1521-0103/373/1/1–9$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.119.263145
J Pharmacol Exp Ther 373:1–9, April 2020

Leukotriene B_4 Receptor Type 2 Accelerates the Healing of Intestinal Lesions by Promoting Epithelial Cell Proliferation[S]

Yui Matsumoto, Yukiko Matsuya, Kano Nagai, Kikuko Amagase, Kazuko Saeki, Kenjiro Matsumoto, Takehiko Yokomizo, and Shinichi Kato

Division of Pathological Sciences, Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Kyoto, Japan (Yui.M, Yuk.M., K.N., K.A., K.M., S.K.); Laboratory of Pharmacology and Pharmacotherapy, College of Pharmaceutical Sciences, Ritsumeikan University, Shiga, Japan (K.A.); and Department of Biochemistry, Juntendo University Graduate School of Medicine, Tokyo, Japan (K.S., T.Y.)

Received October 8, 2019; accepted January 2, 2020

ABSTRACT

Leukotriene B_4 receptor type 2 (BLT2) is a low-affinity leukotriene B_4 receptor that is highly expressed in intestinal epithelial cells. Previous studies demonstrated the protective role of BLT2 in experimentally induced colitis. However, its role in intestinal lesion repair is not fully understood. We investigated the role of BLT2 in the healing of indomethacin-induced intestinal lesions in mice. There was no significant different between wild-type (WT) and BLT2-deficient (BLT2KO) mice in terms of the development of indomethacin-induced intestinal lesions. However, healing of these lesions was significantly impaired in BLT2KO mice compared with WT mice. In contrast, transgenic mice with intestinal epithelium–specific BLT2 overexpression presented with superior ileal lesion healing relative to WT mice. An immunohistochemical study showed that the number of Ki-67–proliferative cells was markedly increased during the healing of intestinal lesions in WT mice but significantly attenuated in BLT2KO mice. Exposure of cultured mouse intestinal epithelial cells to CAY10583, a BLT2 agonist, promoted wound healing and cell proliferation in a concentration-dependent manner. Nevertheless, these responses were abolished under serum-free conditions. The CAY10583-induced proliferative effect was also negated by Go6983, a protein kinase C (PKC) inhibitor, U-73122, a phospholipase C (PLC) inhibitor, LY255283, a BLT2 antagonist, and pertussis toxin that inhibits G protein–coupled receptor signaling via $G_{i/o}$ proteins. Thus, BLT2 plays an important role in intestinal wound repair. Moreover, this effect is mediated by the promotion of epithelial cell proliferation via the $G_{i/o}$ protein–dependent and PLC/PKC signaling pathways. The BLT2 agonists are potential therapeutic agents for the treatment of intestinal lesions.

SIGNIFICANCE STATEMENT

The healing of indomethacin-induced Crohn's disease–like intestinal lesions was impaired in mice deficient in low-affinity leukotriene B_4 receptor type 2 (BLT2). They presented with reduced epithelial cell proliferation during the healing. In contrast, healing was promoted in mice overexpressing intestinal epithelial BLT2. In cultured intestinal epithelial cells, the BLT2 agonist CAY10583 substantially accelerated wound repair by enhancing cell proliferation rather than migration. Thus, BLT2 plays an important role in the intestinal lesions via acceleration of epithelial cell proliferation.

Introduction

Leukotriene B_4 receptor type 2 (BLT2) is a low-affinity leukotriene B_4 receptor activated by various eicosanoids, including 12(*S*)-hydroxyeicosatetraenoic acid, 15(*S*)-hydroxyeicosatetraenoic acid, and 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT) (Yokomizo et al., 2001; Okuno et al., 2008). The 12-HHT is a byproduct of thromboxane A_2 via cyclooxygenase (COX) followed by thromboxane synthase. It is an endogenous BLT2 ligand (Okuno et al., 2008). BLT2 is highly expressed in the epithelia of the small intestine and colon and in skin keratinocytes (Iizuka et al., 2005; Liu et al., 2014). BLT2-deficient (BLT2KO) mice presented with aggravation of dextran sulfate sodium–induced colitis via impairment of the epithelial barrier (Iizuka et al., 2010). BLT2 overexpression in Madin-Derby canine kidney II epithelial cells exhibited increased transepithelial electrical resistance and decreased fluorescein isothiocyanate–dextran leakage via upregulation of the major tight junction component claudin 4 (Ishii et al., 2016). Thus, BLT2 helps maintain epithelial barrier function and integrity.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport, and Culture of Japan [Grant 19K07109 to K.M., Grant 18K06692 to K.A., Grant 18K6923 to K.S., Grant 15H05904, and Grant 18H02627 to T.Y.] and the Takeda Science Foundation (to K.M.).
Y.M., Y.M., and K.N. contributed equally to this work.
The authors have no conflicts of interest to disclose.
https://doi.org/10.1124/jpet.119.263145.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: 12-HHT, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid; BLT2, leukotriene B_4 receptor type 2; BLT2KO, BLT2 deficient; COX, cyclooxygenase; EGF, epidermal growth factor; IGF, insulin-like growth factor; IL, interleukin; MPO, meloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; TBP, TATA-binding protein; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; villin-BLT2-Tg, transgenic with intestinal epithelium–specific overexpression of BLT2; WT, wild type; YAMC, young adult mouse colon.

Earlier studies disclosed that skin wound healing was delayed in BLT2KO mice relative to wild-type (WT) mice. A synthetic BLT2 agonist accelerated skin wound healing in both normal and diabetic *db/db* mice (Liu et al., 2014). An in vitro scratch assay on primary keratinocytes and keratinocyte cell lines showed that BLT2 activation accelerated re-epithelialization by promoting cell migration (Liu et al., 2014). BLT2 overexpression and activation enhanced bronchial epithelial cell proliferation and migration (Liu et al., 2018). COX inhibition by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and diclofenac inhibited 12-HHT synthesis and delayed skin and corneal wound healing (Liu et al., 2014; Iwamoto et al., 2017). These findings suggest that BLT2 plays important roles in skin and bronchium healing by promoting epithelial cell proliferation and migration.

The intestinal epithelium maintains mucosal homeostasis and regulates inflammation and wound repair and healing. Disruption of these functions results in inflammatory bowel disease and chronic mucosal inflammation. Efficient epithelial repair and healing are vital for the reversal of mucosal inflammation and the recovery of homeostasis (Leoni et al., 2015a,b). Epithelial cell migration and proliferation are closely associated with physiologic remodeling and wound healing after tissue injury (Yamaoka et al., 2014; Seidelin et al., 2015).

NSAIDs are commonly prescribed for the treatment of various inflammatory diseases. However, they may injure the stomach and small intestine (Bjarnason et al., 1993; Yamada et al., 1993; Konaka et al., 1999). There are pathomorphological similarities between human Crohn's disease and indomethacin-induced small intestinal lesions in rodents (Anthony et al., 2000). Thus, indomethacin-induced mouse intestinal lesions may be one of only a few animal models for intestinal lesions. We reported that daily NSAID administration impaired healing in indomethacin-induced intestinal lesions by inhibiting COX and reducing prostaglandins biosynthesis (Takeuchi et al., 2007, 2010). We hypothesized that 12-HHT/BLT2 and prostaglandin/E-type prostaglandin (EP) receptors may participate in the healing of intestinal lesions.

Here, we focused on the role of BLT2 in the healing of intestinal lesions by using BLT2KO mice and transgenic mice with intestinal epithelium–specific BLT2 overexpression (villin-BLT2-Tg mice) in an indomethacin-induced Crohn's disease–like ileitis model. We also cultured murine intestinal epithelial cells in an in vitro wound healing model.

Materials and Methods

Animals. BLT2-deficient mice were generated as previously described and backcrossed with BALB/c mice for >10 generations (Iizuka et al., 2010). Villin-BLT2-Tg mice were generated by expressing FLAG-tagged murine BLT2 under the control of the villin-promoter as previously described. They were backcrossed with C57BL/6 mice (Ishii et al., 2016). The BALB/c and C57BL/6 mice were purchased from SLC (Shizuoka, Japan) and served as the respective wild types. Male BLT2KO and WT mice weighing 24–28 g and male villin-BLT2-Tg and WT mice weighing 22–25 g were used in the experiments. All mice were maintained in plastic cages at 22 ± 1°C under a 12-hour light/dark cycle and had free access to food and water. They were separated using the pharmacological random comparison group method. All animal experiments were approved by the Committee on the Ethics of Animal Research of Kyoto Pharmaceutical University (PETH-19-004).

Indomethacin-Induced Intestinal Lesions. The mice were fasted for 18 hours, allowed free access to food for 2 hours, and administered subcutaneous indomethacin (Sigma-Aldrich Corp., St. Louis, MO). Indomethacin was suspended in saline with a drop of Tween 80 (Wako Pure Chemical Industries Ltd., Osaka, Japan). The dosages were at 8 mg/kg for BALB/c mice and 10 mg/kg for C57BL/6 mice. Normal mice received vehicle only (Tween 80/saline). To highlight hemorrhagic intestinal lesions, each mouse was intravenously injected with 100 μl of 1% (w/v) Evans blue solution at 30 minutes before sacrifice (Kawahara et al., 2011). The mice were sacrificed at 24, 48, 72, and 96 hours after indomethacin injection. Their small intestines were excised, fixed in 2% (v/v) formalin, opened longitudinally, and examined for lesions under a dissecting microscope (S6D; Leica, Wetzlar, Germany) at ×10. The areas (square millimeter) of the visible lesions were measured after blinding the investigators to the group assignments. The intestinal tissues were fixed in 10% (v/v) neutralized formalin, embedded in paraffin, sectioned at 4 μm thickness, and stained with H&E. Histologic injury was observed under a microscope (BX51; Olympus, Tokyo, Japan) at ×100.

Determination of Myeloperoxidase Activity. Myeloperoxidase (MPO) activity was determined using a previously reported method (Kato et al., 2012) with modifications. The animals were sacrificed 72 hours after indomethacin injection, and their small intestines were excised. The tissues were homogenized in 50 mM phosphate buffer containing 0.5% (w/v) hexadecyltrimethylammonium bromide (pH 6.0; Wako Pure Chemical Industries Ltd.). The homogenized samples were subjected thrice to freeze-thawing and centrifuged at 2000*g* and 4°C for 10 minutes. MPO activity was determined by adding 5 μl supernatant to 45 μl of 10 mM phosphate buffer (pH 6.0) and 50 μl of 1.5 M *o*-dianisidine HCl (Sigma-Aldrich Corp.) containing 0.0005% (v/v) hydrogen peroxide. Changes in absorbance at λ = 450 nm were recorded on a MULTISKAN 60 (Thermo Fisher Scientific Inc., Waltham, MA). The quantities of MPO were interpolated from a standard curve for commercial MPO (Sigma-Aldrich Corp.). Protein content was determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). The MPO activity was recorded in micromoles H_2O_2/mg protein.

Determination of Cytokine Expression by Quantitative Reverse-Transcription Polymerase Chain Reaction. The animals were sacrificed 72 hours after indomethacin injection, and their small intestines were excised. Total RNA was extracted from the tissues with Sepasol RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan), reverse-transcribed with PrimeScript RT Master Mix (TaKaRa Bio Inc., Kusatsu, Shiga, Japan), and analyzed by quantitative polymerase chain reaction on a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.) with SYBR Premix ExTaq II (TaKaRa Bio Inc.). Predesigned primer sets for mouse interleukin (IL)-1β (MA025939), tumor necrosis factor (TNF)-α (MA097070), insulin-like growth factor (IGF)-1 (MA118254), epidermal growth factor (EGF) (MA114367), vascular endothelial growth factor (VEGF)-A (MA165085), and TATA-binding protein (TBP) (MA050367) were obtained from the Perfect Real-Time Supporting System (TaKaRa Bio Inc.). The specific primer set used for BLT2 detection was forward, 5′-ACAGCCTTGGCTTTCTTCAG-3′, and reverse, 5′-TGCCCCATTACTTTCAGCTT-3′. The mRNA expression level was standardized to that of TBP.

Determination of Intestinal Epithelium Proliferation. The proliferation of the intestinal epithelium was determined immunohistochemically. The animals were sacrificed 72 hours after indomethacin injection, and their small intestines were excised. The tissues were immersed in 4% (v/v) paraformaldehyde at 4°C for 2 hours. They were cryoprotected overnight in 20% (w/v) sucrose solution before being embedded in optimal cutting temperature compound mounting medium (Sakura Finetek, Tokyo, Japan). They were sectioned to 30 μm thickness with a cryostat (Leica Instruments, Nussloch, Germany) and thaw-mounted onto Superfrost Plus slides (Matsunami, Osaka, Japan). Immunohistochemical procedures were performed as previously described (Matsumoto et al., 2018).

Proliferative cells were detected using anti–Ki-67 monoclonal antibody (eBiosciences, San Diego, CA) and Alexa Fluor 594 donkey anti-rat IgG secondary antibody (Thermo Fisher Scientific Inc.). Sections were viewed under a confocal microscope (A1R$^+$; Nikon, Tokyo, Japan). Images were captured by Nikon NIS-Elements AR version 4.20.00 (Nikon). Multiple Z-stacked images were projected onto a single plane and reconstructed with NIS-Elements AR version 4.20.00. To count Ki-67–positive cells, tissue sections were viewed under a confocal microscope at ×200. Quantitative determinations were made using three random locations per mouse. Measurements were performed after blinding the investigator to the animal group assignments.

Cell Culture. Young adult mouse colon (YAMC) epithelial cells were kindly provided by Dr. Robert Whitehead (Vanderbilt University, Nashville, TN) (Whitehead et al., 1993). The YAMC cells were cultured at 33°C in RPMI 1640 (Wako Pure Chemical Industries Ltd.) containing 5% (v/v) FBS (HyClone, GE Healthcare UK Ltd., Little Chalfont, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (Nacalai Tesque, Tokyo, Japan), insulin-transferrin-selenium premix (Wako Pure Chemical Industries Ltd.), and 5 U/ml murine interferon-γ (Invitrogen, Grand Island, NY).

Wound Healing and Cell Proliferation Assays. YAMC cells were cultured in 24-well microplates at a density of 5×10^5 cells/well for 24 hours before the wound healing assay. Each well contained the appropriate additive-free medium. Wounds were inflicted on the cell monolayers with 200-μl pipette tips. Wound closure was evaluated 6 and 24 hours later under a microscope (CK40; Olympus Corporation) connected to the MC120 HD camera (Leica Microsystems, Tokyo, Japan). The wound areas were measured with ImageJ version 1.50i (National Institutes of Health, Bethesda, MD). The wound healing rates were expressed as percent reductions in the wounded areas relative to the initial wound areas. The wound healing rate was equal to (1 − wound area 6 or 24 hours after wound infliction/initial wound area) × 100. The BLT2 agonist CAY10583 (30–1000 nM; Cayman Chemical Co., Ann Arbor, MI) was added immediately after wound infliction.

Cell proliferation was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (EMD Millipore Corp., Temecula, CA). YAMC cells were incubated at a density of 1000 cells/well for 4 hours in culture medium containing the MTT reagents. HCl-isopropanol solution was added to the wells to stop the reactions. Cell proliferation activity was measured at $\lambda = 570$ nm in a MULTISKAN 60 (Thermo Fisher Scientific) and expressed as a percent of the control (vehicle only). The BLT2 agonist CAY10583 (30–1000 ng/ml) was added to the MTT reagents. The BLT2 antagonist LY255283 (30 μM; Tocris Bioscience, Bristol, UK), the protein kinase C (PKC) inhibitor Go6983 (10 μM; Cayman Chemical Co.), proteins pertussis toxin (PTX; 100 ng/ml; R&D Systems Inc., Minneapolis, MN) that inhibits G protein–coupled receptor signaling via $G_{i/o}$ proteins, the phospholipase C (PLC) inhibitor U-73122 (10 μM; Cayman Chemical Co.), and its inactive analog LY-73343 (10 μM; Cayman Chemical Co.) were preincubated for 24 hours before adding the CAY10583. All reagents were first dissolved in DMSO and added to the medium to make up the desired concentration (final DMSO concentration = 0.1%).

Statistical Analysis. Data are presented as means ± S.E.M. Statistical analyses were performed with GraphPad Prism version 6.0h (GraphPad Software, La Jolla, CA). Multiple groups were compared by one-way or two-way ANOVA followed by Holm-Sidak's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

BLT2KO Delayed the Healing of Indomethacin-Induced Small Intestine Lesions. Within 24 hours, a single indomethacin injection caused Evans blue–stained hemorrhagic lesions from the distal jejunum to the ileum. The lesions extended to their maximum area by 48 hours after the injection. However, the severity of the lesions was nearly the same in both WT and BLT2KO mice (Fig. 1, A and B). In WT mice, the lesions fully healed within 96 hours after indomethacin injection. Maximum lesion areas were observed at 48 hours after indomethacin injection. However, they were significantly smaller by 72 and 96 hours after indomethacin injection. In contrast, healing of these lesions was impaired in BLT2KO mice. Their lesions areas at 72 and 96 hours were significantly greater than those in WT mice at the same time points. Histologic examinations disclosed that the lesions had reached the muscularis mucosa 48 hours after indomethacin injection in both WT and BLT2KO mice (Fig. 1C). Whereas the histologic injury fully healed within 96 hours after indomethacin injection in WT mice, it had not improved in the BLT2KO mice by that time point.

BLT2KO Impaired Restoration of the Increase in MPO Activity and Cytokine Expression 72 hours after Indomethacin Injection. A single indomethacin injection increased intestinal MPO activity and TNF-α and IL-1β mRNA expression after 24 and 48 hours (data not shown). In WT mice, MPO activity, TNF-α and IL-1β mRNA expression had returned to their normal level within 72 hours after indomethacin injection (Fig. 2), whereas, in BLT2KO mice, the MPO activity had a higher tendency, and TNF-α and IL-1 mRNA expression was significantly higher in the intestine 72 hours after indomethacin than the control intestine (without indomethacin). These observations suggest that the restoration of the increase in MPO activity, TNF-α and IL-1β mRNA expressions were apparently impaired in BLT2KO mice.

BLT2 Attenuated Increases in Intestinal Crypt Cell Proliferation 72 hours after Indomethacin Injection. Ki-67 protein is a marker of proliferative cells. Immunohistochemical observation with anti–Ki-67 antibody revealed that Ki-67–positive proliferative cells were localized to the crypts of the small intestine (Fig. 3A). The number of proliferative cells was nearly the same in both WT and BLT2KO mice (Fig. 3B). A single indomethacin injection increased the number of Ki-67–positive proliferative cells around the edges of the intestinal lesions in WT mice. In contrast, the indomethacin-induced increase in Ki-67–positive proliferative cells was significantly impaired in BLT2KO mice.

There Was No Difference between WT and BLT2KO Mice in Terms of Growth Factor Upregulation. IGF-1, EGF, and VEGF-A in the small intestine were markedly upregulated 72 hours after indomethacin injection (Fig. 4, A–C). However, there was almost no difference between WT and BLT2KO mice in terms of the expression levels of these growth factors. On the other hand, BLT2 mRNA was upregulated in the small intestine of WT mice in response to indomethacin injection (Fig. 4D). BLT2 expression could not be detected in BLT2KO mice.

Intestinal BLT2 Overexpression Promoted the Healing of Indomethacin-Induced Small Intestine Lesions. A single indomethacin injection induced severe intestinal lesions in both WT and villin-BLT2-Tg mice (Fig. 5). Maximum lesion areas were observed at 48 hours after indomethacin injection. The severity of the lesions was almost the same for both WT and BLT2KO mice. Healing of the indomethacin-induced intestinal lesions was more strongly promoted in villin-BLT2-Tg than in WT mice. Lesion severity at 72 and 96 hours after indomethacin injection was significantly lower in villin-BLT2-Tg than in WT mice.

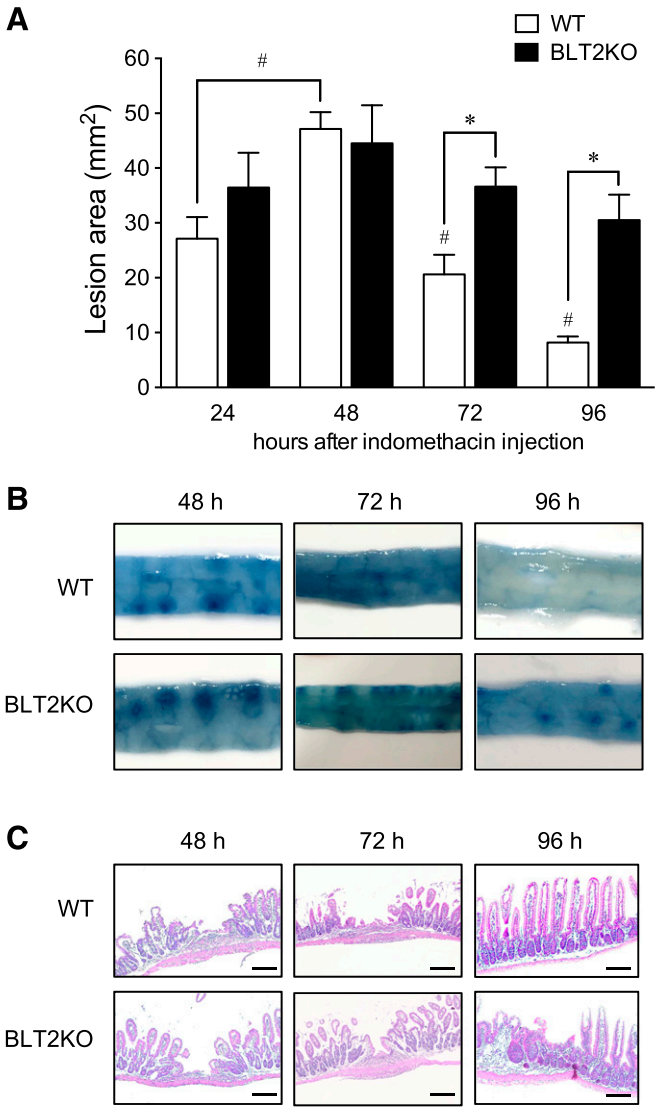

Fig. 1. Time-course changes in indomethacin-induced intestinal lesions in WT and BLT2KO mice. Animals were subcutaneously injected with indomethacin (10 mg/kg), and their intestinal lesions were examined 24, 48, 72, and 96 hours after drug administration (A). Data are means ± S.E.M. (n = 6–8); *$P < 0.05$ compared with WT mice; #$P < 0.05$ compared with 24 hours postinjection. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test. Typical macroscopic (B) and histologic observations (C) of indomethacin-induced intestinal lesions at 48, 72, and 96 hours. Scale bar, 50 μm.

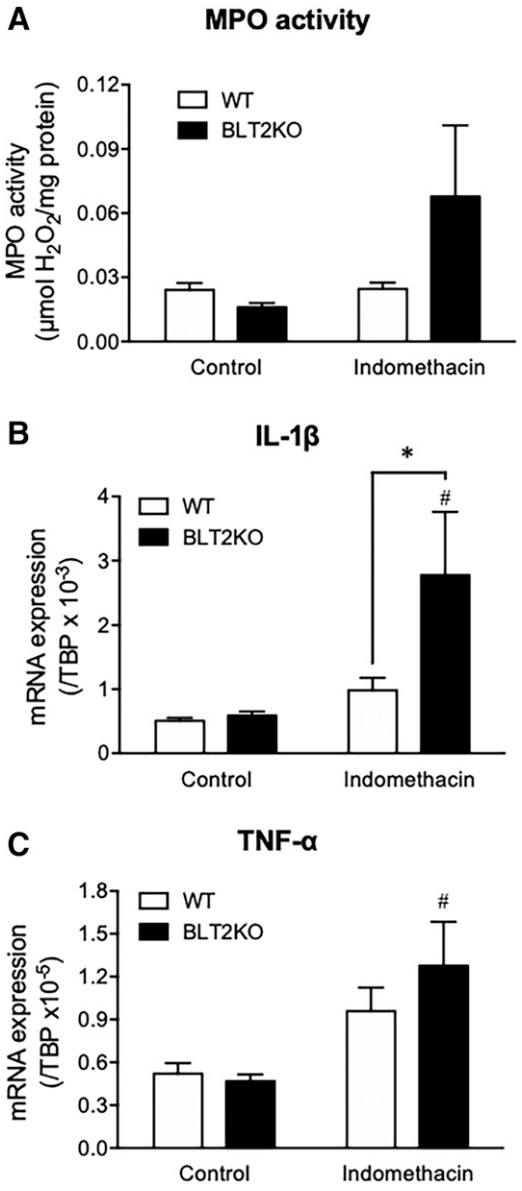

Fig. 2. Changes in MPO activity and cytokine expression in the intestine during the healing of indomethacin-induced intestinal lesions in WT and BLT2KO mice. Animals were subcutaneously injected with indomethacin (10 mg/kg). MPO activity was determined by the o-dianisidine method (A). IL-1β (B) and TNF-α (C) mRNA expression levels (standardized to TBP) were determined by quantitative real-time reverse-transcription polymerase chain reaction 72 hours after indomethacin administration. Data are means ± S.E.M. (n = 7 to 8); *$P < 0.05$ relative to WT mice; #$P < 0.05$ compared with indomethacin-free control. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test.

BLT2 Activation Promoted Wound Healing in YAMC Cells. We assessed the effect of the BLT2 agonist CAY10583 on YAMC cell wound healing. In the presence of FBS and IFN-γ (serum (+)), CAY10583 exposure promoted wound closure in the YAMC monolayer in a concentration-dependent manner (Fig. 6). A significant promoting effect was observed 3 hours after 100 nM CAY10583 treatment and 6 hours after 300 nM CAY10583 treatment. However, no CAY10583-induced promotion of healing was observed in the absence of FBS and IFN-γ (serum (−)).

BLT2 Activation Promoted YAMC Cell Proliferation. We examined the effect of the BLT2 agonist CAY10583 on YAMC cell proliferation. In the presence of FBS and IFN-γ ((serum (+)), CAY10583 exposure promoted YAMC cell proliferation in a concentration-dependent manner (Fig. 7A). A significant promoting effect was observed in response to 300 nM CAY10583. However, no CAY10583-induced promoting effect was observed in the absence of FBS and IFN-γ (serum (−)) (Fig. 7B). Cell proliferation induced by 300 nM CAY10583 in the presence of FBS and IFN-γ ((serum (+)) was almost entirely abrogated by LY255283 (30 μM), a BLT2 antagonist, Go6983 (10 μM), a PKC inhibitor, PTX (100 ng/ml) that inhibits G protein–coupled receptor signaling via $G_{i/o}$ proteins, U-73122 (10 μM), the PLC inhibitor, but not its inactive analog U-73343 (10 μM) (Fig. 7C). The control cell proliferation action (without CAY10583) was significantly inhibited by

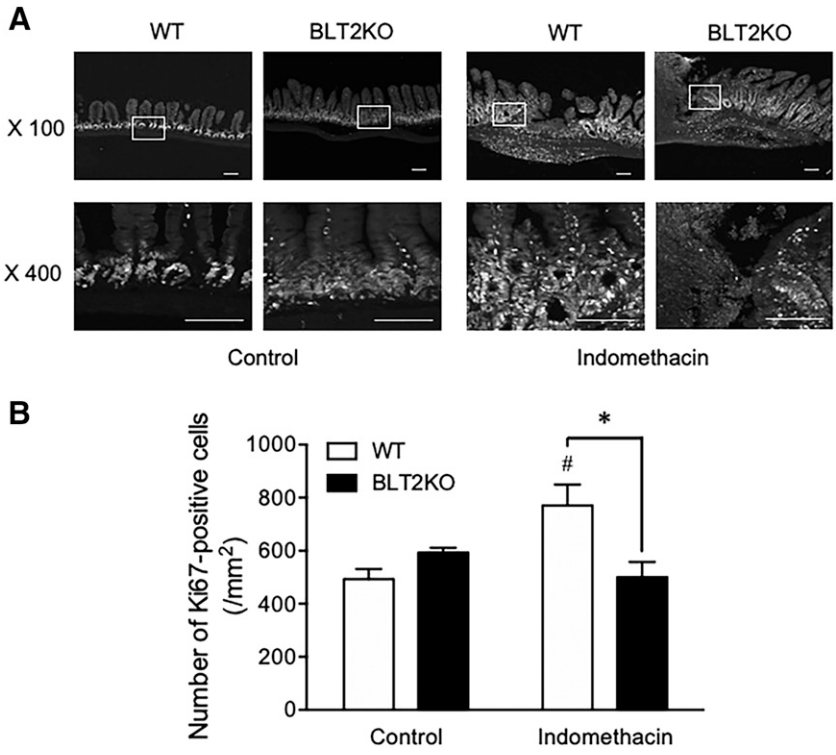

Fig. 3. Changes in intestinal epithelial cell proliferation during the healing of indomethacin-induced intestinal lesions in WT and BLT2KO mice. Animals were subcutaneously injected with indomethacin (10 mg/kg). Cell proliferation was determined immunohistochemically using anti–Ki-67 antibody 72 hours after indomethacin administration. Typical immunohistochemical images of intestinal tissues around the lesions. Original magnification, 100× and 300× (A). Scale bar, 100 μm. Ki-67–positive cells were counted around the lesions (B). Data are means ± S.E.M. ($n = 5$); $^{*}P < 0.05$ relative to WT mice; $^{\#}P < 0.05$ compared with indomethacin-free control. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test.

LY255283 and U-73122, but other inhibitors had no effect on control cell proliferation.

Discussion

Previous studies demonstrated that BLT2 is highly expressed and regulates epithelial barrier functions in the colon (Iizuka et al., 2010). It also promotes wound repair and healing in skin keratinocytes and in bronchial and corneal epithelial cells (Liu et al., 2014, 2018; Iwamoto et al., 2017). We hypothesized that BLT2 may also regulate repair and healing responses in the intestinal tract.

Here, we observed that a single indomethacin injection induced multiple hemorrhagic lesions in the small intestines of mice within 24 hours. The lesions reached their maximum size by 48 hours after the indomethacin injection. There was

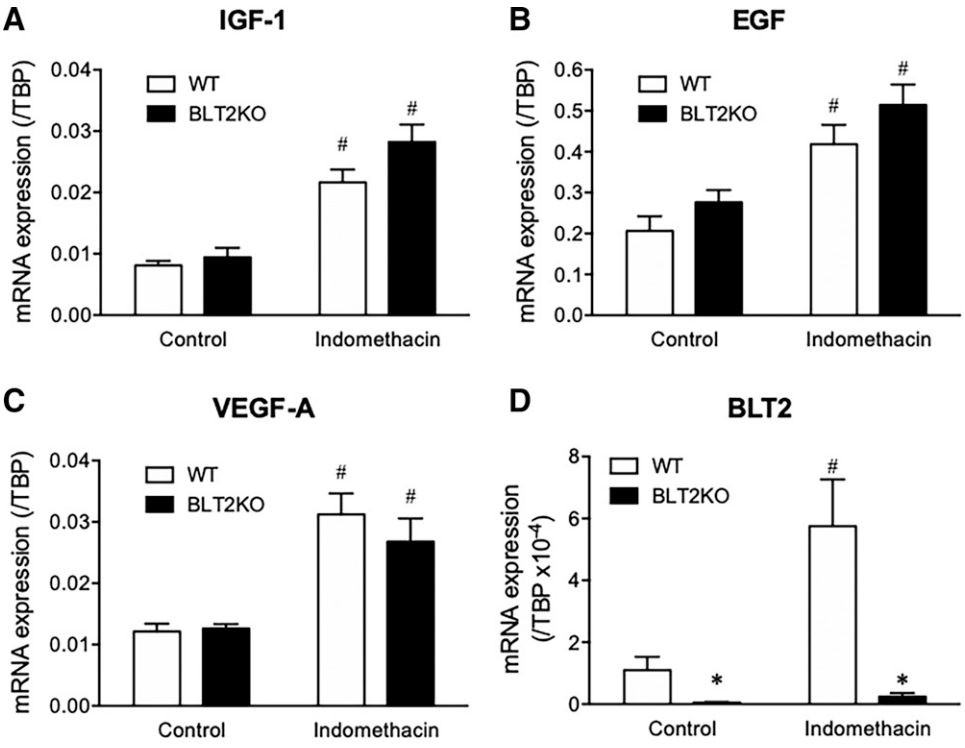

Fig. 4. Changes in intestinal growth factor and BLT2 expression levels during the healing of indomethacin-induced intestinal lesions in WT and BLT2KO mice. Animals were subcutaneously injected with indomethacin (10 mg/kg). mRNA expression levels (standardized to TBP) of IGF-1 (A), EGF (B), VEGF-A (C), and BLT2 (D) were determined by quantitative real-time reverse-transcription polymerase chain reaction 72 hours after indomethacin administration. Data are means ± S.E.M. ($n = 7$ to 8); $^{*}P < 0.05$ relative to WT mice; $^{\#}P < 0.05$ compared with indomethacin-free control. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test.

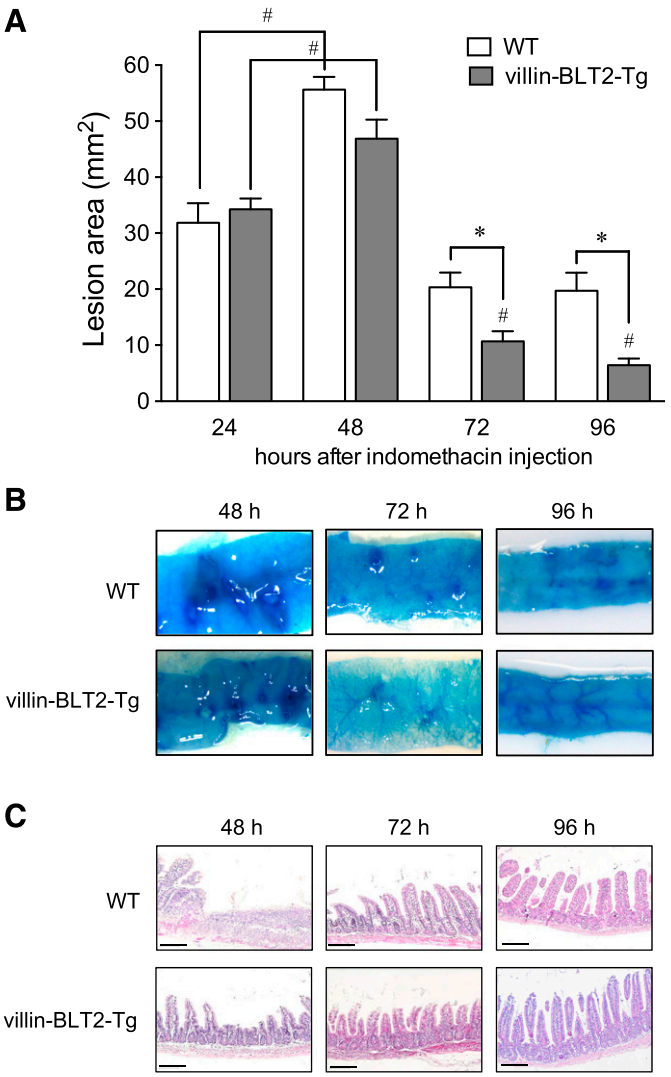

Fig. 5. Time-course changes in indomethacin-induced intestinal lesions in WT and villin-BLT2-Tg mice. Animals were subcutaneously injected with indomethacin (8 mg/kg). Intestinal lesions were examined 24, 48, 72, and 96 hours after indomethacin administration (A). Data are means ± S.E.M. (n = 6–8); *$P < 0.05$ relative to WT mice; $^{\#}P < 0.05$ compared with indomethacin-free control. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test. Typical macroscopic (B) and histologic observations (C) of indomethacin-induced intestinal lesions at 48, 72, and 96 hours. Scale bar, 50 μm.

no significant difference between WT and BLT2KO mice in terms of lesion development. However, lesion healing was significantly impaired in BLT2KO mice compared with WT mice. Several studies indicated that MPO activity and inflammatory cytokine expression were upregulated in a manner dependent on the severity of the indomethacin-induced intestinal lesions (Konaka et al., 1999; Yasuda et al., 2011). In the present study, we observed that indomethacin-induced increases in MPO activity and IL-1β and TNF-α expression were restored to normal levels 72 hours after indomethacin injection in WT mice. However, this recovery was impaired in BLT2KO mice. These findings suggest that compared with the WT, BLT2KO mice exhibit delayed healing of indomethacin-induced intestinal lesions.

To confirm the importance of BLT2 in the healing of indomethacin-induced intestinal lesions, we examined the effects of BLT2 overexpression on villin-BLT2-Tg mice. They expressed FLAG-tagged mouse BLT2 under the control of the villin-promoter active in the entire intestinal epithelium (Ishii et al., 2016). BLT2 was upregulated in the epithelia of the small intestines of villin-BLT2-Tg mice (Supplemental Table 1). Relative to the WT, healing of indomethacin-induced intestinal lesions was promoted in villin-BLT2-Tg mice. Nevertheless, BLT2 overexpression had no significant influence on lesion development. Therefore, BLT2 probably plays a vital role in the healing of indomethacin-induced intestinal lesions.

Iizuka et al. (2010) reported that dextran sulfate sodium–induced colitis was more severe in BLT2KO mice than the WT. In contrast, we observed no differences among the WT, BLT2KO, and villin-BLT2-Tg mice in terms of the development of indomethacin-induced intestinal lesions. We previously showed that a single 10 mg/kg indomethacin injection inhibited COX-dependent prostaglandin E2 synthesis for ≥24 hours (Kunikata et al., 2001). Moreover, this inhibition was reversed until 48 hours after the indomethacin injection (Hatazawa et al., 2006). The endogenous BLT2 ligand 12-HHT is generated via COX (Okuno et al., 2008). Thus, 12-HHT production may also be reduced for 24 hours after indomethacin injection. The COX inhibitor acetylsalicylic acid reduced 12-HHT production in epidermal wound fluids (Liu et al., 2014). Therefore, decreased 12-HHT production may not cause any significant differences between WT and BLT2KO mice in terms of the development of indomethacin-induced intestinal lesions.

Healing of tissue injury involves multiple steps, including angiogenesis and re-epithelialization. These are regulated by growth factors such as VEGF, EGF, and IGF-1 (Jones et al., 1999; Hatazawa et al., 2006; Tarnawski and Ahluwalia, 2012). NSAID-induced COX inhibition suppressed re-epithelialization and angiogenesis by attenuating epithelial cell migration/proliferation and endothelial cell proliferation, respectively. These responses impaired healing (Schmassmann et al., 1995; Szabó et al., 2002). Here, Ki-67–positive immunostaining revealed that epithelial cell proliferation was markedly augmented around the edges of the lesions in WT mice during healing. However, this process was dramatically inhibited in BLT2KO mice. IGF-1, EGF, and VEGF were upregulated during the healing of intestinal lesions in both WT and BLT2KO mice. Therefore, the BLT2-mediated healing response in indomethacin-induced intestinal lesions promotes epithelial cell proliferation. The BLT2-mediated healing response may not be the result of the upregulation of the aforementioned growth factors. Rather, it may be directly induced by epithelial cell proliferation.

Prostaglandins are synthesized along with 12-HHT via COX. They are key mediators of healing in gastrointestinal disease (Halter et al., 2001). Höper et al. (1997) and Sonoshita et al. (2001) reported that prostaglandin E2 upregulated VEGF via EP_4 receptors. We previously disclosed that daily EP_4 antagonist administration impaired healing of indomethacin-induced intestinal lesions by attenuating angiogenesis and downregulating VEGF (Takeuchi et al., 2007, 2010). Prostaglandin E2/EP_4 receptor signaling may play a vital role in the angiogenesis that occurs during healing. The 12-HHT and prostaglandin E2 produced by COX may participate in the healing of intestinal lesions by promoting epithelial proliferation via BLT2 and angiogenesis via EP_4 receptors. BLT2

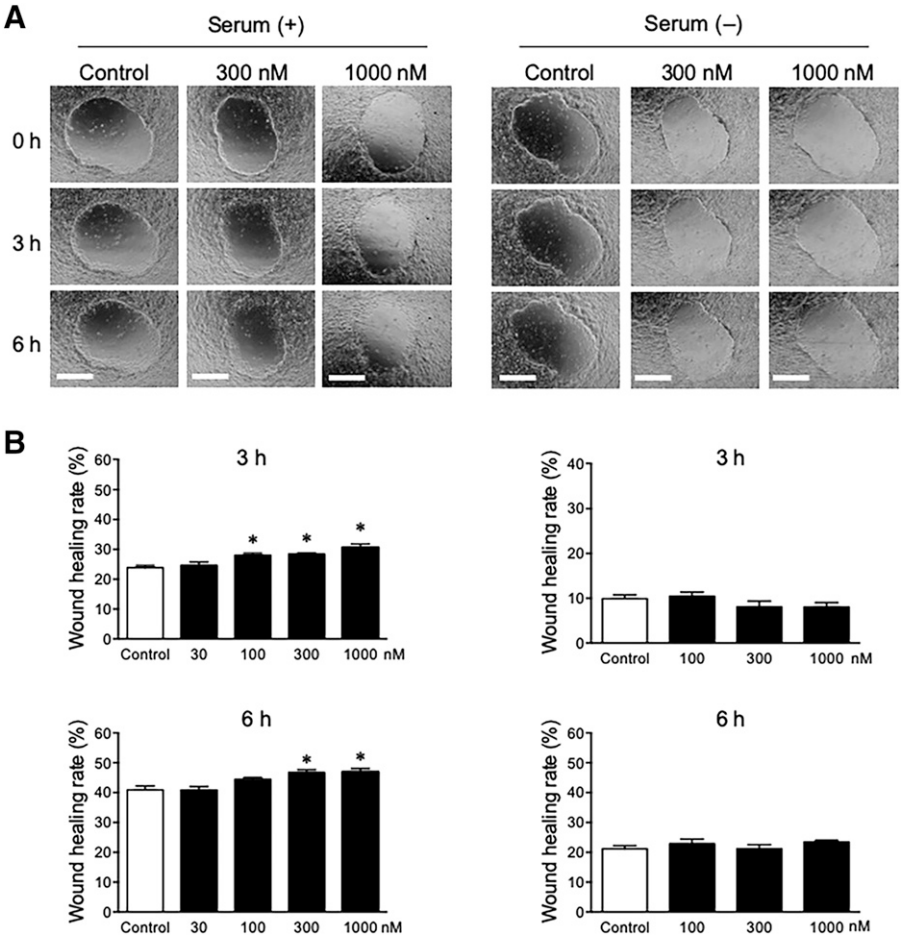

Fig. 6. Effect of the BLT2 agonist CAY10583 on wound repair in YAMC cells. A wound was made in the YAMC cell monolayer using a pipette tip. Wound closure was observed 3 and 6 hours after injury. CAY10583 (30–1000 nM) was added to the cells immediately after wound infliction. Typical microscopic observation (A) and quantitative results of wound repair in YAMC cells (B). Wound repair rate was calculated as the percent decrease in the initial wound area in the presence of serum (+) and in its absence (−). Scale bar, 500 μm. Data are means ± S.E.M. ($n = 5$); $^*P < 0.05$ compared with control. Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test.

was expressed in vascular endothelial cells and contributed to VEGF-induced angiogenesis (Kim et al., 2009). Thus, BLT2 may be associated with angiogenesis during the healing of indomethacin-induced intestinal lesions. However, further research is needed to elucidate its role in this process.

We evaluated the influences of BLT2 activation on wound repair in cultured intestinal epithelial cells. BLT2 activation by the agonist CAY10583 significantly promoted wound closure in YAMC cells in the presence of serum. On the other hand, this response was not observed in the absence of serum. We previously showed that the promotion of healing via YAMC cell migration could be detected even in the absence of serum (Tsukahara et al., 2017). Thus, the BLT2-promoted healing response may be induced by accelerating cell proliferation rather than cell migration. The MTT assay revealed that CAY10583 significantly promoted YAMC cell proliferation in the presence of serum but not in its absence. CAY10583-promoted cell proliferation was abrogated by the BLT2 antagonist LY255283. These findings suggest that promotion of healing by BLT2 is mediated by an increase in the proliferation of intestinal epithelial cells. BLT2 accelerated the migration but not the proliferation of epidermal keratinocytes (Liu et al., 2014). In contrast, BLT2 activation by overexpression or by 12-HHT or CAY10583 treatment promoted human bronchial epithelial Human Bronchial Epithelial Cell Line (16HBE) cell proliferation and migration (Liu et al., 2018). Several studies demonstrated that BLT2 is upregulated and participates in pancreatic and colorectal cancer pathogenesis by inducing cancer cell proliferation (Hennig et al., 2008; Park et al., 2019). Here, we detected BLT2 upregulation in the small intestine during the lesion healing process there. Thus, the functional roles of BLT2 may vary with cell type.

In the present study, we found that the promoting effect of CAY10583 on YAMC cells was attenuated by PTX as well as PKC and PLC inhibition. BLT1 and BLT2 couple to the PTX-sensitive $G_{i/o}$-like G protein and repress adenylate cyclase (Yokomizo, 2015). For signaling purposes, these receptors couple to at least three classes of G proteins such as $G_{i/o}$, Gq-like, and Gz (Yokomizo et al., 2000, 2018). Thus, BLT2-promoted proliferation of intestinal epithelial cells may be mediated by $G_{i/o}$ and Gq-like G protein signaling pathways. Because Go6983 is a broad PKC inhibitor, various isoforms of PKC, including PKCα, PKCβ, PKCγ, PKCδ, and PKCμ, may be involved in this response. We further observed that LY255283 and U-73122 significantly reduced cell proliferation even in the absence of CAY10583. The previous studies showed that 10% fetal bovine serum contains about 15 nM of 12-HHT (Matsunobu et al., 2013). Thus, the proliferation of YAMC cells may be stimulated by 12-HHT in serum-containing culture medium.

BLT2 plays an important role in the healing of intestinal lesions. It may promote epithelial cell proliferation via PTX-dependent $G_{i/o}$ and PLC/PKC-mediated Gq-like G protein signaling pathways. Therefore, BLT2 activation by

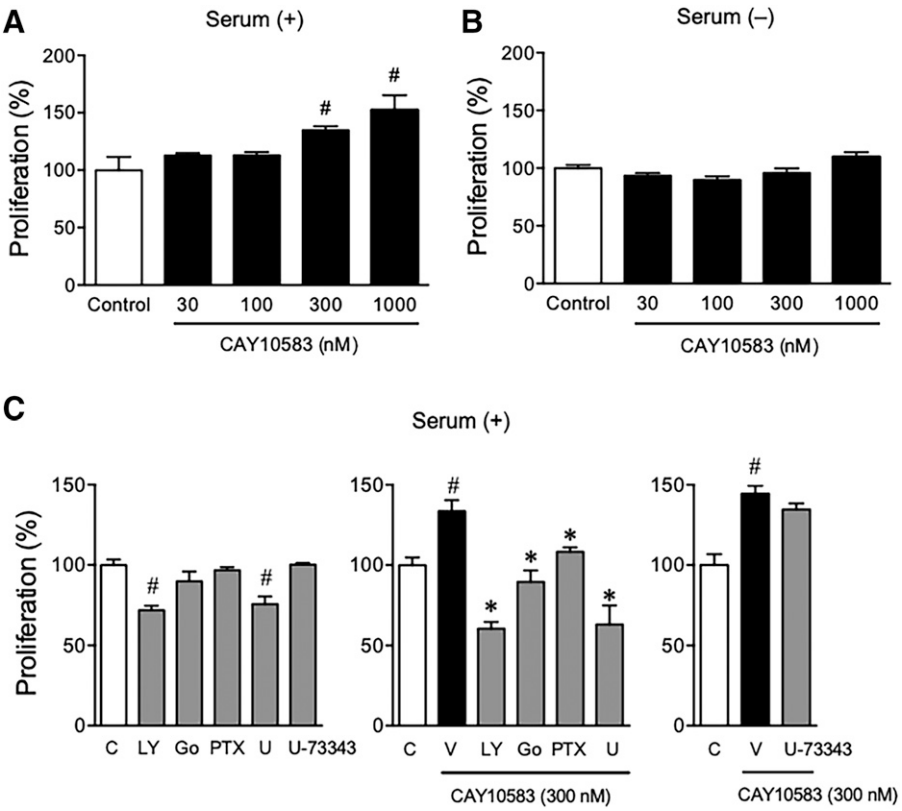

Fig. 7. Effect of BLT2 agonist CAY10583 on YAMC cell proliferation as determined by MTT assay. The concentration-dependent effect of CAY10583 (30–1000 nM) was evaluated in the presence of serum (+) and in its absence (−) (A). Effect of the BLT2 antagonist LY255283 (LY; 30 μM), the PKC inhibitor Go6983 (Go; 10 μM), the G_i protein inhibitor PTX (100 ng/ml), the PLC inhibitor U-73122 (U; 10 μM), and its inactive analog U-73343 (10 μM) on cell proliferative action with (B) or without 300 nM CAY10583 (C) was assessed in the presence of serum. Proliferation rate was calculated as a percent of the control (vehicle only). Data are means ± S.E.M. (n = 5 to 6); $^{\#}P < 0.05$ relative to the control; $^{*}P < 0.05$ compared with CAY10583 alone (V). Analyzed by ANOVA followed by Holm-Sidak's multiple comparison test.

pharmacological agonists may be a promising approach toward the treatment of intestinal lesions.

Acknowledgments

The authors thank Dr. Takuya Tsukahara for technical assistance and Dr. Robert Whitehead for providing the YAMC cells.

Authorship Contributions

Participated in research design: Saeki, K. Matsumoto, Yokomizo, Kato.

Conducted experiments: Y. Matsumoto, Matsuya, Nagai, Amagase.

Performed data analysis: Amagase, Kato.

Wrote or contributed to the writing of the manuscript: Yokomizo, Kato.

References

Anthony A, Pounder RE, Dhillon AP, and Wakefield AJ (2000) Similarities between ileal Crohn's disease and indomethacin experimental jejunal ulcers in the rat. *Aliment Pharmacol Ther* **14**:241–245.

Bjarnason I, Hayllar J, MacPherson AJ, and Russell AS (1993) Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* **104**:1832–1847.

Halter F, Tarnawski AS, Schmassmann A, and Peskar BM (2001) Cyclooxygenase 2-implications on maintenance of gastric mucosal integrity and ulcer healing: controversial issues and perspectives. *Gut* **49**:443–453.

Hatazawa R, Ohno R, Tanigami M, Tanaka A, and Takeuchi K (2006) Roles of endogenous prostaglandins and cyclooxygenase isozymes in healing of indomethacin-induced small intestinal lesions in rats. *J Pharmacol Exp Ther* **318**:691–699.

Hennig R, Osman T, Esposito I, Giese N, Rao SM, Ding XZ, Tong WG, Büchler MW, Yokomizo T, Friess H, et al. (2008) BLT2 is expressed in PanINs, IPMNs, pancreatic cancer and stimulates tumour cell proliferation. *Br J Cancer* **99**:1064–1073.

Höper MM, Voelkel NF, Bates TO, Allard JD, Horan M, Shepherd D, and Tuder RM (1997) Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP. *Am J Respir Cell Mol Biol* **17**:748–756.

Iizuka Y, Okuno T, Saeki K, Uozaki H, Okada S, Misaka T, Sato T, Toh H, Fukayama M, Takeda N, et al. (2010) Protective role of the leukotriene B4 receptor BLT2 in murine inflammatory colitis. *FASEB J* **24**:4678–4690.

Iizuka Y, Yokomizo T, Terawaki K, Komine M, Tamaki K, and Shimizu T (2005) Characterization of a mouse second leukotriene B4 receptor, mBLT2: BLT2-dependent ERK activation and cell migration of primary mouse keratinocytes. *J Biol Chem* **280**:24816–24823.

Ishii Y, Saeki K, Liu M, Sasaki F, Koga T, Kitajima K, Meno C, Okuno T, and Yokomizo T (2016) Leukotriene B4 receptor type 2 (BLT2) enhances skin barrier function by regulating tight junction proteins. *FASEB J* **30**:933–947.

Iwamoto S, Koga T, Ohba M, Okuno T, Koike M, Murakami A, Matsuda A, and Yokomizo T (2017) Non-steroidal anti-inflammatory drug delays corneal wound healing by reducing production of 12-hydroxyheptadecatrienoic acid, a ligand for leukotriene B_4 receptor 2. *Sci Rep* **7**:13267.

Jones MK, Tomikawa M, Mohajer B, and Tarnawski AS (1999) Gastrointestinal mucosal regeneration: role of growth factors. *Front Biosci* **4**:D303–D309.

Kato S, Matsuda N, Matsumoto K, Wada M, Onimaru N, Yasuda M, Amagase K, Horie S, and Takeuchi K (2012) Dual role of serotonin in the pathogenesis of indomethacin-induced small intestinal ulceration: pro-ulcerogenic action via 5-HT3 receptors and anti-ulcerogenic action via 5-HT4 receptors. *Pharmacol Res* **66**:226–234.

Kawahara R, Yasuda M, Hashimura H, Amagase K, Kato S, and Takeuchi K (2011) Activation of α7 nicotinic acetylcholine receptors ameliorates indomethacin-induced small intestinal ulceration in mice. *Eur J Pharmacol* **650**:411–417.

Kim GY, Lee JW, Cho SH, Seo JM, and Kim JH (2009) Role of the low-affinity leukotriene B4 receptor BLT2 in VEGF-induced angiogenesis. *Arterioscler Thromb Vasc Biol* **29**:915–920.

Konaka A, Kato S, Tanaka A, Kunikata T, Korolkiewicz R, and Takeuchi K (1999) Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. *Pharmacol Res* **40**:517–524.

Kunikata T, Araki H, Takeeda M, Kato S, and Takeuchi K (2001) Prostaglandin E prevents indomethacin-induced gastric and intestinal damage through different EP receptor subtypes. *J Physiol Paris* **95**:157–163.

Leoni G, Neumann PA, Kamaly N, Quiros M, Nishio H, Jones HR, Sumagin R, Hilgarth RS, Alam A, Fredman G, et al. (2015a) Annexin A1-containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. *J Clin Invest* **125**:1215–1227.

Leoni G, Neumann PA, Sumagin R, Denning TL, and Nusrat A (2015b) Wound repair: role of immune-epithelial interactions. *Mucosal Immunol* **8**:959–968.

Liu M, Saeki K, Matsunobu T, Okuno T, Koga T, Sugimoto Y, Yokoyama C, Nakamizo S, Kabashima K, Narumiya S, et al. (2014) 12-Hydroxyheptadecatrienoic acid promotes epidermal wound healing by accelerating keratinocyte migration via the BLT2 receptor. *J Exp Med* **211**:1063–1078.

Liu M, Shen J, Yuan H, Chen F, Song H, Qin H, Li Y, Xu J, Ye Q, Li S, et al. (2018) Leukotriene B4 receptor 2 regulates the proliferation, migration, and barrier integrity of bronchial epithelial cells. *J Cell Physiol* **233**:6117–6124.

Matsumoto K, Yamaba R, Inoue K, Utsumi D, Tsukahara T, Amagase K, Tominaga M, and Kato S (2018) Transient receptor potential vanilloid 4 channel regulates vascular endothelial permeability during colonic inflammation in dextran sulphate sodium-induced murine colitis. *Br J Pharmacol* **175**:84–99.

Matsunobu T, Okuno T, Yokoyama C, and Yokomizo T (2013) Thromboxane A synthase-independent production of 12-hydroxyheptadecatrienoic acid, a BLT2 ligand. *J Lipid Res* **54**:2979–2987.

Okuno T, Iizuka Y, Okazaki H, Yokomizo T, Taguchi R, and Shimizu T (2008) 12(S)-Hydroxyheptadeca-5Z, 8E, 10E-trienoic acid is a natural ligand for leukotriene B4 receptor 2. *J Exp Med* **205**:759–766.

Park J, Jang JH, and Kim JH (2019) Mediatory role of BLT2 in the proliferation of KRAS mutant colorectal cancer cells. *Biochim Biophys Acta Mol Cell Res* **1866**: 329–336.

Schmassmann A, Tarnawski A, Peskar BM, Varga L, Flogerzi B, and Halter F (1995) Influence of acid and angiogenesis on kinetics of gastric ulcer healing in rats: interaction with indomethacin. *Am J Physiol* **268**:G276–G285.

Seidelin JB, Larsen S, Linnemann D, Vainer B, Coskun M, Troelsen JT, and Nielsen OH (2015) Cellular inhibitor of apoptosis protein 2 controls human colonic epithelial restitution, migration, and Rac1 activation. *Am J Physiol Gastrointest Liver Physiol* **308**:G92–G99.

Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, and Taketo MM (2001) Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. *Nat Med* **7**:1048–1051.

Szabó IL, Pai R, Jones MK, Ehring GR, Kawanaka H, and Tarnawski AS (2002) Indomethacin delays gastric restitution: association with the inhibition of focal adhesion kinase and tensin phosphorylation and reduced actin stress fibers. *Exp Biol Med (Maywood)* **227**:412–424.

Takeuchi K, Kato S, and Amagase K (2010) Prostaglandin EP receptors involved in modulating gastrointestinal mucosal integrity. *J Pharmacol Sci* **114**:248–261.

Takeuchi K, Tanaka A, Kato S, Aihara E, and Amagase K (2007) Effect of (S)-4-(1-(5-chloro-2-(4-fluorophenyoxy)benzamido)ethyl) benzoic acid (CJ-42794), a selective antagonist of prostaglandin E receptor subtype 4, on ulcerogenic and healing responses in rat gastrointestinal mucosa. *J Pharmacol Exp Ther* **322**:903–912.

Tarnawski AS and Ahluwalia A (2012) Molecular mechanisms of epithelial regeneration and neovascularization during healing of gastric and esophageal ulcers. *Curr Med Chem* **19**:16–27.

Tsukahara T, Hamouda N, Utsumi D, Matsumoto K, Amagase K, and Kato S (2017) G protein-coupled receptor 35 contributes to mucosal repair in mice via migration of colonic epithelial cells. *Pharmacol Res* **123**:27–39.

Whitehead RH, VanEeden PE, Noble MD, Ataliotis P, and Jat PS (1993) Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *Proc Natl Acad Sci USA* **90**: 587–591.

Yamada T, Deitch E, Specian RD, Perry MA, Sartor RB, and Grisham MB (1993) Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. *Inflammation* **17**:641–662.

Yamaoka H, Sumiyoshi H, Higashi K, Nakao S, Minakawa K, Sumida K, Saito K, Ikoma N, Mabuchi T, Ozawa A, et al. (2014) A novel small compound accelerates dermal wound healing by modifying infiltration, proliferation and migration of distinct cellular components in mice. *J Dermatol Sci* **74**:204–213.

Yasuda M, Kawahara R, Hashimura H, Yamanaka N, Iimori M, Amagase K, Kato S, and Takeuchi K (2011) Dopamine D_2-receptor antagonists ameliorate indomethacin-induced small intestinal ulceration in mice by activating α7 nicotinic acetylcholine receptors. *J Pharmacol Sci* **116**:274–282.

Yokomizo T (2015) Two distinct leukotriene B4 receptors, BLT1 and BLT2. *J Biochem* **157**:65–71.

Yokomizo T, Kato K, Hagiya H, Izumi T, and Shimizu T (2001) Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. *J Biol Chem* **276**:12454–12459.

Yokomizo T, Kato K, Terawaki K, Izumi T, and Shimizu T (2000) A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J Exp Med* **192**:421–432.

Yokomizo T, Nakamura M, and Shimizu T (2018) Leukotriene receptors as potential therapeutic targets. *J Clin Invest* **128**:2691–2701.

Address correspondence to: Shinichi Kato, Division of Pathological Science, Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto 607-8414, Japan. E-mail: skao@mb.kyoto-phu.ac.jp