Role of microsomal prostaglandin E synthase-1 in the facilitation of angiogenesis and the healing of gastric ulcers

Takako Ae, Takashi Ohno, Youichiro Hattori, Tsutomo Suzuki, Kanako Hosono, Tsutomu Minamino, Takehito Sato, Satoshi Uematsu, Shizuo Akira, Wasaburo Koizumi, and Masataka Majima

1Department of Pharmacology, Kitasato University School of Medicine, 2Department of Molecular Pharmacology, Graduate School of Medicine, 3Department of Gastroenterology, Kitasato University School of Medicine, Kanagawa; and 4Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

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It is well known that NSAIDs, such as aspirin and indomethacin, not only damage the gastrointestinal mucosa but impair the healing of preexisting ulcers as well (10, 15, 33). NSAIDs are believed to reduce the levels of prostaglandins (PGs) due to their inhibition of cyclooxygenase (COX). Angiogenesis is the process involving the formation of new vasculature and is recognized as an important mechanism in tumor development and in chronic inflammation (16, 17). It is also accepted that the healing process of gastric ulcers is highly dependent on angiogenesis (22). We have reported previously that endogenous prostaglandins, especially PGE2, play a significant role in the enhancement of angiogenesis during tumor development and wound healing processes (2, 13). Mizuno et al. (19) first demonstrated that both COX-2 mRNA and its protein were expressed strongly in mouse stomachs in which ulcers had been induced. They also revealed that COX-2 inhibition noticeably delayed the healing process of gastric ulcers. These results suggested that the prostaglandin responsible for the enhancement of gastric ulcer healing might be PGE2.

Recent advances have led to the molecular identification and characterization of various enzymes involved in the biosynthesis of PGE2, including PLA2, COX, and terminal PG synthase (PGES) (20, 26). Each of the three enzymes is involved in steps that can be rate limiting for PGE2 biosynthesis and that involve multiple enzymes/isozymes that can act during different phases of pathological conditions. PGES, which catalyzes the conversion of PGH2 to PGE2, exists as both membrane-associated and cytosolic forms. There are two membrane-bound enzymes designated as mPGES-1 and mPGES-2 (25), of which mPGES-1 is a glutathione (GSH)-requiring perinuclear protein belonging to the MAPEG (membrane-associated proteins involved in eicosanoid and GSH metabolism) family (25). This enzyme is induced strongly by proinflammatory stimuli, is downregulated by anti-inflammatory glucocorticoids, and is coupled functionally with COX-2 in marked preference to COX-1. In fact, the expression of mPGES-1 is partly regulated by the mitogen-activated protein kinase pathways (11), where the kinases may switch on the inducible transcription factor Egr-1 that, in turn, binds to the proximal GC box in the mPGES-1 promoter, leading to mPGES-1 transcription (21). Induction of mPGES-1 expression has also been observed in various systems in which COX-2-derived PGE2 is thought to play a critical role, such as inflammation, fever, pain, female reproduction, tissue repair, and cancer (25).

The importance of PGE2 in various pathophysiological events emphasizes the need to understand the role of each PGES enzyme in vivo; however, there has been no report, so far, on the functional relevance of mPGES-1 in the healing of gastric ulcers and in angiogenesis, which is indispensable to the healing process. Since there are no inhibitors capable of acting on this enzyme with enough selectivity, we developed mPGES-1 knockout (KO) mice to analyze the pathophysiological roles of mPGES-1 (32). We have used this KO mouse to show that mPGES-1 plays an essential role in lipopolysaccharide (LPS)-stimulated delayed PGE2 production by macrophages, although these mice are fertile, develop normally after birth, and retain the LPS-stimulated production of various cytokines (32). In this study, we have clarified for the first time the role of mPGES-1 in ulcer-associated angiogenesis and in...
the healing processes using mPGES-1 KO mice. This study addresses the possible adverse effects in gastric tissues when a selective mPGES-1 inhibitor is used to treat patients with inflammation or cancers.

MATERIALS AND METHODS

Animals. We used 9-wk-old male mPGES-1 KO mice developed by us and backcrossed with C57BL6 and with their wild-type (WT) counterparts (C57BL6). All mice were kept in a room maintained at a constant temperature (25 ± 1°C) and humidity (60 ± 5%) throughout the experimental period and were allowed free access to normal chow and water. All experimental protocols were approved by Experimental Animal Ethics Committee at Kitasato University School of Medicine.

Induction of gastric ulcers. Gastric ulcers were induced by the serosal application of 100% acetic acid, as reported by us previously (22). The serosal area exposed to 100% acetic acid was 50 mm². The animals were fed normally thereafter.

Measurement of ulcerated area. The stomachs were removed and opened along the greater curvature. The ulcerated area was determined by use of ImageJ software in a blind manner.

Measurement of PGE₂. Levels of PGE₂ were measured as in our previous report (28). Briefly, the stomachs were removed and were immediately put into the liquid nitrogen. The frozen stomachs with ulcer or without ulcer were pulverized within a stainless cylinder cooled with liquid nitrogen. The powder of the stomach was immediately put into 10 ml of 80% ethanol containing 10⁻⁵ M indomethacin without thawing and mixed well. After centrifugation at 13,000 g for 30 min at 4°C, the supernatant was dried with reduced pressure. The residue was resuspended with distilled water (5 ml) and was acidified to pH 3 with addition of 1 N HCl. Acidified samples were applied to the SepakC18, and PG fractions were extracted with ethylacetate (7 ml) after washing of the column with distilled water and ethanol. The extract was dried under reduced pressure, and the residue was dissolved with assay buffer. The levels of PGE₂ were determined with EIA kit (Cayman Chemical) according to manufacturer’s instructions.

Immunohistochemistry. The tissues including the ulcer were immediately fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated with a graded series of ethanol solutions, and embedded in paraffin (30). We then cut 3-μm sections from the paraffin-embedded tissue. For the evaluation of angiogenesis, immunohistochemical staining was performed by using a polymer-based detection system with secondary antibodies and reporter molecules attached to an inert polymer backbone (DAKO). In brief, following hydration, specimens were treated with 0.03% H₂O₂, and then slides were autoclaved in Tris-EDTA buffer (pH 9.0) for CD31 staining or in sodium citrate buffer (pH 6.0) for mPGES-1 staining at 121°C for 15 min for antigen retrieval. After preincubating with 10% bovine serum albumin for 60 min, the specimens were incubated with anti-CD31 rabbit polyclonal antibody (Abcam; 1:400 dilution) or anti-mPGES-1 rabbit polyclonal antibody (Cayman Chemical; 1:1,600 dilution) at 4°C overnight. Immunoreactive signals were detected with 3,3'-diaminobenzine. The specimens were counterstained with Mayer’s hematoxylin and were dehydrated, cleared, and mounted.

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Microvessel density in the ulcer granulation tissues was assessed as a parameter of angiogenesis according to established methods described previously (34). In brief, after the area of highest microvessel density was identified at low power, the individual microvessels were counted on a x400 field in this area.

Real-time PCR. Transcripts encoding mPGES-1, cyclooxygenase (COX)-2, CD31, basic fibroblast growth factor (bFGF), transforming growth factor-β₁ (TGF-β₁), connective tissue growth factor (CTGF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR analysis. The expression of each mRNA at a particular time point after induction of the gastric ulcer was determined as follows: total RNA from the granulation tissue was extracted by use of TRIzol reagent (Invitrogen), and the amount of RNA was measured by BioPhotometer (Eppendorf). Subsequently, we performed first-strand cDNA synthesis from total RNA with 1 μg of total RNA, 200 U of ReverTra Ace (Reverse transcriptase, Toyobo), 40 nmol dNTP mixture (Toyobo), 20 U of RNase inhibitor (Toyobo), and 20 pmol of oligo(dT)₁₂₋₁₈, in a total volume of 40 μl. The reactions were incubated initially at 30°C for 10 min and then at 42°C for 40 min, followed by inactivation of the reaction at 99°C for 5 min. The real-time PCR primers were designed using Primer3 software (http://primer3.sourceforge.net/) based on the GenBank data. The primers for real-time PCR were shown in Table 1. The expression of each mRNA in gastric mucosa without ulcer was measured as control.

ELISA. Gastric ulcer samples were homogenized with 0.1% Tween 20 in PBS containing Complete Protease Inhibitor Cocktail (Roche Diagnostics) and centrifuged at 13,000 g for 5 min. Supernatants were used to determine active TGF-β₁ levels with ELISA kits (R&D System) and bFGF levels with ELISA kits (RayBiotech), according to the manufacturer’s instructions. All levels were normalized to total protein concentrations as determined by Quant-iT assay kit (Invitrogen).

Statistical analysis. Data are shown as means ± SE. Comparisons among multiple groups were performed by factorial ANOVA followed by Bonferroni t-test. Comparisons between two groups were performed with Student’s t-test. Statistic analyses were carried out with SPSS (version 17). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Time course of the healing processes in acetic acid-induced ulcers and upregulations of COX-2 and mPGES-1 in ulcer lesions. Mucosal damage was induced when 100% acetic acid was exposed briefly to the serosa of the stomach on day 0 in WT C57Bl6 mice. The average ulcerated area on day 3 in WT mice was 38.80 ± 3.19 mm² (n = 6), and the area was gradually reduced thereafter (Fig. 1A). By day 14, the size of the ulcer was markedly reduced to 1.03 ± 0.21 mm² (n = 8) in WT mice, less than 3% of the area exposed to acetic acid (Fig. 1A).

Real-time PCR was performed to determine the expression of two enzymes that are related to biosynthesis of PGE₂, COX-2, and mPGES-1 (Fig. 1, B and C). In WT mice at day 3,

Table 1. Sequences of primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tr>
<td>mPGES-1</td>
<td>5'-AGATGCGTGGAAAGTTGGAAGC-3'</td>
<td>5'-CAGGAGGAGGGAGGCTATG-3'</td>
</tr>
<tr>
<td>COX-2</td>
<td>5'-TGGTGCTAGAGAAAATAGGGG-3'</td>
<td>5'-CATCATATTTGAGGCTTGGG-3'</td>
</tr>
<tr>
<td>CD31</td>
<td>5'-AGACAGCGAGAGATTGGGAGG-3'</td>
<td>5'-GCAACTATTAAAGTGGCAGG-3'</td>
</tr>
<tr>
<td>bFGF</td>
<td>5'-GGCTGTGCTGCTTCTTAAAGG-3'</td>
<td>5'-TCTGCGCTGAGCTAAGTATG-3'</td>
</tr>
<tr>
<td>TGF-β₁</td>
<td>5'-AACAAATTTGCTGGTTGACCT-3'</td>
<td>5'-TGTATCCGTCTCTGTGGCTT-3'</td>
</tr>
<tr>
<td>CTGF</td>
<td>5'-AACCCGGGAAGGGAATTAATAGG-3'</td>
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<tr>
<td>GAPDH</td>
<td>5'-ACATCAAGAAAGGGTGGAAGG-3'</td>
<td>5'-AACGGTCAAGATGCGAGTGG-3'</td>
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in WT mice was markedly upregulated in the ulcer tissues compared with normal gastric tissues without ulcers, and the induced levels were kept at fairly high levels throughout the experimental periods (Fig. 1C).

Localization of mPGES-1 in gastric ulcer lesions. To see the localization of mPGES-1 in the ulcer tissues, we performed immunohistochemistry using a mPGES-1 antibody. Figure 2 show the representative immunohistochemical staining for mPGES-1 in gastric ulcer lesions at day 7 and day 14. The mPGES-1-positive cells (brown) are located mainly in the ulcer bed, which was spread under the epithelial cells under the process of reepithelialization. There are more mPGES-1-positive cells at day 7 than day 14. This was consistent with real-time PCR results shown in Fig. 1C. The mPGES-1-positive cells were mononuclear cells accumulated in the ulcer base granulation tissues. Interestingly, there were not mPGES-1-positive endothelial cells under the process of angiogenesis.

Increase in the level of PGE2. The level of PGE2 in WT mice without ulcers was $353.4 \pm 111.5$ pg/ml ($n = 6$). The induction of gastric ulcer at day 3 increased the level of PGE2 in WT mice by 16.7 times (WT with ulcers $5,908.0 \pm 1,108.5$ pg/ml; $n = 6$), and that in KO mice with ulcers was less than in WT with ulcers (KO with ulcers $735.9 \pm 160.0$ pg/ml, $n = 6$) (Fig. 3).

Delay in healing of acetic acid-induced ulcers in mPGES-1 KO mice. At day 3, the average ulcerated area in WT mice was $38.80 \pm 3.19$ mm$^2$ ($n = 6$), and that in KO mice was $46.8 \pm 6.44$ mm$^2$ ($n = 5$). The difference between WT mice and KO mice is not statistically significant at day 3. When measured at day 7, compared with WT, the healing process of acetic acid-induced ulcers in mPGES-1 KO mice was significantly delayed (Fig. 4A, WT $14.7 \pm 1.1$ mm$^2$, KO $23.9 \pm 3.8$ mm$^2$). This delay was also seen in mice at day 10 and at day 14 (day 10; WT $4.42 \pm 0.68$ mm$^2$, KO $9.19 \pm 1.58$ mm$^2$, $n = 11–12$, $P < 0.01$, day 14; WT $1.04 \pm 0.21$ mm$^2$, KO $3.63 \pm 0.56$ mm$^2$, $n = 6–8$, $P < 0.01$). The levels of COX-2 expression at day 7 with ulcers were higher than controls both in WT mice and in KO mice. The levels of COX-2 expression at day 7 in gastric tissue with ulcer in WT mice and mPGES-1 KO mice were not different (Fig. 4B). This was also true in mice at day 14. On the other hand, mPGES-1 expression at day 7 in mPGES-1 KO mice was negligible, although the substantial expression of mPGES-1 was detectable in WT mice (Fig. 4C). At day 14, mPGES-1 expression was also quite low in mPGES-1 KO mice in spite of the substantial expression in WT mice. Figure 4D shows the typical appearance of gastric ulcers in WT and mPGES-1 KO mice at day 7. The damaged area was greater in mPGES-1 KO mice than in WT. Figure 4E shows hematoxylin and eosin staining results for ulcers in WT and mPGES-1 KO mice at day 7. The size of ulcer in mPGES-1 KO mice was larger than that in WT mice, judging from the defects of gastric mucosal epithelium. The thickness of the ulcer granulation tissues was small in mPGES-1 KO mice compared with WT mice. The same was true in mice at day 14. Sometimes we observed the hepatic tissues adhered to the bottom of ulcer beds more frequently in mPGES-1 KO mice.

Suppressed angiogenesis in ulcer granulation tissue in mPGES-1 KO mice. The expression of CD31, used as a marker for angiogenesis in the ulcer granulation tissues in WT mice, was markedly elevated at day 7 compared with that of control

Figure 1. Time course in the healing process of acetic acid-induced ulcers and upregulation of cyclooxygenase (COX)-2 and microsomal PGE synthase 1 (mPGES-1) in ulcer lesions in wild-type (WT) mice. A: time course of changes in ulcer area in WT mice. Values are means ± SE for WT ($n = 7–13$). It took about 14 days after acetic acid-induced ulcers induction to heal macroscopically in WT. B: time course of changes in COX-2 mRNA levels in ulcer lesions and normal mucosa in WT mice ($n = 7–8$). Values are ratios of COX-2 to GAPDH mRNA and represent means ± SE. The ANOVA was used to evaluate the significance of differences between measurements ($^*P < 0.05$, $^{**}P < 0.01$). C: time course of changes in mPGES-1 mRNA levels in ulcer lesions and normal mucosa in WT mice ($n = 7–8$). Values are ratios of mPGES-1 to GAPDH mRNA and represent means ± SE. The ANOVA was used to evaluate the significance of differences between measurements ($^{**}P < 0.01$).
in WT. The expression of CD31 in the ulcer granulation tissues in mPGES-1 KO mice was markedly suppressed at day 7 compared with that in WT (Fig. 5A). Immunohistochemistry, using an anti-CD31 antibody, revealed that a microvessel density of 46.2 ± 3.8 vessels/×400 field (n = 5) in the ulcer granulation tissues in mPGES-1 KO mice at day 7 was significantly less (P < 0.01) than that in WT (68.2 ± 3.8 vessels/×400 field, n = 5; Fig. 5B). Figure 5C shows a typical CD31 immunostaining result from acetic acid-induced ulcer tissues at day 7. The endothelial cells in the vessels are stained brown. We observed microvessels in the granulation tissues in WT mice more than those in mPGES-1 KO mice (Fig. 5C).

Reduced expression of proangiogenic growth factors in ulcer granulation tissues from mPGES-1 KO mice. Real-time PCR was performed to determine the expression of proangiogenic growth factors in ulcer granulation tissues (Fig. 6, A–C). The expressed levels of the growth factors including TGF-β1 (Fig. 6A), bFGF (Fig. 6B), and CTGF (Fig. 6C) in the ulcer granulation tissues at day 7 were markedly elevated compared with controls both in WT mice and in KO mice. The expressed levels of the growth factors in the ulcer granulation tissues in mPGES-1 KO mice at day 7 were markedly suppressed compared with those in WT mice.

ELISA was performed to determine the expression of active TGF-β1 and bFGF at day 7. The protein level of active TGF-β1 at day 7 were significantly greater in WT mice compared with KO mice (WT 15.30 ± 0.47 pg/mg of total protein, n = 10; KO 12.67 ± 0.77 pg/mg of total protein, n = 7; P < 0.05, Fig. 6D). The protein level of bFGF at day 7 were significantly greater in WT mice compared with KO mice (WT 1,277.2 ± 88.7 pg/mg of total protein, n = 10; KO 945.1 ± 107.4 pg/mg of total protein, n = 7; P < 0.05, Fig. 6E).

DISCUSSION

In the experiments described in this report, we used mPGES-1 KO mice, developed in our laboratory, to show for the first time that the expression of mPGES-1 is upregulated in ulcer tissue and that it plays significant roles in the facilitation of angiogenesis in the granulation tissue formed at the base of ulcers and in the facilitation of gastric ulcer healing.

It has been shown that mPGES-1, an inducible PGE synthase, is linked to inflammation (5, 35), pain (31), fever (7, 12), stroke (12), and tumorigenesis (6, 36). Thus mPGES-1 could be a novel therapeutic target in the treatment of various pathologies, allowing the development of alternatives to the classical NSAIDs and selective COX-2 inhibitors. Although the constitutive expression of mPGES-1 in the gastric mucosa has been reported (4), we have shown here that mPGES-1 is upregulated at ulcer sites (Fig. 1C). The expression of mPGES-1 has been reported in human gastric mucosa (9); however, its precise roles in the maintenance of the integrity of the gastric mucosa and in protecting against the substances that elicited the mucosal damage have not been fully elucidated. A role for mPGES-1 in ulcer healing is certainly anticipated; however, there is no reliable inhibitor that selectively inhibits mPGES-1 activities.
In this study, the difference between the average ulcered area at day 3 in WT mice and in KO mice is not statistically significant. Tarnawski (29) reported that granulation tissue develops at the ulcer base within 48–72 h after ulceration. As our laboratory reported previously (22), the angiogenic responses were switched on at the early stage of the ulcer healing. Although not statistically significant, it is not so much surprising that the ulcer area is tended to be larger in mPGES-1 KO mice. Furthermore, we incorporated the temporal results on ulcer healing processes. mPGES-1 KO mice allowed us to study the role of mPGES-1 in the ulcer healing processes. Although it was reported that COX-2 was involved in the repair process of gastric ulcer lesions in mice (19), the particular PGs involved had not been identified. The present study has clarified that PGE2 may be the predominant molecule in the enhancement of ulcer healing, judging from the results obtained from mPGES-1 KO mice.

Ulcer healing, a genetically programmed repair process includes several events including inflammation, cell proliferation, reepithelialization, formation of granulation tissue, angiogenesis, interactions between various cells and the matrix, and tissue remodeling, all of which are also seen in wound healing processes (2, 13). Angiogenesis is also important in ulcer healing. These events are controlled by cytokines and growth factors (EGF, PDGF, HGF, TGF-β, VEGF, angiopoietins) and by transcription factors activated by tissue injury in a spatially and temporally coordinated manner. These growth factors trigger mitogenic and survival pathways utilizing Ras, MAPK, PI-3K/Akt, PLC-γ, and Rho/Rac/actin signaling.

TGF-β is well known as a multifunctional cytokine that regulates many biological processes, including cell proliferation, differentiation, adhesion, intercellular signaling, and the accumulation of extracellular matrix proteins and thus has an essential role during wound healing and tissue repair (23, 24). Many studies over recent years have suggested that TGF-β plays a crucial role in both inflammation and tissue injury repair. Polonikov et al. (23) showed that TGF-β may play an essential role in peptic ulcer disease, and proposed that the biological effects of TGF-β, an isoform of TGF-β, are divided into four groups. Firstly, TGF-β is likely to be needed to restrict local damage to the gastric mucosa after acute injury. Secondly, TGF-β1 impairs cell growth and proliferation and directly stimulates angiogenesis. Thirdly, TGF-β1 increases immunosuppression, and fourthly, TGF-β1 repairs tissue injury and promotes ulcer healing. One of the properties of TGF-β1 is its ability to induce fibrosis and promote the accumulation of the extracellular matrix (3, 18, 23, 24). TGF-β1 also induces fibroblast proliferation and stimulation of collagen synthesis, and it enhances extracellular matrix accumulation via the induction of CTGF (23, 27). TGF-β1 can inhibit matrix metalloproteinases and promotes the formation of elastin. All of
these processes lead to scar tissue formation, fibrosis, and ulcer healing (23).

Amagase et al. (1) showed previously that bFGF is essential for angiogenesis in the healing of gastric ulcers induced by alendronate. In addition, Ernst et al. (8) demonstrated that local injection of bFGF to the base of gastric ulcers significantly accelerated healing, in association with an increase in the amount of microvasculature and mucosal blood flow in the ulcerated area. Moreover, they showed that the neutralization of endogenous bFGF, using a specific antibody, caused a marked delay in gastric ulcer healing, accompanied by reduced angiogenesis.

CTGF is reported to be a downstream mediator for TGF-β. The expression of TGF-β, CTGF, and Type III collagen mRNA in indomethacin-induced gastric ulcers in rat was investigated previously. In situ hybridization revealed CTGF mRNA-positive cells in fibroblast-like cells and in some of the blood vessels on days 1, 3, and 7 after ulcer induction. The reported findings indicated that growth factor CTGF, together with TGF-β, participates in gastric ulcer healing by regulating connective tissue formation and angiogenesis (14). Our present results also indicate that the expression of CTGF is regulated by mPGES-1 activity. Taken together, the results suggest that these growth factors upregulate angiogenesis, a process that is indispensable to ulcer healing.

In conclusion, our present data suggest that mPGES-1 enhances ulcer healing and angiogenesis, which is crucial to ulcer healing. We observed the upregulation of TGF-β, bFGF, and CTGF in the ulcer granulation tissues, which implies that these factors enhance angiogenesis. A selective mPGES-1 inhibitor, if developed in the near future, should be used carefully in patients with gastric ulcers.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Majima M, Hayashi I, Muramatsu M, Katada J, Yamashina S, Katori M. Cyclo-oxygenase-2 enhances basic fibroblast growth factor-induced angiogenesis in ulcer granulation tissues and in mucosa without ulcer (control) in mPGES-1 KO mice and WT mice at day 7. Values are ratios of TGF-β1 against GAPDH mRNA and represent means ± SE (n = 7–8, *P < 0.05, **P < 0.01, ANOVA). B: expression of connective tissue growth factor (CTGF) in ulcer granulation tissues and in mucosa without ulcer (control) in mPGES-1 KO and WT mice at day 7. Values are ratios of CTGF to GAPDH mRNA and represent means ± SE (n = 7–8, *P < 0.05, **P < 0.01, ANOVA). C: expression of basic fibroblast growth factor (bFGF) in ulcer granulation tissues and in mucosa without ulcer (control) in mPGES-1 KO mice and WT mice at day 7. Values are ratios of bFGF to GAPDH mRNA and represent means ± SE (n = 7–8, *P < 0.05, Student’s t-test).


