptors in liver fibrosis and regeneration following carbon tetrachloride (CCl4) hepatic injury has also been reported in SIP2 knockout mice, characterized by reduced hepatic myofibroblasts numbers and proliferation in response to SIP.22 We hypothesized that the TGFβ-Smad3 pathway, SPHK1, RhoA, PI3K, Akt, p-Akt, and p-mTOR may act as a complex signaling network, with extensive crosstalk and strong effects on intestinal fibrosis. Therefore, in this study, we evaluated the in vivo expression of all these molecules in a murine model of Dextran Sulphate Sodium (DSS)-induced colonic fibrosis, and in particular the interaction between SPHK1 and TGFβ-Smad pathways.

Materials and Methods

The experimental colitis was carried out at the Pasteur Institute of Lille, France Care Facility (Pasteur Institute of Lille, France) as per the governmental guidelines and approved by the Ethics Committee for Animal Experimentation of the region Nord-Pas de Calais-France (Comité d’Ethique en Expérimentation Animale Nord-Pas de Calais; CEEA n.75).

Animals

Twenty C57BL/6 wild-type mice were included in the study (Janvier, Le Genest-St-Ise, France). All animals were kept in a pathogen-free environment at constant room temperature, with 12h/12h light/dark cycle and food and water ad libitum.

Experimental design, induction and evaluation of chronic colitis

The mice were randomly divided into two groups: control (H2O) n=5 and DSS n=15. Chronic colitis and fibrosis were induced in mice by oral administration of 2.5% (w/v) DSS (MW: 36,000-40,000, purchased from TdB Consultancy, Uppsala, Sweden) solubilized in autoclaved tap water and administered ad libitum for three cycles (5 days DSS followed by 7 days of tap water) while control groups received only tap water. Animals were monitored daily for fluid intake, weight changes, and examined for signs of colitis such as weight loss, diarrhea (scored on a 0-2 scale, as follows: 0, absence; 1, mild; 2, severe), rectal bleeding, assessed with the ColoScreen III Lab Pack (Elitech, Salon-de-Provence, France) and rectal prolapse, (scored as 0, absence; 1, presence).

Samples recovery and preparation

Four days after the last DSS cycle administration, the animals of each group were sacrificed through cervical dislocation after CO2 anaesthesia. Following laparotomy, colon and rectum were identified and examined for signs of colitis such as ulceration, hemorrhage (scored on a 0–2 grade), depth of lesions (0, absent; 1, mild; 2, moderate; 3, severe), extent of ulcerations (0, absent; 1, small ulcers; 2, big ulcers), degree of inflammation (0, absent; 1, mild; 2, moderate; 3, severe). The sum of these scores was expressed as total microscopic score as previously reported.23 Intestinal inflammation was evaluated as absent, mild, moderate or severe according to the density and the extent of the inflammatory infiltrate, the loss of goblet cells, and the bowel wall thickening.

Masson’s Trichrome allowed to stage the deposition of connective tissue and fibrosis, using H&E, in double-blind, according to the presence of ulcerations (0, absent; 1, small ulcers; 2, big ulcers), degree of inflammation (0, absent; 1, mild; 2, moderate; 3, severe), degree of fibrosis (0, absent; 1, mild; 2, moderate; 3, severe). The sum of these scores was expressed as total microscopic score as previously reported.23 Histological sections were counterstained with Mayer’s Hematoxylin, mounted and observed under the Olympus BX51 Light Microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Two pathologists (AV and RS) independently examined and scored all histological sections of the colonic samples in double-blind, according to the presence of ulcerations (0, absent; 1, small ulcers; 2, big ulcers), degree of inflammation (0, absent; 1, mild; 2, moderate; 3, severe), depth of the lesions (0, absent; 1, lesions extending in the submucosa; 2, lesions in the muscularis propria; 3, lesions in the serosa) and degree of fibrosis (0, absent; 1, mild; 2, moderate; 3, severe). The sum of these scores was expressed as total microscopic score as previously reported.23

Immunohistochemistry

Colonic samples, were also promptly fixed in 4% buffered formalin in PBS for 3 h at room temperature, dehydrated in graded ethanol and embedded in low-melt- ing paraffin. Sections of 3-μm in thickness were incubated for 40 min in methanol and in 3% hydrogen peroxide solution for 5 min. The specimens were incubated overnight at 4°C with specific antibodies (Table 1). The samples were washed in PBS for 5 min and incubated with streptavidin-biotin-peroxidase conjugated secondary antibody (K0675, Dako-Cytomation, Milano, Italy). After rinsed in PBS for 10 min the sections were incubated with 3,3-diaminobenzidine-tetrahydrochloride for 1-3 min. Finally, the sections were counterstained with Mayer’s Hematoxylin, mounted and observed under the Olympus BX51 Light Microscope (Olympus, Optical Co. Ltd., Tokyo, Japan).

To control the specificity of the immune reaction, negative and positive controls were performed for all immunoreactions (Table 1). For negative controls the sections were incubated omitting the primary antibody.
Quantitative digital image analysis of immunohistochemical staining and statistical analyses

Quantitative comparison of immunohistochemical staining was measured by ImageJ, a digital image analysis public domain software (U.S. National Institutes of Health, Bethesda, MD; available at: https://imagej.nih.gov/ij/) for all antibody tested in the study. Five microscopic fields were selected from control and DSS groups, photographed at the same magnification and then subjected to IHC profiler software analysis. The immunopositivity was expressed as a percentage of the total software-classified areas and the data obtained were plotted as histograms. Results were expressed as means ±SD; a P-value <0.05 was considered statistically significant.

Results

Clinical and macroscopic findings of chronic colitis induced by DSS

Administration of DSS has led to a moderate body-weight loss and mortality in 13% of the animals.

The colon of DSS treated mice appeared increased in weight (referred to the last 8 cm of the distal large bowel) whereas colon length was reduced compared to control mice (Figure 1). In DSS group the ratio weight/length of the colon and the major macroscopic findings of fibrosis as dilation, thickness, stenosis and adhesion were significantly increased compared to untreated mice (Table 2).

Immunohistochemistry

The main fibrotic markers Collagen I-III and α-SMA resulted mild expressed in...
control mice (Figure 3 A,D), while there was a significant increase in immunopositivity in DSS mice (Figure 3 B,E) as confirmed by quantitative data (Figure 3 C,F).

In particular, in DSS treated group, α-SMA staining was not limited to the smooth muscle cells of the muscularis mucosa and muscularis propria but it was also present in lamina propria, submucosa and serosa (Figure 3E). In control group the expression was restricted to typical layers (Figure 3D).

Immunopositivity for TGF-β and phosphorylated Smad3 resulted slightly expressed in untreated control mice (Figure 4 A,D), while they were markedly increased in DSS group (Figure 4 B,E). Concerning Smad7 inhibitor protein, the immunopositivity was significantly higher in control respect to DSS (Figure 4 G,H). These data were confirmed by quantitative analysis (Figure 4 C,F,I).

Immunohistochemical and quantitative analysis for Sphingosine Kinase 1 (SPHK1) showed an increased expression in DSS mice (Figure 5 B,C) compared to control group (Figure 5 A,C), according to the fact that TGF-β is able to activate and upregu-

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**Table 2. Clinical findings and macroscopic features of DSS-induced chronic colitis.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>H2O</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of DSS administration (days)</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Number of mice</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Mortality (n.mice)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Body weight (gr)</td>
<td>28.44±0.51</td>
<td>26.20±0.31**</td>
</tr>
<tr>
<td>Colon weight (gr)</td>
<td>0.187±0.006</td>
<td>0.305±0.008**</td>
</tr>
<tr>
<td>Colon length (cm)</td>
<td>8.78±0.015</td>
<td>6.8±0.19**</td>
</tr>
<tr>
<td>Colon weight/size ratio (fold change)</td>
<td>1±0.042</td>
<td>2.113±0.05**</td>
</tr>
<tr>
<td>Dilatation</td>
<td>np</td>
<td>1.2±0.22**</td>
</tr>
<tr>
<td>Tickness</td>
<td>np</td>
<td>0.76±0.2*</td>
</tr>
<tr>
<td>Stenosis</td>
<td>np</td>
<td>0.92±0.23*</td>
</tr>
<tr>
<td>Adhesions</td>
<td>np</td>
<td>0.73±0.22 np</td>
</tr>
<tr>
<td>Total macroscopic score</td>
<td>np</td>
<td>3.64±0.435**</td>
</tr>
</tbody>
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Data are expressed as mean±SEM; np, not present; *P<0.05 vs H2O; **P<0.01 vs H2O.
late this molecule in fibrotic condition. Above all, SPHK1 positivity was predominantly localized in mucosa layer and in submucosa of colon from DSS mice.

RhoA and PI3K staining resulted slightly expressed in control mice (Figure 5 D,G), while was highlightly increase in DSS treated group, mainly in mucosa (strongly in the epithelium, Supplementary Figure 1), but also in submucosa and in serosa (Figure 5 E,H). These data were confirmed by quantitative analysis (Figure 5 F,I). A marked increase of both Akt and phosphorylated Akt was observed in mucosa, submucosa and serosa of DSS treated mice (Figure 6 B,E) compared to untreated mice (Figure 6 A,D) has also shown by quantitative assessment (Figure 6 C,F).

Phosphorilated m-TOR staining was mildly expressed in mucosa of control mice (Figure 6G), while was increased in mucosa (specially in the epithelial cells, Supplementary Figure 1), in submucosa and in serosa of mice receiving DSS (Figure 6H). The different levels of immunopositivity between the two groups were also highlighted by quantitative analysis (Figure 6I).

Discussion

The activation of SPHK1 and generation of S1P, the bioactive lipid of sphingomyelin, plays an important role in a variety of physiologic and pathologic cellular processes in many organs.9,13,17 Several evidences suggest a pro-fibrogenic role of SPHK1 pathway carried out through different mechanisms including the influence on epithelial barrier function, the inflammatory cell recruitment and the activation of myofibroblast (migration, proliferation, differentiation and contraction).

In the present study we investigate the in vivo interaction between TGF-β and SPHK1 signaling in a mouse model of DSS-induced chronic colitis, in order to verify whether SPHK1 and its effectors were able to interact with TGF-β/Smad signaling and if they were involved in the induction and progression of the intestinal fibrosis. Clinical signs observed in DSS-receiving mice were coherent with the development of colonic fibrosis, with a significant increase of the total macroscopic score and histological parameters, com-

Figure 4. Immunohistochemistry (original magnification 10x). TGF-β, p-Smad3, are significantly increased in DSS mice (B,E) while they are mild expressed in control mice (A,D). The inhibitor protein Smad7 shows a lower positivity in DSS mice (H) respect to control (G).
pared to control mice. Immunohistochemical evaluations demonstrated overexpression of the main marker of myofibroblasts activation, with consequent accumulation of collagen in lamina propria and submucosal layer altering the normal architecture of colonic wall with respect to the normal morphology of the control group.

According to other research group, which have previously shown the potential profibrotic role of SPHK1 in pulmonary, cardiac, liver and kidney fibrosis, we demonstrate a close relationship between TGF-β and SPHK1 expressions in mice developing intestinal fibrosis. A concomitant upregulation of TGF-β1, p-Smad3, SPHK1, RhoA, PI3K was found in DSS mice compared to controls. These findings demonstrated that SPHK1 and RhoA are mediators of inflammation and fibrosis. As it occurs in other organs,24,25 the increasing expression of SPHK1 could influence the upregulation of RhoA responsible for inflammatory gene expression subsequently leading to activation of autocrine inflammatory intestinal signaling. We have also observed an increased expression of PI3K, AKT, p-AKT and p-m-TOR in all colonic layers of DSS treated mice compared to controls. TGF-β can activate PI3K/AKT/m-TOR signaling and RhoA may be an upstream mediator of AKT activation that can be also involved in the EMT.26,27

SPHK1 appears to facilitate also in the intestine the action of the critical pro-fibrotic factor TGF-β as occur in other organs, promoting transformation of fibroblasts and epithelial cells into activated myofibroblasts.28

These in vivo data support the hypothesis that TGF-β stimulates production of S1P via increased SPHK1 activity which is then released and acts on the S1P2 receptor to promote pro-fibrotic cellular transformation and function. Therefore, the development of intestinal fibrosis could be influenced not only by TGF-β/Smad pathway but also by crosstalk between TGF-β/SPHK1/S1P signaling by influencing the expression of profibrotic gene as collagen and α-SMA levels (Figure 7).

Despite more detailed investigations, such as in vitro studies, are needed to elucidate the involvement of the different S1P receptors in chronic intestinal inflammation and fibrosis, these preliminary data support...
Figure 6. Immunohistochemistry (original magnification 10x). A marked increase of both Akt and phosphorilated Akt is observed in mucosa, submucosa and serosa of DSS treated mice (B,E) compared to controls (A,D). Phosphorilated m-TOR staining is mild expressed in mucosa of control mice (G), while the immunopositivity is increased in epithelial cells (supplementary Figure 1), in submucoasa and in serosa of DSS treated mice (H).

Figure 7. Schematic representation of TGF-β dependent signaling implicated in fibrosis process. TGF-β upon biding its receptors, activates both canonical Smads pathway and noncanonical pathway (SPHK1/S1P/mTOR), inducing transcription of pro-fibrotic target genes.
the hypothesis of a possible interaction between the above-mentioned molecules in the development of colorectal fibrosis in mice. Development of molecules to regulate the synthesis of SIP and to selectively block or stimulate different SIP receptor subtypes could be an attractive target for the development of new pharmacological treatments of intestinal fibrosis.29

References


