Thrombospondin 1 and Its Mimetic Peptide ABT-510 Decrease Angiogenesis and Inflammation in a Murine Model of Inflammatory Bowel Disease

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Key Words

Thrombospondin 1 • Inflammatory bowel disease • Metalloproteinase 2 • Apoptosis • Angiogenesis • VEGF • Basic FGF

Abstract

Objective: Vascular abnormalities and expression of proangiogenic factors have been repeatedly reported in inflammatory bowel disease (IBD). Thrombospondin 1 (TSP-1) is a protein well known for its antiangiogenic and anti-inflammatory properties. Using the dextran sulfate sodium (DSS) model, the role of TSP-1 in IBD has been investigated in vivo.

Methods: TSP-1-deficient mice (TSP-1^{-/-}) and WT mice were treated with DSS for 7 days. Disease activity indices, myeloperoxidase activity (MPO) and histology were analyzed. Microvascular density (MVD) was quantified using immunohistochemistry (IMH) with CD31 antibody. TGF- β_1 , basic FGF, VEGF, TNF- α and MMPs protein levels were evaluated by IMH and enzyme-linked immunoabsorbent assay (ELISA). Mice were treated with ABT-510 (Abbott Laboratories), an antiangiogenic TSP peptide, using miniosmotic pumps for 7 days. **Results:** TSP-1^{-/-} mice had a worse clinical outcome and exhibited severe signs of rectal bleeding compared to the WT controls. The TSP-1^{-/-} mice showed a higher level of crypt damage and deeper lesions. The grade of inflamma-

tion and the levels of MPO activity were also significantly higher in colons of TSP-1^{-/-} mice. TSP-1^{-/-} mice displayed higher MVD in focal areas of the colon after only 3 days of DSS treatment. Furthermore, clinical severity of the colitis and angiogenesis was significantly diminished when mice was treated with ABT-510. **Conclusions:** These findings directly link TSP-1 as a protective factor in IBD and suggest antiangiogenesis treatment, including compounds such as ABT-510 as an adjuvant therapy for IBD.

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Introduction

Inflammatory bowel disease (IBD) is a major health problem affecting nearly 14 million Americans. Its two principal conditions are Crohn's disease and ulcerative colitis. Both are chronic inflammatory diseases affecting people of any age or gender and both pathologies significantly increase the occurrence of colorectal carcinomas.

Although the pathophysiology of IBD is still obscure, strong evidence indicates that these entities result from anomalous immune responses. Any inflammatory response has a significant vascular component. Activated endothelium directly regulates the influx of leukocytes

and the production of cytokines and chemokines in damaged tissue. Vascular alterations in colons from patients with IBD have been well documented [1–3] and proangiogenic factors such as VEGF and hypoxia-inducible factor-1 α (HIF-1 α) have been found to be significantly elevated in ulcerative colitis [4–11]. More recently, antiangiogenesis has emerged as a novel therapeutic approach for IBD [12–15].

Thrombospondin 1 (TSP-1) is an endogenous inhibitor of angiogenesis secreted by activated platelets, immune cells such as monocytes and macrophages, as well as a variety of epithelial cells. The thrombospondin family consists of 5 different members with common and specific structures [16]. TSP-1 and TSP-2 are the best characterized members and their functions in angiogenesis in vivo and in vitro have been well investigated. TSP-1 is an endothelial regulator since it modulates adhesion, suppresses migration of endothelial cells, inhibits their proliferation and induces apoptosis through CD36 activation [17, 18]. TSP-1 also regulates metalloproteinases (MMPs) and through the same mechanism modulates endothelial migration and vessel formation [19].

Studies have suggested that TSP-1 plays an active role in the resolution of the inflammatory response after mucosal damage [20]. One of the possible mechanisms by which TSP-1 regulates inflammation is through interaction with transforming growth factor beta 1 (TGF- β_1) [21]. Once activated, TGF- β_1 executes important regulatory effects on cell proliferation and immune response in tissues. Besides its role in TGF- β_1 activation, TSP-1 binds to integrin-associated protein (IAP), also called CD47 [22]. IAP is a 50-kDa glycoprotein present in all mammalian cells, which regulates integrin function and directly activates immune responses in vivo. Therefore, TSP-1 can, for example, inhibit dendritic cell activation by immobilizing IAP [23, 24].

TSP-1^{-/-} mice display inflammation-related abnormalities such as leukocytosis, spontaneous pneumonia and inflammatory changes in the pancreas [20]. Based on this evidence, the role of TSP-1 in IBD has been investigated using dextran sulfate sodium (DSS). The DSS model is widely and reliably employed. Furthermore, DSS-induced colitis shares common features with human IBD, including late carcinogenesis [24, 25]. The second type I repeat of TSP-1 directly binds to CD36, blocking angiogenesis and modulates endothelial cell chemotaxis. Using the above model, the antiangiogenic effects of ABT-510, a TSP mimetic peptide containing the internal sequence from this domain [26], were also investigated. Elevated circulating endothelial cells in TSP-1 null mice were re-

stored to WT normal levels after 5 days of treatment with ABT-510 [27].

Results of this study point out for the first time that TSP-1 is a regulator of angiogenesis and inflammation in the DSS model, and to TSP-1 fragments or their mimetics as potential therapeutic alternatives for IBD.

Materials and Methods

Mice and Induction of Colitis

TSP-1-null mice on a 129Sv background were backcrossed 8 times to the C57BL/6 background [28]. They were hosted and bred at the vivarium facility of the Department of Pharmaceutical Sciences at Wilkes University. WT mice from the same background and age were purchased directly from Jackson Laboratories 2 weeks before the start of the trials. The Institutional Animal Care and Use Committee at Wilkes University approved all experiments performed with these mice. DSS (MP Biomedicals, Aurora, Ohio, USA) was administered to TSP-1^{-/-} 6-week-old males (n = 18) and WT mice (n = 13). TSP-1^{-/-} mice, which were drinking only distilled water, served as controls (n = 16). Except for the controls, mice were treated with 5% DSS dissolved in drinking water for 7 consecutive days. DSS consumption was monitored daily. Only bottles of the same capacity (4 oz) and antidrip system (Criticter Canteen, Pet International, Elk Grove Village, Ill., USA) were used for DSS treatment.

Pump Implantation and Peptide Treatment

WT mice (n = 13) and TSP-1^{-/-} mice (n = 11) were anesthetized and osmotic minipumps containing a solution with ABT-510 peptide were subcutaneously implanted. Pumps delivered ABT-510 peptide solution at controlled rates (0.5 μ l/h) and the dose was 60 mg/kg/day dissolved in 5% glucose sterile aqueous solution. Pumps containing only 5% glucose solution (5 W) as vehicle were also implanted in WT mice (n = 8). Twenty-four hours after the pump implantations mice drank 5% DSS for 7 days.

Disease Activity Indices

The clinical severity of the colitis was determined by disease activity indices daily according to the following parameters: stool consistency [formed feces (1), soft consistency (2), and liquid feces (3)], presence of blood in the feces [negative by guaiac paper (1), positive only with guaiac paper (2) and bloody feces (3)], and weight loss [absence of weight loss (0), <3 g (1), 3–6 g (2) and > 6 g (3)].

Histology and Immunohistochemistry

Colon tissues were fixed in 6% zinc formalin overnight and paraffin embedded. Incubation with purified rat anti-mouse CD31 (BD Pharmingen, San Diego, Calif., USA) was performed for 1 h. Slides were incubated with biotinylated goat anti-rat IgG (Vector Laboratories, Burlingame, Calif., USA) for 30 min, rinsed again and incubated with streptavidin-horseradish peroxidase (BD Pharmingen). For TSP-1 (Santa Cruz Biotechnologies, Santa Cruz, Calif., USA) and CD36 (Abcam, Boston, Mass., USA) a goat anti-mouse antibody was used followed by horseradish peroxidase or alkaline phosphatase (Santa Cruz Biotechnologies). The antibody recognizing TGF- β_1 (Santa Cruz Biotechnologies) was

already conjugated with horseradish peroxidase. Color was developed using a DAB substrate kit (BD Pharmingen) or alkaline phosphatase substrate kit (Vector Laboratories).

CD36 immunohistochemistry (IMH) was scored in a semi-quantitative fashion using both the intensity and the distribution of specific staining. From each 200 $\times$ field a percentage of positively stained cells was recorded as follows: negative staining was scored as 0, weak as 1, moderate as 2 and intense as 3. A total score (Hscore) was obtained by summing the percentage of positive cells. The resulting number was multiplied by the intensity of the staining. Epithelial and endothelial cells as well as the leukocytic infiltrate were evaluated separately.

Histopathologic Evaluation

The following parameters were measured: inflammation [none (0), mild (1), moderate (2) and severe (3)] and depth of the lesion [none (0), mucosal (1), mucosal and submucosal (2) and transmural (3)]. Crypt and villus damage was determined based on the depth of distortion and complete or incomplete lack of glandular and/or surface epithelium. It was graded independently of the grade of inflammation as none (0), basal (1), two thirds of damage (2), and complete loss of crypts and epithelium (3). Each section was also scored for focal or extensive ulceration. Lesions were defined as small and focal [two or less different lesions in 100 $\times$ power fields (1)], multifocal [more than two lesions in 100 $\times$ power fields (2)] or extensive [the ulceration extends throughout the whole 100 $\times$ field (3)].

Quantification of Microvascular Density

Microvascular density (MVD) was determined in colon sections from mice drinking DSS for 3 and 7 days. Immunostained sections were screened at low magnification ($\times 40$) to detect areas with high vascularization. Serial pictures were taken from these areas at high power ($\times 400$), covering both mucosa and submucosa. At least 5 microphotographs were taken from each area. Computer-digitized images were evaluated using a color digital camera (Olympus, Tokyo, Japan). The number of vessels/field (measured as mean vascular density) was assessed counting the vessels positive for CD31. All the sections were double-coded before taking pictures and analyzed by three authors (L.S.G., S.P. and L.D.).

Measurement of Apoptosis

Apoptotic cells were detected in colon samples; TUNEL labeling was performed according to a published method [20]. Briefly, tissue sections were incubated with 2 μ g/ml of trypsin (Sigma) for 10 min at room temperature. Sections were then covered with a buffer containing 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, and 1 mM CoCl₂. An aliquot of 0.2 μ l containing terminal deoxynucleotidyl transferase (Promega, Madison, Wisc., USA) and 10 μ M biotinylated 16-dUTP (Roche Diagnostics, Indianapolis, Ind., USA) was added to the sections. The slides were incubated in a humidified chamber at 37°C for 60 min. After that, slides were washed with a solution containing 5 M NaCl and 500 mM Na citrate. The sections were then incubated for 30 min with streptavidin-horseradish peroxidase (Vector Laboratories) and DAB was used as the chromogen. The apoptotic index was obtained counting the number of apoptotic cells per field. At least 5 different fields per section were evaluated in a blinded fashion.

Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) was assessed by rinsing colons with cold phosphate-buffered saline and stored at -70°C. Specimens were freeze-thawed 3 times. Tissues were suspended in a solution of 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma, St. Louis, Mo., USA) in 50 mM potassium phosphate buffer at pH 6.0 and sonicated. Suspensions were centrifuged at 40,000 g for 15 min and the supernatant assayed. 0.1 ml were mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma, St. Louis, Mo., USA) and 0.0005% hydrogen peroxide (H₂O₂). One unit of MPO activity was defined as the amount needed to degrade 1 μ mol of peroxide/min.

Determination of Protein Levels in Colons by ELISA

Immunoassays

Colon tissues were collected and cultured according to a published protocol [29]. Levels of VEGF, FGF, TGF- β ₁ activation, tumor necrosis factor alpha (TNF- α), interleukin-1 α (IL-1 α), interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), Fas ligand (FasL), MMPs (MMP-2, MMP-3, MMP-9) protein were determined in 100- μ l supernatants using commercially available enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, Minneapolis, Minn., USA) and following the manufacturer's instructions.

Statistical Analysis

Data were analyzed for significance by a one-way analysis of variance (ANOVA). Calculations were performed using the Stat-View system for Macintosh (Abacus Concepts, Berkeley, Calif., USA). $p < 0.05$ was considered significant. Where appropriate, values are expressed as the mean $\pm$ standard deviations.

Results

Clinical Severity of DSS-Induced Colitis

The clinical severity of the colitis was assessed by grading the presence of diarrhea, blood in feces and weight loss. Bleeding was observed in TSP-1^{-/-} as early as the second day of treatment (fig. 1a). Diarrhea also occurred earlier in TSP-1^{-/-} mice than in WT mice (fig. 1b). After 3 days of DSS treatment, TSP-1-deficient mice showed higher scores than WT in both parameters ($p < 0.0001$) with progressively deteriorating conditions (day 7, $p < 0.0001$) (fig. 1a, b). In mice treated with ABT-510 (osmotic pumps), bleeding and diarrhea were delayed and grading was significantly lower ($p < 0.0001$). TSP-1^{-/-} mice displayed more weight loss than WT by the 3rd day ($p < 0.0001$) of DSS treatment. After 7 days, the weight loss was considerably higher in TSP-1^{-/-} compared to WT and TSP-1^{-/-} mice treated with ABT-510 ($p < 0.0001$). WT mice under ABT-510 also showed diminished weight loss compared to WT mice injected with vehicle ($p = 0.0180$) (fig. 1c).

Gross Features of TSP-1 Null Mice Colons and Mice Treated with ABT-510

Generalized bleeding, edema and ulceration were grossly observed in TSP-1^{-/-} mice colons (fig. 1d) compared with the spotty lesions observed in WT mice colons (fig. 1e). TSP-1^{-/-} mice treated with ABT-510 showed spotty lesions, but bleeding and ulceration was clearly ameliorated (fig. 1f). Colonic tissues of TSP-1^{-/-} mice controls drinking only water showed solid feces and no signs of bleeding or ulceration (fig. 1g).

Histopathologic Analysis of DSS-Induced Colitis

The crypt damage was more accentuated in TSP-1^{-/-} ($p = 0.014$), the glandular component of the mucosa was more preserved in WT lesions (fig. 2a, g, arrows); depth of the lesions was also increased in TSP-1^{-/-} mice ($p = 0.0002$) (fig. 2b, g, h; double-headed arrows). The damage is observed along all the layer of the TSP-1-deficient mucosa. Inflammation was more severe and increasingly more intense in the deeper layers of the colonic wall in TSP-1^{-/-} mice ($p < 0.0001$) (fig. 2c). No pathological changes were observed in any of the colon samples from TSP-1^{-/-} mice drinking only water (fig. 2j). However, significant differences were observed in TSP-1^{-/-} treated with ABT-510 (fig. 2i). Crypt damage ($p = 0.0002$), depth ($p = 0.0857$) and inflammation ($p \leq 0.0001$) were significantly diminished in treated TSP-1^{-/-} mice (fig. 2a, b, c, i). Evaluation was also assessed in the small intestine separately. In general, there was less erosion and damage compared to the corresponding colons sections. The mucosa showed little damage with shortening of the villi, mild inflammation and cystic changes in both genotypes.

Fig. 1. Disease activity indices in mice treated with 5% DSS during 7 days. **a** TSP-1^{-/-} mice showed bleeding by the 2nd day of treatment, earlier than controls ($p < 0.0001$). By the 7th day, TSP-1^{-/-} mice showed a higher grade of bleeding and it was diminished in both types of mice treated with ABT-510. **b** Diarrhea also appeared earlier in TSP-1^{-/-} mice and it aggravated during the cycle ($p < 0.0001$). ABT-510 diminished diarrhea in both genotypes. **c** TSP-1^{-/-} mice suffered a major weight loss compared to WT and TSP-1^{-/-} mice treated with ABT-510 ($p < 0.0001$). WT mice also displayed loss of weight compared to WT treated ($p = 0.0180$). Colons from mice drinking DSS for 7 days showed extensive areas of edema and deep hemorrhage (**d**) compared with the WT controls (**e**). **f** Colons from mice treated with ABT-510 showed lesser hemorrhage and damage. **g** Colons from TSP-1^{-/-} mice drinking only water without any signs of bleeding or ulceration. * $p < 0.0001$; ** $p < 0.05$.

No differences were detected between the genotypes and treatments in villus damage. However, the depth of the lesions was increased in TSP-1-deficient colons compared to WT mice ($p = 0.0019$) (fig. 2e, k, l). The severity of the inflammation was higher in TSP-1^{-/-} and lower in TSP-1^{-/-} mice treated with ABT-510 ($p = 0.0153$ and $p = 0.0228$, respectively) (fig. 2f, k, l).

TGF- β , TSP-1 and CD36 IMH in Colons from WT and TSP-1^{-/-} Mice after 7 Days of DSS Treatment

TGF- β expression in colons of mice drinking only distilled water was observed mainly in the apical areas of the epithelium. In colonic tissues from DSS-treated mice, intense extracellular staining was observed, particularly in the submucosa. Cytoplasmic stain was also detected among some cells of the inflammatory infiltrate in both genotypes. Endothelial cells were strongly positive for TGF- β in all colonic layers, especially in the muscle layer in both genotypes. When total and active TGF- β protein levels were measured using ELISA, no statistical differences were observed between both genotypes (data not shown).

TSP-1 staining was localized in the extracellular matrix of submucosa. It was also observed among the leukocytic infiltrate and platelets inside of blood vessels. Areas of hemorrhage and fibrin deposition around blood vessels were also intensely stained. The glandular component showed little or negative stain for TSP-1. Most of the epithelial cells showed mild basal cytoplasmic staining. The stroma without inflammation or hemorrhage was also weakly positive. The intensity was directly related with the grade of inflammation in the tissue. The muscular layer and smooth muscle cells of blood vessels showed moderate staining. Interestingly, we observed decreased staining for the antibody in colons treated with ABT-510. Normal areas were weakly positive or almost negative and no staining for TSP-1 antibody was detected in TSP-1 colons used as negative controls (fig. 3a–d). Since CD36 is a major receptor for TSP-1, its immunolocalization was also determined in this study. CD36 was strongly expressed in normal colons. Epithelial and endothelial cells showed strong cytoplasmic staining. Smooth muscle cells and scattered leukocytes were also positive (fig. 3e). We observed few positive leukocytes among the inflammatory infiltrate in WT (fig. 3f) and TSP-1^{-/-} colons (fig. 3g) with colitis. However, CD36 staining in leukocytes was increased in both types of mice treated with ABT-510 (fig. 3h). Hscores of the leukocytic infiltrate were significantly increased in both types of mice treated with ABT-510 (fig. 4).

Fig. 3. TSP-1 IMH (blue color) was negative in TSP-1-deficient colon used as control (a). The WT colon under normal conditions showed weakly positive stain (b). Strong expression was observed in the extracellular matrix of submucosa. The leukocytic infiltrate, platelets, hemorrhage and fibrin deposition around blood vessels were also intensely positive. The glandular component showed little or negative stain for TSP-1 (c). A diminished TSP-1 staining was noticed in colonic sections from mice treated with ABT-510 (d). CD36 (brown color) was highly expressed in normal colons (e). The glandular component, endothelium, smooth mus-

cle cells as well as scattered leukocytes were positive (e). CD36 expression in WT colons (f) and TSP-1^{-/-} colons (g) with DSS-induced colitis was less intense compared with the staining of TSP-1^{-/-} colons treated with ABT-510 (h). TUNEL (nuclear brown) and CD31 (cytoplasmic blue) double-stained sections displayed apoptotic cells among epithelial cells in WT intestines (i). Few capillaries in the mucosa of WT intestines treated with ABT-510 were TUNEL positive (j). TSP-1^{-/-} colon treated with vehicle (k) and with ABT-510 (l) both displayed TUNEL-positive endothelial cells (arrows).

Fig. 2. Histopathologic evaluations of lesions in mice treated with DSS for 7 days. The crypt damage was more accentuated in TSP-1^{-/-} ($p = 0.008$) (a, g, h) and depth of the lesions was also increased in TSP-1^{-/-} mice ($p < 0.001$) (b, g, h). Overall inflammation was also increased in colons of TSP-1^{-/-} mice ($p < 0.0001$) (c). Crypt damage ($p = 0.0002$), depth ($p = 0.0857$) and overall inflammation was significantly diminished in TSP-1^{-/-} mice treated with ABT-510 compared with mice treated with the vehicle ($p < 0.0001$). Histological sections of TSP-1^{-/-} colons treated with ABT-510 showed less inflammation at all levels and more glandular and luminal epithelium was preserved (h). No lesions were observed in TSP-1^{-/-} mice drinking water (j, k) (100 $\times$ original

magnification). Villi displayed milder pathological features than in colons. In all the genotypes, shortening of the villi and cystic changes were observed in the mucosal compartment without erosions. No differences were observed regarding villus damage (d, k, l). TSP-1^{-/-} small intestines displayed lesions with leukocytic infiltration throughout the intestinal wall (k). TSP-1^{-/-} small intestines displayed deeper lesions compared to WT ($p = 0.0019$) (e). Inflammation was more severe in TSP-1^{-/-} small intestine (k) mice compared to WT (l) ($p = 0.0153$) and lesser in TSP-1^{-/-} intestines treated with ABT-510 ($p = 0.0228$) (f). * $p < 0.001$; ** $p < 0.05$. v = Vehicle; A = ABT-510.

Fig. 4. Analysis of CD36 in colons from WT and TSP-1^{-/-} mice with DSS-induced colitis treated or not with ABT-510. CD36 Hscores of the leukocytic infiltrate in WT colons with colitis were similar to Hscores detected in colons without disease. CD36 Hscores were significantly elevated in colonic tissues from WT and TSP-1^{-/-} mice treated with ABT-510 ($p = 0.0142$ and 0.0031 , respectively). v = Vehicle. ** $p < 0.05$.

Apoptotic Indices

Apoptosis was observed mainly at the mucosal and submucosal colonic layers and numerous TUNEL-positive nuclei were detected among the leukocytic infiltrate and luminal exudates. In the villi TUNEL-positive cells were seen among the epithelial cells and scattered leukocytes among the inflammatory infiltrate. Endothelial cell apoptosis was also assessed (fig. 3i–l). Many endothelial cells were TUNEL-positive in mice treated with ABT-510 (fig. 3j, l). However, the apoptotic indices were similar in WT or TSP-1^{-/-} mice treated or not with ABT-510.

Myeloperoxidase

MPO levels were higher in TSP-1^{-/-} mice ($p = 0.0184$) compared to WT mice under DSS and TSP-1^{-/-} mice treated with ABT-510 ($p = 0.0271$). No differences were detected in WT mice treated with ABT-510 compared with the WT without treatment (fig. 5a).

Protein Quantification of VEGF, Basic FGF, TNF- α , INF- γ , GMS, IL-1 α , IL-6, MMPs and FasL

Supernatants of colons of TSP-1^{-/-} mice drinking 5% DSS for only 3 days secreted highest levels of TNF- α ($p = 0.0493$) (fig. 5b). Levels of INF- γ , GM-CSF, IL-1 α , and IL-6 were all decreased in WT mice with only 3 days of colitis, but they did not reach significance (fig. 5c–f). The levels of these factors as well as the ones for basic FGF (bFGF), VEGF, MMP-3, MMP-9 and FasL in colons with 7 days of colitis did not show significance (data not shown). However, MMP-2 levels were significantly elevated in TSP-1^{-/-} mice at day 3 ($p = 0.0217$) (fig. 6a). At day 7, TSP-1-deficient colons secreted significant amounts of MMP-2 compared with WT ($p = 0.0025$) and were significantly lower after treatment with ABT-510 ($p = 0.0023$). Lower levels were also detected in WT mice after ABT-510 treatment, but they did not reach statistical significance (fig. 6c).

MVD in DSS-Induced Colitis in TSP-1^{-/-} Mice and Mice Treated with ABT-510 Peptide

In order to evaluate angiogenesis before extensive breakage and erosions develop, the colons from mice that were given 5% DSS for only 3 days were evaluated. This time limit was chosen because TSP-1^{-/-} mice started to bleed on the 2nd day. Results indicate that at this point in time, a higher number of blood vessels were observed in TSP-1^{-/-} mice ($p = 0.0118$) (fig. 6b, e) compared to WT colons at the same time point (fig. 6b, f). The evaluation of colon samples from these mice indicates that angiogenesis and leukocyte migration through blood vessels were focally located. These changes occurred at very early stages even before erosions were observed. At day 7 TSP-1^{-/-} mice also showed increased MVD (fig. 6d, g) compared with WT colons (fig. 6f, h). MVD was also significantly decreased in TSP-1^{-/-} colons after treatment with ABT-510 ($p < 0.0001$) (fig. 6f, i). No changes in MVD were observed in TSP-1^{-/-} mice drinking water (fig. 6j) and TSP-1^{-/-} treated with ABT-510 (without disease) ($n = 5.7 \pm 1.56$). Also no changes were detected between TSP-1^{-/-} and WT mice drinking water ($n = 5.7 \pm 2.45$).

Discussion

In this study, TSP-1-deficient mice were tested in a model of intestinal injury-inflammation. Using DSS as the inducer of colitis, TSP-1^{-/-} mice displayed higher MPO activity, more crypt damage and deeper lesions. The effects of the mimetic peptide ABT-510 in DSS-

Fig. 5. MPO assay and inflammation grading of colons from mice treated with DSS for 7 days (a). MPO levels were higher in TSP-1^{-/-} mice compared to WT mice under DSS and TSP-1^{-/-} treated with ABT-510: TSP-1^{-/-} vs. TSP-1^{-/-}/A, $p = 0.0271$; TSP-1^{-/-} vs. WT, $p = 0.0184$. TNF- α levels were higher in TSP-1^{-/-} colons after 3 days of DSS treatment ($p = 0.0493$) (b). Levels of IL-6 (c), INF- γ (d), IL-1 α (e) and GM-CSF (f) were increased in TSP-1^{-/-} but without statistical significance. A = ABT-510. ** $p < 0.05$.

induced colitis were also determined. ABT-510 is a nine-amino acid peptide closely related to an internal sequence from the second type I repeat of TSP-1. It is an antiangiogenic agent showing preclinical antitumor activity in both mouse models and spontaneous tumors in dogs and is in clinical trials as a cancer therapeutic [35–37]. These results indicate that this peptide ameliorates the clinical evolution of the disease in WT and TSP-1^{-/-} mice under DSS. Using the same concentrations of DSS, bleeding was significantly delayed and the overall severity of disease

was improved. Inflammation grading and angiogenesis were also significantly diminished.

Previous evidence indicates important anti-inflammatory functions of TSP-1 in vivo [20]. The TSP-1-deficient mice have shown leukocytosis and abnormal inflammatory cell infiltrates in the lung and pancreas. A possible explanation for these results could be the activation of TGF- β_1 by TSP-1. However, our results for protein quantification by ELISA and IMH indicated a similar grade of TGF- β_1 activation in the TSP-1^{-/-} mice as in

Fig. 6. Protein levels of MMP-2 and MVD. TSP-1^{-/-} colons secreted higher levels of MMP-2 by day 3 of DSS treatment ($p = 0.0217$) (**a**). MMP-2 was also elevated in these mice after 7 days of 5% DSS ($p = 0.0025$) and decreased with the treatment of ABT-510 ($p = 0.0023$) (**c**). Higher MVD were assessed in colitis lesions from TSP-1^{-/-} mice after 3 and 7 days of drinking DSS ($p = 0.0118$ and 0.0008 , respectively) (**b** and **d**, respectively). Fewer vessel counts were detected in TSP-1^{-/-} mice treated with ABT-510 compared to

TSP-1^{-/-} mice treated with vehicle ($p < 0.0001$) and WT ($p < 0.0001$) (**d**). CD31 IMH showed numerous microvessels (arrows) in TSP-1^{-/-} colonic lesions compared with the WT at day 3 (**e** and **f**, respectively) and day 7 (**g** and **h**, respectively). TSP-1^{-/-} mice treated with ABT-510 for 7 days displayed lesser microvessels in the lesions (**i**). No significant changes in vascular density were detected in TSP-1^{-/-} mice without colitis (**j**). * $p < 0.0001$; ** $p < 0.05$. A = ABT-510; v = vehicle.

the controls. TGF- β_1 activation may occur in colons of TSP-1^{-/-} mice by other compensatory mechanisms.

Alterations in the inflammatory response in TSP-1^{-/-} mice may also be the result of interactions between TSP-1 and CD47. CD47 or IAP is a widely expressed plasma membrane protein that modulates cell adhesion. CD47

null mice have a severe defect in leukocyte migration [30]. CD47 binds to TSP-1 as well as to integrins and receptors SIRP α_1 and SIRP β [31]. The binding of SIRP receptors in T cells with CD47 located in antigen-presenting cells directly enhances T cell proliferation. The TSP-1/CD47 axis modulates vascular-induced migration of monocytes to

the injured tissues, regulating the expression of ICAM-1 by endothelial cells [22]. TSP-1 secreted by dendritic cells can negatively regulate the secretion of cytokines during early activation through interactions with CD47 and CD36. These bindings can also inhibit early T cell activation, promoting naive T cell anergy [23].

Recently, CD36 has been reported to be downregulated in DSS-induced colitis [12]. Our findings indicate that protein expression is also diminished in colitis lesions and that this expression is inversely correlated to the grade of inflammation in the tissue. CD36 was intensely expressed in the normal colon of both genotypes. The damaged epithelium in DSS colitis was also positive for CD36. However, most of the leukocytic infiltrate showed little or no staining at all. Moreover, colons treated with ABT-510 showed a higher expression of CD36 when compared with TSP-1^{-/-} colons.

TSP-1 has been found to be upregulated in DSS-induced colitis [12]. These data indicate that TSP-1 in normal colon is barely noticed. However, strong expression of TSP-1 was observed in colons with DSS-induced colitis. TSP-1 was localized in leukocytes, hemorrhage and thrombus, important elements of any colitis; thus, the rise of TSP-1 in these tissues seems logical. A reduced expression of TSP-1 in WT colons treated with ABT-510 was also noticed. TSP-1 may be secreted at lower levels when compounds such as ABT-510 are available, reducing the angiogenic stimulus. It has been suggested that angiogenesis in DSS-induced colitis occurs through 'loss of angiogenic inhibition' [12]. These results demonstrate that the lack of TSP-1, a natural angiogenic inhibitor, significantly aggravates the colitis and underline the regulatory functions of TSP-1 which are critical for restoring homeostasis after tissue injury and disease.

The axis TSP-1/CD36 inhibits angiogenesis by inducing apoptosis in endothelial cells. Using TUNEL assay, positive cells were detected among epithelial cells, the leukocytic infiltrate, smooth muscle and endothelium. This is a model in which injury, inflammation and mucosal loss are produced by toxicity. TSP-1^{-/-} mice especially suffered a major damage in all the intestinal layers. Epithelial and endothelial cells will still undergo apoptosis or necrosis anyway, which may have affected our results. Some vessels negative for CD31 were observed in the vicinity of positive-stained ones. Weak or negative expression of CD31 and/or TUNEL-positive endothelial cells may be the consequence of necrosis due to intense vasculitis or ABT-510-mediated apoptosis. It has been reported that ABT-510 induces endothelial cell apoptosis by increasing FasL [34]. However, no differences in the soluble form of FasL were detected

among the groups, which may suggest a Bcl-2-mediated mechanism or one using another death receptor.

In vivo, mice with a targeted deficiency of TSP-1 have shown a lower number of blood vessels in the retina than their WT counterparts [32]. Tumors implanted in these mice displayed higher blood vessel numbers and vascular morphological abnormalities [33]. In this study, these mice developed a more severe clinical disease, early bleeding and intense deep hemorrhage. The earlier bleeding in TSP-1 mice seems to be a consequence of increased angiogenesis at earlier stages of DSS injury. Histological evaluation of samples from mice given DSS for 3 days indicates that the angiogenesis is an early event, which precedes any other morphological change. Previous studies have shown focal increase of HIF-1 α and VEGF as well as changes in the vascular permeability before morphological changes occur [4–6]. Recent data have demonstrated that active angiogenesis is critical in experimental colitis and antiangiogenic agents may be considered as new strategies for IBD treatment [15]. In this study, the secreted levels of angiogenic factors such as VEGF and bFGF were also analyzed but no significant changes were detected among the groups. However, TSP-1^{-/-} intestines from mice under multiple cycles of 2% DSS secrete higher levels of both VEGF and bFGF [38]. These results possibly suggest that VEGF and bFGF may be more critical in chronic inflammation.

Cytokines involved in mucosal barrier function and inflammation were investigated as well. As early as at 3 days significantly higher levels of TNF- α were detected in colitis lesions of TSP-1-deficient mice. TNF- α is secreted for many cells including endothelial cells. This cytokine may be the primary stimulus initiating early inflammatory events that will also increase angiogenesis and will maintain the inflammation-angiogenesis cycle.

Elevated levels of MMP-2 were found in TSP-1^{-/-} mice at days 3 and 7 after DSS treatment. Furthermore, significantly decreased levels of MMP-2 were also observed after 7 days of treatment with ABT-510. MMP-2 is a powerful angiogenic and mitogenic protein, which is secreted by activated T cells. This MMP plays an important role in leukocyte migration to injured tissues and angiogenesis. MMP-2 is activated by TSP-1 and TSP-2 [39], but they also promote the clearance of its proactivated form [40]. Moreover, TSP-1^{-/-} endothelial cells express high levels of MMP-2 [41]. MMP-2 expression has been previously described in IBD [42]. Interestingly, MMP-2 as TSP-1 expression is also localized in the extracellular matrix of inflamed submucosa. The results of this study strongly suggest a cross-talk between these factors in experimental colitis.

In conclusion, this work demonstrates the regulatory functions of TSP-1 in tissue injury, angiogenesis and inflammation and is the first report evaluating its role in an IBD model. TSP-1-derived peptides may be particularly helpful in Crohn's disease, preventing and targeting the most frequent transmural inflammation, delaying and ameliorating many of its clinical complications. These data certainly point out angiogenesis as an early pathological mechanism and the use of antiangiogenic peptides such as ABT-510 as a promising adjuvant therapy for IBD.

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