BMP signaling in rats with TNBS-induced colitis following BMP7 therapy

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1Department of Anatomy and 2Department of Physiology and Immunology, Faculty of Medicine, University of Rijeka, and 3Department of Internal Medicine, Clinical Hospital Rijeka, Rijeka; 4Psychiatric Hospital Rab, Rab; 5Department of Pathology, Faculty of Medicine, University of Rijeka, Rijeka; and 6Laboratory of Mineralized Tissues, Center for Translational and Clinical Research, School of Medicine, University of Zagreb, Zagreb, Croatia

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Marie I, Kucic N, Turk Wensveen T, Smoljan I, Grahovac B, Zoricic Cvek S, Celic T, Bobinac D, Vukicevic S. BMP signaling in rats with TNBS-induced colitis following BMP7 therapy. Am J Physiol Gastrointest Liver Physiol 302: G1151–G1162, 2012. First published February 23, 2012; doi:10.1152/ajpgi.00244.2011.—Beyond stimulating bone formation, bone morphogenetic proteins (BMPs) are important in development, inflammation, and malignancy of the gut. We have previously shown that BMP7 has a regenerative, anti-inflammatory, and antiproliferative effect on experimental inflammatory bowel disease (IBD) in rats. To further investigate the BMP signaling pathway we monitored the effect of BMP7 therapy on the BMP signaling components in the rat colon during different stages of experimentally induced colitis by 2,4,6-trinitrobenzene sulfonic acid (TNBS). The results showed a significantly decreased BMP7 expression in the acute phase, followed by a significantly increased BMP2 and decreased BMP6 expression during the chronic phase of colitis. BMP7 therapy influenced the expression of several BMPs with the most prominent effect on downregulation of BMP2 and upregulation of BMP4 in the chronic phase of colitis. Importantly, connective tissue growth factor and noggin expression were elevated in the acute stage and significantly decreased upon BMP7 therapy. BMP receptor I expression was unchanged, whereas BMP receptor II was decreased at day 2 and increased at days 14 and 30 of TNBS inflammation. However, an opposite pattern of expression following BMP7 therapy has been observed. BMP7 increased the expression of BR-Smad including Smad3 and Smad4. Inhibitory Smads were increased at colitis and significantly decreased following BMP7 therapy at later stages of the disease. We suggest that BMP signaling was altered during TNBS-induced colitis and was recovered with BMP7 administration, suggesting that IBD is a reversible process.

Bone morphogenetic proteins; inflammatory bowel disease; Smad proteins

BONE MORPHOGENETIC PROTEINS (BMPs) comprise the largest subgroup of the transforming growth factor (TGF-β) superfamily of molecules. They were discovered and named based on their osteoinductive ability. Today we know that BMPs have an essential role in embryonic development and tissue differentiation, regulation of cell survival, and apoptosis, as well as in the organogenesis of skeletal and nonskeletal tissues (14, 45, 60, 67, 68, 69, 74). BMPs transduce their signals by binding to two different serine/threonine kinase receptors, type I (BMPRIA and BMPRII) and type II (BMPRII), triggering the phosphorylation and activation of the BMPRI by the BMPRII and activating the downstream Smad signaling molecules through Smad-dependent and Smad-independent pathways (36, 47, 48, 59). The activated type I receptor phosphorylates R-Smads and this permits their association with Smad4. The R-Smad family includes the BMP-specific receptor-regulated Smad subfamily (BR-Smads, Smad1/5/8) and activin/TGF-β-specific receptor-regulated Smad subfamily (AR-Smads, Smad2/3). R-Smad forms a complex with Co-Smad (Smad4) and transduces to the nucleus, where the transcription of TGF-β/BMP target genes is determined (41).

The specific expression pattern of BMPs and the components of the BMP signaling pathway that were observed in all three germ layers pointed to the essential role of BMPs in normal gastrointestinal tract development (4, 18, 31, 37). Germline mutations in BMPRI and Smad4 have been identified in patients with juvenile polyposis (JP), a rare autosomal dominant syndrome characterized by the development of multiple hamartomatous polyps in the gastrointestinal tract (11, 32). Additionally, several molecular pathways have been identified that control crypt fission, an important element underlying the clonal expansion of intestinal tumors (27, 30). Target inactivation of BMPRIA in epithelial cells results in the expansion of the intestinal stem cell population and growth of polyps in the small intestine (32). A phenotype similar to human JP has been created in villin-noggin mice by the inhibition of BMP activity in the intestinal epithelium via transgenic expression of noggin (4, 27). There is also increasing evidence that BMPs and their signaling pathway have an important role in intestinal carcinoma, particularly in tumor progression, invasion, and metastasis, as described for TGF-β and BMP7 (52). Recently, researchers have shown that BMPs act mainly as tumor suppressor factors in intestine tumorigenesis, as evidenced by the loss of BMP signaling pathway in the colorectal cancer (28, 29, 35). Smad5 gene downregulation was observed in human inflammatory bowel disease (IBD), which leads to higher susceptibility to experimental colitis (2). Conditional mutations of the BMPRRII in mice lead to appearance of epithelial hyperplasia and hamartomatous polyps (5). BMPs are important in enteric gliogenesis with enhancement of the glial differentiation and expression of ErbB3 receptor and their ligands in enteric crest-derived cells (12).

In our previous study we showed the expression profile of proinflammatory cytokines, BMP7, and its receptors in the colon during different stages of experimental IBD. BMP7 when applied prophylactically or therapeutically reduced the inflammation due to a decrement of proinflammatory cytokine, especially IL-6 (44). To further characterize the role of BMPs in the intestine, in this study we examined the expression of various BMPs, their receptors, and downstream molecules of BMP signaling (Smad family members) in rats with IBD. We
showed that BMPs and BMP pathway components are preserved in the colon of diseased animals and the alterations in their expression during IBD are recovered by the BMP7 therapy.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 230–250 g were used. The rats were housed in standard cages and fed with standard laboratory chow and tap water ad libitum. During the experiments the fundamental ethical principles of working with animals were respected. All animal procedures were approved by the Animal Ethics Committee of the Faculty of Medicine, University of Rijeka, Croatia.

Experimental colitis and study design. We used the experimental model of IBD as described (51). Animals were deprived of food for 24 h before the induction of colitis. Under light ether anesthesia, colitis was induced by intracolonic installation of a solution containing hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS; 30 mg; Sigma-Aldrich, Taufkirchen, Germany) in 50% ethanol. TNBS-solution was introduced into the colon through a plastic catheter at a distance of ~8 cm from the anus. The animals were maintained in a head-down position for some time to prevent the leakage of TNBS solution. During the study the animals were monitored for signs of colon inflammation, including diarrhea and rectal bleeding. The rats were randomized into four experimental groups, as follows: control animals without treatment (normal group; n = 10); rats receiving 50% vol/vol ethanol alone (ethanol control; n = 40); rats receiving TNBS enema (TNBS group; n = 40), and rats receiving TNBS enema plus BMP7 (TNBS/BMP7 group; n = 40). BMP7 was applied intravenously in a dose of 100 μg/kg at 1, 2, 3, 5, 7, 14, 20, and 25 days after colitis induction. BMP7 was administered systemically according to the results of Grгуurevic et al. (26), who showed the presence of intact BMP signaling in the serum 30 min after its administration. For the time-course study, we euthanized 10 animals on days 2, 5, 14, and 30 by colitis in TNBS and TNBS/BMP7-treated rats, respectively.

Assessment of colonic damage. The animals were weighed at the beginning of the experiment as well as on the day of termination to determine alterations in body weight. The distal 8 cm of the rat colon was dissected, rinsed with saline, and reviewed for the existence of colitis. After these procedures were completed, the colon was examined in a blind fashion and scored for macroscopically visible damage according to criteria described previously (Table 1) (10, 44, 72). Briefly, the scoring on a scale from 0 to 14 included the appearance of bleeding and diarrhea, ulcer with/without inflammation at one or more sites, as well as the size of extending ulceration and the presence of adhesions. After the completion of the macroscopic scoring, three colon samples with visible inflammation were taken for microscopic analyses. In the absence of inflammation the samples were taken 2, 4, and 6 cm proximal from the anus. Tissue sections were examined by two independent observers (T. Turk Wensveen and I. Maric) and scored on a scale from 0 to 5, including extension of the focal ulceration and the presence of transmural inflammation (Table 2) (44, 62). The scored indexes are expressed as a median (range).

Subsequently, the samples of colon tissue were stored in the liquid nitrogen for further analyses.

Immunohistochemistry. For immunohistochemistry, colons were fixed in 4% formalin, embedded in paraffin and sectioned in serial 4-μm slices. Immunostaining was performed by standard procedures. Briefly, sections were incubated for 30 min in methanol and 3% hydrogen peroxide. Antigen retrieval was performed in citrate buffer (pH = 6) at 100°C for 15 min and cooled for 10–20 min at room temperature. After blocking in 3% BSA-PBS TWEEN, the sections were incubated overnight at 4°C with a polyclonal antibody to pSmad1/5/8, which recognizes the doubly phosphorylated forms of Smad1 (Ser463/465), Smad5 (Ser463/465), and Smad8 (Ser426/428) at a concentration 1:100 (Cell Signaling Technology, Beverly, MA). Dako EnVision+ System-HRP detection system was used as a secondary reagent following the manufacturer’s instruction. Liquid Dab+ substrate (Dako, Carpinteria, CA) was used as a chromogen. Slides were counterstained with hematoxylin. To determine pSmad1/5/8 expression in colon samples, the positive cells were counted on 10 high-power fields (×400 magnification) in the mucosa and submucosa of each animal (n = 5 per experimental group). Cell counting was performed by use of an image analyzer system equipped with a software package (Issa, VAMS, Zagreb, Croatia) by two independent observers (I. Smoljan and T. Celic). The staining score of counted cells revealed a significant interobserver concordance (Pearson’s r = 0.683, P < 0.05). The results are expressed as a mean ± SD of pSmad1/5/8-positive cells per high-power field.

Western blot analysis. The colon proteins were extracted from the rat colons by homogenization in RIPA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, Germany) and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). Tissue homogenates were centrifuged and prepared in the SDS sample buffer. The samples (50 μg protein) were subjected to electrophoresis on 10% SDS-PAGE and blotted onto a PVDF Western blotting membrane (Roche Diagnostics). To detect pSmad1/5/8 immunoreactivity, the membrane was incubated with a rabbit polyclonal antibody to pSmad1/5/8 (Cell Signaling Technology) diluted 1:500 overnight at 4°C with agitation. After washing with TBS-T buffer (TBS with 0.1% Tween 20), the membrane was incubated with anti-rabbit IgG-horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) diluted 1:1,000 for 1 h at room temperature. Blots were then stripped and reblotted with a rabbit β-actin antibody (Sigma-Aldrich) diluted 1:200 for 1 h at room temperature. Blots were visualized by using SuperSignal West Pico Chemiluminescent Substrate; the bands were intensified with little effort)

Table 1. Criteria for macroscopic scoring of colonic lesions (49, 77)

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<th>Criteria</th>
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<td>Normal tissue appearance</td>
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<tr>
<td>1</td>
<td>Damage of surface epithelium</td>
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<tr>
<td>2</td>
<td>Focal ulceration limited to mucosa</td>
</tr>
<tr>
<td>3</td>
<td>Focal, transmural ulceration and inflammation</td>
</tr>
<tr>
<td>4</td>
<td>Extensive transmural ulceration and inflammation bordered by normal mucosa</td>
</tr>
<tr>
<td>5</td>
<td>Extensive transmural ulceration and inflammation involving entire section</td>
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Table 2. Criteria for microscopic scoring of colonic lesions (49, 67)

<table>
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<td>No adhesions</td>
</tr>
<tr>
<td>2</td>
<td>Minor adhesions (colon can be separated from other tissue</td>
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<tr>
<td>3</td>
<td>Major adhesions</td>
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### Oligonucleotide primer sequences used in the study

Table 3.

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<td>BMP7</td>
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RT-PCR analysis. Total RNA extraction from the colons was performed by homogenization with QIAzol lysis reagent (1 mg/100 mg of tissues; Qiagen) by the acid guanidinium thiocyanate phenol-chloroform method as described (18). The total RNA from the cells was extracted with NucleoSpin RNA II kit (Machnery-Nagel, Düren, Germany) according to the manufacturer’s instructions. The integrity of each sample was verified by electrophoresis in a 1% denatured agarose gel. Reverse transcription was performed by use of a Takara RNA PCR kit (AMV) ver.3.0. (Takara Bio, Shiga, Japan). The primer sequences and product size are shown in Table 3. PCR products were separated by agarose gel electrophoresis with ethidium bromide. Using a 100-bp DNA ladder, we confirmed the predicted sizes of the obtained PCR products. The intensity of bands was quantified by a calibrated imaging densitometer (Kodak Image Station 440, LabImage).

LPMC isolation and cell culture. Lamina propria mononuclear cells (LPMC) were isolated from the freshly dissected colons as described (25, 71). Briefly, the colon samples were flushed with ice-cold PBS and the Peyer’s patches were removed. The colon was opened longitudinally, cut into 5-mm pieces, and incubated in HBSS free of calcium and magnesium (HBSS-CMF) containing 1 mmol/l of DTT for 30 min at room temperature. Subsequently, to remove free of calcium and magnesium (HBSS-CMF) containing 1 mmol/l of EDTA were collected and processed for immunofluorescence and RT-PCR analysis.

Immunofluorescent cell staining. LPMC cells, from a suspension containing 5 × 10^7 cells, were centrifuged on a glass slide in a cyto spin centrifuge. The attached cells were first washed with PBS and then fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature. After blocking in 2% bovine serum albumin in PBS, the slides were incubated at 4°C overnight with a primary antibody against pSmad1/5/8 in dilution 1:500. The bound antibodies were visualized by incubation with a FITC-conjugated goat anti-rabbit IgG as secondary antibody (dilution 1:300) (Invitrogen). At the same time, the cells were stained by Evans blue and analyzed by brightfield microscopy (Olympus BX51).

Statistical methods. Results were analyzed by use of the Statistica 10 software package (StatSoft, Tulsa, OK). The data were expressed as means ± SD or medians (range). Comparison of parametric data was performed by Student’s t-test for unpaired observations and nonparametric data by the Wilcoxon rank sum test.
RESULTS

Macroscopic and microscopic signs of colitis in experimental groups. Animals receiving the TNBS/ethanol enema had clinical signs of colitis characterized by rectal bleeding and diarrhea. During the acute phase of TNBS colitis the body weight was lower by 7.4–13%, whereas during the chronic phase of the disease animals gained weight (0.92–8.14%), with the exception of ethanol-treated rats, which did not show body weight changes during the acute phase. Also, BMP7 therapy did not influence animals' weight (data not shown). Macroscopic observations of colons isolated from the rats with TNBS colitis more frequently revealed the presence of cobblestone-like lesions, larger ulcers, and adhesions with scored index 9 (5–12), 7 (4–9), 6 (4–12), and 4.5 (3–7) on days 2, 5, 14, and 30, respectively (Table 4). The TNBS/BMP7-treated group showed lower index scoring for the same data points, which were 7 (5–10), 6 (4–8), 4 (3–6), and 3 (3–4), with significant difference on days 14 and 30. Microscopic analyses showed diminished surface epithelium and colon crypts with transmural infiltration at the acute phase of TNBS colitis with scored index 4.5 (3–5) and 3 (2–4) for TNBS group on days 2 and 5, respectively; and 3 (3–5) for TNBS/BMP7 group at both time points. On days 14 and 30, the scored microscopic index was 3.5 (2–5) and 2.5 (2–4) for TNBS group, and 2 (1–3) and 1.5 (1–2) for TNBS/BMP7 group, which was significantly different (Table 4). The colons of ethanol-treated animals had no macroscopic changes, with minor damage of the surface epithelium during the acute phase of colitis (data not shown).

Expression of BMPs in the TNBS-induced colonic inflammation. We determined the expression of BMP2, 4, 6, and 7 in colon during acute (2nd and 5th day of colitis) and chronic phase (14th and 30th day of colitis) of TNBS colitis by RT-PCR. Expression of all BMPs was detected with a significantly increased expression in the chronic stage (Fig. 1). The basal level of CTGF was also present in the normal colon and ethanol control. The expression of the BMP antagonist noggin was increased in colons of TNBS rats, showing a significant increment compared with controls and was also reduced upon BMP7 therapy.

BMP signaling pathway is preserved in TNBS rat model of IBD. BMPRs were expressed in all examined samples with a constitutive expression of BMPRIA, whereas the expression of BMPRIIB was significantly decreased during TNBS colitis and following BMP7 therapy, compared with the ethanol-treated rats (Fig. 2). Expression of BMPRII showed an opposite pattern in BMP7-treated rats with a significantly higher receptor expression level in the chronic stage of colitis.

Analysis of the BMP/Smad signaling system including R-Smads (Smad1/5/8) and Co-Smad (Smad4) showed the strongest expression of Smad1, whereas the expression of Smad5 was low (Fig. 3). BMP7 therapy significantly increased the expression of R-Smads, particularly Smad1 in the acute and Smad8 in the chronic stage of colitis. R-Smads were significantly decreased in the ethanol-treated rats compared with TNBS/BMP7-treated animals. Expression of Smad4 was mostly unchanged throughout the whole observation period, with the exception of day 5 where a significant decrement in the TNBS colitis group was found. The TGF-β-responsive Smad2 and -3 were increased in the colon of BMP7-treated rats with significantly increased Smad3 expression on days 5 and 30 (Fig. 3). During TNBS-induced colitis, the expression of Smad2 and Smad3 showed concurrent enhancement on days 2 and 14 of colitis, which represents the beginning of the acute or chronic stage of colon inflammation. It was also noticed that the ethanol control rats showed a significantly lower expression of Smad2, compared with BMP7-treated rats. Regarding the inhibitor Smads, BMP7 treatment markedly decreased the expression level of Smad6 on day 30 and Smad7 on day 14 of TNBS colitis.

Shift of BMP signaling in the regenerating colon. To further investigate the activity of BMP signaling pathway in TNBS colitis, we determined the presence of phosphorylated Smad1, 5, and 8 (pSmad1/5/8). Immunohistochemical staining showed the presence of pSmad1/5/8 in colons taken at days 2 and 5 after the colitis induction in both diseased and BMP7-treated colons with different staining patterns (Fig. 4A). On day 2 of TNBS colitis, pSmad1/5/8-positive staining was present in the crypt epithelium located in the deeper parts of the mucosa and was almost absent in the surface epithelium, whereas in BMP7-treated animals, pSmad1/5/8-positive staining was observed in the glandular epithelium of the superficial crypts. Ethanol-treated rats showed a positive staining in the glandular epithelium of colon crypts and in the lamina propria. On day 5, pSmad1/5/8-positive staining was present in irregularly shaped crypts and in the superficial epithelium. The BMP7 therapy resulted in the recovery of crypts, characterized by the formation of numerous crypts with a regular shape. Besides the crypt and surface epithelium, pSmad1/5/8-positive staining was additionally detected in the lamina propria. The same staining pattern was observed in the ethanol-treated rats. There was no difference in the number of pSmad1/5/8-positive cells between the groups during the acute stage of TNBS colitis. On day 14 of TNBS colitis, the nuclear pSmad1/5/8 staining was localized in colons of TNBS rats, especially on the day 2 after induction of colitis, whereas the BMP7 treatment significantly reduced its expression (Fig. 1). The basal level of CTGF was also present in the normal colon and ethanol control. The expression of the BMP antagonist noggin was increased in colons of TNBS rats, showing a significant increment compared with controls and was also reduced upon BMP7 therapy.

Table 4. Effects of BMP7 treatment on macroscopic and microscopic signs of colitis

<table>
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<th>Experimental Group</th>
<th>Day of TNBS Colitis</th>
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<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>TNBS group</td>
<td>9 (5–12)</td>
</tr>
<tr>
<td>TNBS/BMP7 group</td>
<td>7 (5–10)</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>1 (0–1)</td>
</tr>
<tr>
<td>TNBS group</td>
<td>4.5 (3–5)</td>
</tr>
<tr>
<td>TNBS/BMP7 group</td>
<td>3 (3–5)</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>1 (0–1)</td>
</tr>
</tbody>
</table>

Data are expressed as median (range); n = 10. *Macroscopic damage score from 0 to 14; †Microscopic damage score from 0 to 5; ‡Ethanol control is significantly different from both groups; *P < 0.01; †P < 0.05.
in the crypt epithelium, lamina propria, and submucosa, which led to significantly higher number of pSmad1/5/8-positive cells in BMP7-treated rats (95.2 ± 21.2 vs. 55.2 ± 19.12 in TNBS colitis; *P* < 0.001). At a later stage of colitis, pSmad1/5/8 staining was slightly increased in the lining epithelium, probably because of its recovery on day 30. In addition, pSmad1/5/8 staining was also present in the crypt epithelium and the lamina propria; however, at this time point it was almost absent in the submucosa. The ethanol group showed an intense staining in the crypt epithelium and in the lamina propria (56.1 ± 19.68 vs. 28.66 ± 10.31 in TNBS control and 32.86 ± 12.31 in TNBS/BMP7 group). In all colon sections, a strong pSmad1/5/8 staining was present in the enteric plexus located between the muscle layers (Fig. 4A, arrowhead).

To quantify pSmad1/5/8 in the colon samples on different days of TNBS colitis, we performed Western blot analysis from whole colon lysates. The expression of pSmad1/5/8 was significantly higher in the samples from BMP7-treated rats on days 5 and 30 from the TNBS group and ethanol control rats with the exception of day 14 of colitis (Fig. 4B). The recovery of the BMP signaling activity was observed from day 14 of TNBS colitis or ethanol-treated rats.

**BMP signaling pathway is active in LPMC.** To validate the importance of LPMC in the BMP signal transduction we examined the expression of pSmad1/5/8 in LPMC isolated from the colon at different time points of colitis. pSmad1/5/8 were present in all examined samples as a punctuate pattern localized in the cytoplasm by green immunofluorescence (Fig. 3C). Next, LPMCs extracted from normal and inflamed colons were stimulated with BMP7 and analyzed for BMP-responsive Smads by RT-PCR. Smad1/5/8 were barely detectable in LPMCs in untreated as well as in BMP7-treated cells from

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**Fig. 1. Expression of bone morphogenetic proteins (BMPs) and BMP antagonists in the rat colon following 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and BMP7 treatment.** A: RT-PCR analyses of BMP ligands, CTGF and noggin in BMP7-treated and control rats on 2nd, 5th, 14th and 30th day following induction of colitis. B: BMPs and BMP antagonists levels were measured by densitometry from day 2 to day 30. Data represent mean values ± SD; net intensity is expressed as no. × 10^6; n = 6 or 7 rats at each time point. *P* < 0.05 vs. TNBS group and TNBS/BMP7 group, #P < 0.05 vs. TNBS group and ethanol control, §P < 0.05 vs. TNBS/BMP7 group and ethanol control, †P < 0.05 vs. TNBS group and normal group, ‡P < 0.05 vs. TNBS/BMP7 group and normal group, +P < 0.05 vs. ethanol control and normal rats.
normal colons. As shown in Fig. 1C, Smad1/8 were marginally detected in unstimulated LPMCs from TNBS animals, whereas after BMP7 treatment their expression was increased. Smad5 was undetectable in both normal and inflamed colons. In addition, LPMCs isolated from the colons of both normal and TNBS rats demonstrated a three times higher cell number 24 h following BMP7 therapy. This indicates that BMP7 influenced the proliferation of LPMCs (Fig. 4C).

DISCUSSION

In the present study we showed that BMP-Smad signaling is altered in the colon during TNBS-induced colonic inflammation and is characterized by a decreased expression of BMPs, BMPRs, and Smads, except BMP2, BMPRII, and Smad6. Exogenously applied BMP7 restored the components of the BMP signaling pathway and decreased the expression of CTGF and noggin. The activity of the BMP pathway shown by the presence of pSmad1/5/8 was observed mostly in the crypt epithelium and the LPMC of the colon.

Several studies have indicated the importance of BMPs in gastrointestinal development and diseases (27, 28, 48). During intestinal morphogenesis, BMPs are important mediators of numerous epithelial-mesenchymal interactions (57). Analysis of BMP gene expression in the colon revealed the presence of Bmp1, 2, 5, 7, and SMAD7 and BmprII in the top part of the colon crypt (37). Recent studies showed the perturbation of BMP signaling pathway in hereditary gastrointestinal diseases and the efficacy of growth factor
therapy along with the conventional treatment of IBD (27, 38). Downregulation of BMP2 and BMP4 because of the mutation of Fkh/FoxL1 or disruption of Nkx2–3 transcription factors affects the villus morphology because of increased epithelial proliferation (32, 54). Karlsson et al. (34) observed BMP2 and BMP4 expression in the villus cluster and subsequent epithelial proliferation limited to the inter-villus and prospective crypt regions, whereas our results show that BMP2 and BMP4 are expressed in the colon of both, normal and TNBS rats. It was also shown that BMP2 is produced in the gut by epithelial surface and its secretion is increased by colonic myofibroblasts in the response to the

<table>
<thead>
<tr>
<th>A</th>
<th>TNBS colitis</th>
<th>TNBS colitis/ BMP7 treatment</th>
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<td>GAPDH</td>
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Fig. 3. Expression of Smad molecules in rat the colon during TNBS colitis and BMP7 therapy. A: RT-PCR analyses of Smads (Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, Smad8) in colon samples from diseased, BMP7-treated, and control rats (ethanol control and normal colon) on 2nd, 5th, 14th, and 30th day following induction of colitis. B: densitometric analysis of the expression intensity were performed. Data represent mean values (SD), net intensity is expressed as no. × 10^6, n = 6 or 7 rats at each time point. *P < 0.05 vs. TNBS group and TNBS/BMP7 group, #P < 0.05 vs. TNBS group and ethanol control, §P < 0.05 vs. TNBS/BMP7 group and normal group.
calcium-sensing receptor activation (29, 55). We showed a marked increase of BMP2 expression in TNBS colitis and its reduction following BMP7 treatment. Increased expression of BMP2 in TNBS-induced colonic inflammation could be associated with a reduced sensitivity or loss of inhibitory factors, resulting in an increased epithelial cell proliferation. BMP7 decreased the BMP4 expression in MC3T3-E1 cells but maintained the capacity for promoting the osteoblastic phenotype (45), whereas we showed here that BMP7 treatment restored the colonic BMP4 expression in TNBS colitis.
BMP6 shares some identical features with BMP7, like the affinity to BMP receptors and mechanisms for osteoblastic differentiation of human mesenchymal stem cells (41, 48, 66). BMP6 is present in the gastrointestinal smooth muscle and in the pancreas during development and its misexpression leads to agenesis of the pancreas and reduced stomach size (19, 56). The liver is probably the source of endogenous BMP6, which regulates the expression of hepcidin and iron absorption in the gut regulating its concentration in the serum (3, 66). Also, BMP-hepcidin axis is a potential therapeutic target in IBD because it is upregulated during the disease (73). Blocking hepcidin expression by anti-BMP6 reagents leads to restoring IBD-associated anemia and to decreased IL-17 expression. Overexpression of BMP7 accompanied with several profibrotic growth factors was found in strictestrustructed segments of human Crohn’s disease intestine, whereas in the experimental model of IBD the antifibrotic property of BMP7 had been observed (9, 23). Our results showed that both BMP6 and BMP7 were expressed in the normal colon. Expression of BMP7 in the acute stage and BMP6 in the chronic stage of colitis are lower in these respective stages of colitis and were improved by BMP7 therapy. This finding suggested a synergistic and/or complementary effect of BMP6 and -7 during healing. It is known that BMP6 has 20 times higher affinity to BMPRIA and is more resistant to noggin inhibition than BMP7 (61).

CTGF was identified as being frequently upregulated in IBD and diabetic nephropathy, which are accompanied by fibrosis (9, 53). It was shown that upregulated CTGF inhibits BMP4 and BMP7 activity leading to deficiency of BMP signaling (1, 53, 64). Also, noggin has a reciprocal relationship with BMP2 in colonic myofibroblasts and coexpression with BMP4 in atherosclerotic lesions of human coronary arteries (13, 55). Overexpression of noggin caused the absence of Smad 1/5/8 signaling and a disease similar to JP, whereas its downregulation showed opposite effect on different types of arthritis (27, 43). We found significantly increased CTGF and noggin expression during the acute stage of IBD colitis and their reduction by BMP7. CTGF expression was concomitant with BMP4 expression, whereas a correlation of noggin and examined BMPs was not observed. A balance between different BMPs and their antagonists is present in the normal gut but is also subject to change upon inflammation.

JP is another disease associated with the dysfunction of BMP signaling, primarily Smad4 and BMPRIA (11, 32). The inhibition of BMP signaling by transgenic expression of noggin or BMPRII in mice resulted in a disease similar to JP (5, 27). We found preserved expression of BMP receptors in colon samples, especially BMPRIA, whose expression is unchanged and uniform during the TNBS colitis, and upon BMP7 treatment. The BMPRIIB and BMPRII expression is significantly decreased during the acute phase of colitis and normalized following BMP7 treatment. Persistence of BMPRI expression in TNBS-induced colon inflammation suggested that the BMP signaling variations in IBD are reversible. BR-Smads were present in the colon during TNBS colitis, showing lower expression, and were maintained by the BMP7 therapy, which is in accordance with the results of Li et al. (42), showing that the loss of Smad5 leads to an increased susceptibility to experimental colitis, whereas its expression is significantly decreased in human Crohn’s disease and dextran sodium sulfate colitis (2). We found a decreased expression of Smad5 during colitis. Additionally, in LPMC isolated from inflamed colons, the expression of BR-Smads following BMP7 therapy was more pronounced except for the Smad5. In the TNBS colitis we found a preserved but altered activity of BMP signaling (pSmad1/5/8), which was improved in the chronic stage and recovered by BMP7 therapy. Fiocchi (20) described the intestinal inflammation as an interaction between immune and nonimmune cells including epithelial, endothelial, mesenchymal, and nerve cells, with nonimmune cells as an important factor of the inflammatory response. The presence of pSmad 1/5/8 in the crypt epithelium and the lamina propria cells as well as in isolated LPMC revealed the preserved activity of BMP pathway in both the nonimmune and immune intestinal cells during the TNBS colitis. The findings of pSmad 1/5/8 in the enteric plexus pointed to another possible effect of preserved BMP pathway to arrest the smooth muscle dysfunction, which was observed in TNBS colitis as the outcome of both oxidative stress and proinflammatory cytokines, specifically IGF-1 and TGF-β (58). In vivo regulation of Smad molecules is dependent not only on the ligand application but also on the BMP pathway modulation by RAS/ERK, TLR-IL-1R, or Wnt pathways (8, 15, 30, 50). It was also shown that Dragon, repulsive guidance molecule (RGM) family member, enhanced the BMP signaling activity and decreased the IL-6 expression in a BMP-ligand-dependent manner via a non-Smad pathway, like p38MAPK and Erk1/2 (70). Our data show that Smad4 is continuously present during the experimental colitis, suggesting that Smad4 is essential for the transcriptional Smad complex although the TGF-β signaling independently of Smad4 might be possible (17).

The importance of TGF-β1 is well known in the pathogenesis of IBD and its lack is characterized by an inadequate immune response. We have previously reported that TGF-β is upregulated in TNBS model of colitis (44) although TGF-β as a negative regulator of T cell response should have reduced the intestinal inflammation in IBD (7, 9, 49, 50). TNBS inflammation is characterized by hypertrophy of smooth muscle cells in muscularis externa induced by an enhanced level of TGF-β and IGF1 (58). The disruption of TGF-β/Smad signaling pathway in IBD is characterized by high expression of Smad7 and reduced Smad3 phosphorylation, leading to an inadequate inflammatory cell response to TGF-β, and a persisting inflammation, whereas Smad7 inhibition leads to its restoration (7, 49, 50). In Smad3 knockout mice there is an increased staining of TGF-β1 and Smad7 in the intestine with an absence of macroscopic lesions (75) and a reduction of fibrosis (24, 40, 63). We found reduced expression of TGF-β R-Smads in TNBS colitis, especially Smad3, which is significantly increased following BMP7 therapy. Smad7 expression was significantly increased in colons from TNBS rats on day 14 with a prominent decrease after BMP7 therapy. Therefore, we suggest that BMP7 affects healing by inhibition of TGF-β1, rather than via a reduction of the TGF-β/Smad signaling pathway (23, 44). In addition, BMP7 downregulates the expression level of inhibitory Smads, which supports the observation that blocking Smad7 is the key event in the regulation of inflammation in IBD (49).
We have previously demonstrated that BMP7 has an anti-inflammatory effect by decreasing the expression of proinflammatory cytokines, whereas TGF-β1 expression was preserved (44). Here, we show that an anti-inflammatory effect of BMP7 is additionally mediated by the decreased expression of inhibitory Smads, which enables the downstream signaling in the BMP pathway. It is also possible that during IBD other pathways, such as Jak/STAT1 and NF-κB, are activated and BMP7 enhances their cross signaling with the BMP pathway (6, 65). In addition, TGF-β achieves its anti-inflammatory activity through Smad6, which has the ability to bind to adaptor Pellino-1 protein of interleukin 1 receptor (IL-1R)-associated kinase 1 (IRAK1), preventing the formation of IRAK1-Pellino1-TRAF6 complex, which stimulates IkBα degradation, allowing NF-κB translocation into the nucleus and a subsequent expression of the proinflammatory genes (15). Here we showed an increased expression of Smad6 during TNBS colitis, suggesting an increased intestine resistance to inflammation. BMP7 treatment normalizes the Smad6 expression to levels similar to the healthy colon. Although the gastrointestinal tract is characterized by a remarkable ability to maintain the physiological inflammation, when the intestinal homeostasis is corrupted the disease occurs (21). The TNBS experimental model of colitis allows a comparison of the acute and chronic stage of colitis to the “late” stage of human IBD regarding the tissue damage and clinical signs of disease, but missing the “early” stage in humans, which occurs mostly without symptoms (22).

In conclusion, our findings demonstrate the important role of BMP signaling in the intestinal homeostasis and possible therapeutic targets for IBD therapy. Beside macroscopic and microscopic signs of healing, BMP7 treatment restored the components of the BMP pathway characterized by the increased expression of synergistic BMPs, receptor type IB and BR-Smads, and downregulation of BMP inhibitors.

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AUTHOR CONTRIBUTIONS

I.M. designed and performed the research and wrote the first draft of the manuscript; N.K. performed experiments and contributed to the analysis of data; T.T.W. and L.S. performed experiments and assisted in writing of the manuscript; B.G. and S.Z.C. analyzed and interpreted the data; T.C. performed experiments; S.V. initiated the project, critically reviewed and revised the manuscript; D.B. helped in designing experiments and wrote parts of the manuscript; S.Y. initiated the project, critically reviewed and revised the manuscript for important intellectual content, and finally approved the manuscript.

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DISCLOSURES

The authors declare that they have no conflict of interest, financial or otherwise.

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19. Dichmann DS, Miller CP, Jensen J, Scott Heller R, Serup P. Bone morphogenetic protein signaling is preserved (44). Here, we show that an anti-inflammatory effect of BMP7 is additionally mediated by the decreased expression of inhibitory Smads, which enables the downstream signaling in the BMP pathway. It is also possible that during IBD other pathways, such as Jak/STAT1 and NF-κB, are activated and BMP7 enhances their cross signaling with the BMP pathway (6, 65). In addition, TGF-β achieves its anti-inflammatory activity through Smad6, which has the ability to bind to adaptor Pellino-1 protein of interleukin 1 receptor (IL-1R)-associated kinase 1 (IRAK1), preventing the formation of IRAK1-Pellino1-TRAF6 complex, which stimulates IkBα degradation, allowing NF-κB translocation into the nucleus and a subsequent expression of the proinflammatory genes (15). Here we showed an increased expression of Smad6 during TNBS colitis, suggesting an increased intestine resistance to inflammation. BMP7 treatment normalizes the Smad6 expression to levels similar to the healthy colon. Although the gastrointestinal tract is characterized by a remarkable ability to maintain the physiological inflammation, when the intestinal homeostasis is corrupted the disease occurs (21). The TNBS experimental model of colitis allows a comparison of the acute and chronic stage of colitis to the “late” stage of human IBD regarding the tissue damage and clinical signs of disease, but missing the “early” stage in humans, which occurs mostly without symptoms (22).

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AUTHOR CONTRIBUTIONS

I.M. designed and performed the research and wrote the first draft of the manuscript; N.K. performed experiments and contributed to the analysis of data; T.T.W. and L.S. performed experiments and assisted in writing of the manuscript; B.G. and S.Z.C. analyzed and interpreted the data; T.C. performed experiments; D.B. helped in designing experiments and wrote parts of the manuscript; S.Y. initiated the project, critically reviewed and revised the manuscript for important intellectual content, and finally approved the manuscript.

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BMP SIGNALING IN TNBS COLITIS


