Prevention of Trinitrobenzene Sulfonic Acid-Induced Experimental Colitis by Oral Administration of a Poly(lactic-coglycolic Acid) Microsphere Containing Prostaglandin E₂ Receptor Subtype 4 Agonist

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ABSTRACT
Prostaglandin E₂ receptor subtype 4 (EP4) agonists are known to reduce intestinal inflammation and enhance epithelial regeneration. We explored the possibility of colonic delivery of an EP4 agonist, 2-[(4-[(2-((1R,2R,3R)-3-hydroxy-5-oxocyclopentyl)ethyl][sulfanyl]butanoyl)oxy]ethyl nonanoate (ONO-AE2-724), using poly(lactic-coglycolic acid) (PLGA) microspheres. Colitis was induced in mice by the intrarectal administration of trinitrobenzene sulfonic acid (TNBS). ONO-AE2-724-PLGA microspheres (EP4-MS) were prepared by the standard technique. Drug distributions after oral administration of EP4-MS were determined by liquid chromatography-tandem mass spectrometry analysis. To evaluate the protective effect of EP4-MS, animals were orally treated by gavage with single doses of EP4-MS 24 h before TNBS instillation. The changes in body weight, histopathology, immunohistochemistry, and expression of inflammatory cytokines were evaluated. Oral administration of EP4-MS enhanced colonic tissue drug concentration without any increase in the serum concentration during the 48 h after intake. EP4-MS pretreatment, but not unloaded ONO-AE2-724, significantly attenuated TNBS-induced colitis and diminished colonic mRNA expression levels of proinflammatory cytokines. In addition, a significant increase in the expression of CD25 and FoxP3 was found in isolated lamina propria CD4⁺ T cells of EP4-MS-treated mice. Immunohistochemical analysis of Ki-67 and single-stranded DNA revealed that EP4-MS pretreatment significantly suppressed apoptosis of colonic cells and promoted epithelial cell proliferation. These results suggest that EP4-MS protect mice from TNBS-induced colitis by intestinal local ONO-AE2-724 delivery. The EP4-MS may offer a promising new therapeutic strategy to treat inflammatory bowel diseases.

Introduction
Human inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn’s disease, are characterized by chronic and spontaneous inflammation due to dysfunction of mucosal T cells, initiation of innate immunological responses, and triggering of inflammatory cytokine production, further amplified by inflammation brought about by elicitation of adaptive immune responses (Strober et al., 2002; Loftus, 2004). IBDs occur in the younger generation, and symptoms of IBDs such as diarrhea, rectal bleeding, abdominal pain, weight loss, anemia, and leukocytosis are ongoing. Because there is no known radical cure for IBDs with the exception of surgical treatment for UC, long-term drug treatment for the remission of symptoms and the prevention of relapse is usually needed.

Several studies showed that prostaglandin (PG) E₂-PGE₂ receptor subtype 4 (EP4) signaling is an important regulator of mucosal immune responses. The EP4 receptor is expressed in various murine immune cells, including T cells, B cells, and macrophages, related to immune responses (Ozono et al., 2000; Kitahara et al., 2005). EP4 receptor agonists are known to reduce inflammatory bowel diseases and enhance tissue regeneration (Burgos-Vargas et al., 2008). However, EP4 receptor agonists are not effective in clinical practice because of limited oral availability and systemic toxicity (Burgos-Vargas et al., 2010).

Moreover, EP4 receptor agonists are poorly available in the colonic mucosa, and the clinical efficacy of EP4 receptor agonists is limited by their systemic toxicity. The current study therefore explores the possibility of colonic delivery of an EP4 agonist, 2-[(4-[(2-((1R,2R,3R)-3-hydroxy-5-oxocyclopentyl)ethyl][sulfanyl]butanoyl)oxy]ethyl nonanoate (ONO-AE2-724), using poly(lactic-coglycolic acid) (PLGA) microspheres. Colitis was induced in mice by the intrarectal administration of trinitrobenzene sulfonic acid (TNBS). ONO-AE2-724-PLGA microspheres (EP4-MS) were prepared by the standard technique. Drug distributions after oral administration of EP4-MS were determined by liquid chromatography-tandem mass spectrometry analysis. To evaluate the protective effect of EP4-MS, animals were orally treated by gavage with single doses of EP4-MS 24 h before TNBS instillation. The changes in body weight, histopathology, immunohistochemistry, and expression of inflammatory cytokines were evaluated. Oral administration of EP4-MS enhanced colonic tissue drug concentration without any increase in the serum concentration during the 48 h after intake. EP4-MS pretreatment, but not unloaded ONO-AE2-724, significantly attenuated TNBS-induced colitis and diminished colonic mRNA expression levels of proinflammatory cytokines. In addition, a significant increase in the expression of CD25 and FoxP3 was found in isolated lamina propria CD4⁺ T cells of EP4-MS-treated mice. Immunohistochemical analysis of Ki-67 and single-stranded DNA revealed that EP4-MS pretreatment significantly suppressed apoptosis of colonic cells and promoted epithelial cell proliferation. These results suggest that EP4-MS protect mice from TNBS-induced colitis by intestinal local ONO-AE2-724 delivery. The EP4-MS may offer a promising new therapeutic strategy to treat inflammatory bowel diseases.

ABBREVIATIONS: IBD, inflammatory bowel disease; UC, ulcerative colitis; PG, prostaglandin; EP4, PGE₂ receptor subtype 4; 5-ASA, 5-aminosalicylic acid; PLGA, poly(lactic-coglycolic acid); ONO-AE2-724, 2-[(4-[(2-((1R,2R,3R)-3-hydroxy-5-oxocyclopentyl)ethyl][sulfanyl]butanoyl)oxy]ethyl nonanoate; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; ssDNA, single-strand DNA; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; Treg, regulatory T cell.
of mucosal immune response, and EP4 has emerged as an alternative therapeutic target for IBDs (Kabashima et al., 2002; Nitta et al., 2002; Jiang et al., 2007, 2010). EP4 knockout mice are most susceptible to experimental colitis induction by oral intake of 5% dextran sodium sulfate, which is one of the common reproducible murine colitis models of IBDs. Those results suggest that EP4 works to keep mucosal integrity, to suppress the innate immunity, and to down-regulate the proliferation and activation of CD4+ T cells. Agonists for EP4 have also been shown to inhibit the production of chemokines and cytotoxic cytokines from immune cells, which suppress helper T1 cell differentiation and promote epithelial cell survival and growth by activating antiapoptotic and proliferative cellular signaling pathways. Studies in humans revealed that EP4 receptors are constitutively expressed in the colonic epithelium and are up-regulated during IBDs (Wallace, 2001). As an alternative, increased PGE2 levels in inflamed mucosa may protect from exacerbation of IBDs (Wallace, 2001). In a recent study, a clinical trial has shown promising therapeutic effects of an EP4 agonist in 5-aminosalicylic acid (5-ASA) refractory cases (Nakase et al., 2010).

PGE2-EP4 signals also play significant roles in other organs, especially in vascular reactivity; however, therapeutic use of EP4 agonists for such a purpose would be limited by unwanted physiological side effects. Because systemic non-specific distribution leads to a high concentration of the drug in organs and plasma, resulting in toxicity, drug delivery systems that directly increase the drug concentration at the required site of action are needed for the development of new agents to treat chronic diseases.

Poly(lactic-co-glycolic acid) (PLGA) microspheres can be efficiently taken up by intestinal microfold cells (M cells) and macrophages (Tabata et al., 1996). A previous study reported that microspheres containing dexamethasone achieved colonic targeted drug release and treatment in a dextran sodium sulfate-induced mice colitis model (Nakase et al., 2000). Hence, in this study, we loaded the newly synthesized EP4 agonist, 2-[4-[(2-[(1R,2R,3R)-3-hydroxy-4-[[3-(methoxymethyl)phenyl]but-1-enyl]-5-oxocyclopentyl]ethyl]sulfanyl]butanoyloxy]ethyl nonanoate (ONO-AE2-724), into PLGA microspheres (EP4-MS) to investigate the possibility of targeted drug delivery for treatment of IBDs. A trinitrobenzene sulfonic acid (TNBS)-induced mice colitis model produced by intrarectal injection of TNBS is a helper T1 cell-mediated colitis with many features of Crohn’s disease (Strober et al., 2002). The efficacy and the pharmacokinetics of the oral intake of EP4-MS in mice for the prevention of intestinal inflammation were investigated.

**Fig. 1. Structure of ONO-AE2-724.**

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**Materials and Methods**

**Drugs and Reagents.** ONO-AE2-724 (EP4 agonist) was synthesized by Ono Pharmaceutical Co. (Osaka, Japan) according to Sakai et al. (2004) (Fig. 1). ONO-AE2-724 was rapidly de-esterificated at its carboxylic part in serum and transformed into the active form, ONO-AE1-437. The inhibition constant (Ki) values of the EP4 agonist, ONO-AE1-437, were 0.7, 56, and 620 nM for EP4, EP3, and EP2, respectively, and >10,000 nM for the other prostanoid receptors (Miyamoto et al., 2003). PLGA with a lactide/glycolic acid ratio of 75:65 was purchased from Wako Pure Chemicals (Osaka, Japan). TNBS, dichloromethane, polyvinylalcohol, Tween 80, dimethyl sulfoxide, formic acid (HPLC grade), acetonitrile (HPLC grade), and ethanol (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO), Nacalai Tesque Inc. (Kyoto, Japan), Wako Pure Chemicals, Kanto Chemical (Tokyo, Japan), and Kishida Chemical (Osaka, Japan).

**Preparation of EP4 (ONO-AE2-724) Microspheres.** Preparation of EP4 (ONO-AE2-724) microspheres (EP4-MS) was reported previously (Marui et al., 2006). In brief, the EP4 agonist, ONO-AE2-724 (10 mg), and PLGA75-65 (90 mg) were dissolved in 1 ml of dichloromethane as the oil phase. The oil phase was gradually added into aqueous polyvinylalcohol (0.1%) solution under stirring with a turbine-shaped mixer (Homomixer) at 6000 rpm to obtain an oil-in-water emulsion. Then the PLGA microspheres were suspended in a polyvinyl alcohol solution after organic solvent evaporation. Supernatant was discarded and replaced with fresh water or aqueous medium containing 0.2% w/v Tween 80. Washed microsphere precipitation was lyophilized to remove residual organic solvent and water and then dried solid ONO-AE2-724 microspheres were recovered.

**Size and Polydispersion Measurement.** The particle size distribution of EP4-MS and unloaded PLGA microspheres (MS) (placebo) were determined by using a laser diffraction particle size analyzer (SALD-2200; Shimadzu Corporation, Kyoto, Japan) in double-distilled water at 25°C.

**Scanning Electron Microscopy of EP4-MS.** Microspheres were fixed on an aluminum support with carbon-adhesive glue and coated with gold-palladium (30 mA, 40 s) (JSM 6701F; JEOL, Tokyo, Japan). Coated samples were observed using a scanning electron microscope (JFC 1600; JEOL).

**In Vitro Release of ONO-AE2-724 from PLGA Microspheres.** Microspheres were fixed on an aluminum support with carbon-adhesive glue and coated with gold-palladium (30 mA, 40 s) (JSM 6701F; JEOL, Tokyo, Japan). The ONO-AE2-724 concentration of each sample was determined by using a laser diffraction particle size analyzer (SALD-2200; Shimadzu Corporation, Kyoto, Japan) in double-distilled water at 25°C.

**Scanning Electron Microscopy of EP4-MS.** Microspheres were fixed on an aluminum support with carbon-adhesive glue and coated with gold-palladium (30 mA, 40 s) (JSM 6701F; JEOL, Tokyo, Japan). The samples were observed using a scanning electron microscope (JFC 1600; JEOL).

**Efficiency of Association of EP4-MS.** The efficiency of association of ONO-AE2-724 with PLGA microspheres was determined by HPLC. Samples of microspheres containing ONO-AE2-724 were dissolved in acetonitrile containing internal standard (4-hydroxybenzoic acid N-nonyl ester) and centrifuged. A volume of 10 μl of supernatant was transferred into a HPLC vial after filtration. The concentration of ONO-AE2-724 was determined using an analytical curve.

**In Vitro Release of ONO-AE2-724 from PLGA Microspheres.** First, 2.5 mg of EP4-MS was suspended in 0.5 ml of 0.2% w/v SDS containing phosphate-buffered saline (PBS) and incubated at 37°C in a shaking bath. After centrifugation, this suspension was serially sampled (3 h, 6 h, 1 day, 5 days, 7 days, 13 days, 21 days, 28 days, and 35 days after incubation). The ONO-AE2-724 concentration of...
the sample solution was assessed by HPLC as previously reported above.

Animals. All animal care and experimental protocols used in this study were approved by the local ethics committee from Kyoto University Graduate School of Medicine. The experiments were conducted using female BALB/c mice (8–12 weeks of age) obtained from Shimadzu Laboratories Japan, Inc. (Kyoto, Japan). All mice were maintained under specific pathogen-free conditions in wire cages at 22–24°C and 60 ± 5% humidity and were provided with standard laboratory foods (Oriental Yeast Co., Tokyo, Japan).

Concentration of ONO-AE2-724 and Its Active Form of ONO-AE1-437 after Oral Intake of EP4-MS in Mice. For the determination of the colon, intestinal remnants, and blood distributions of ONO-AE2-724, EP4-MS were orally administered once to female BALB/c mice. The concentrations of ONO-AE2-724 and its de-esterificated active form, ONO-AE1-437, were determined in colon tissue, intestinal remnant, and blood at 2, 4, 6, 10, 24, and 48 h after administration (n = 3 at each time point for each treatment) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described below.

According to the results from the entrapment association of ONO-AE2-724 and PLGA microspheres, in 1 mg, EP4-MS contain 0.1 mg of ONO-AE2-724. Therefore, 0.2 ml of 10 mg · kg⁻¹ · ml⁻¹ (1 mg of ONO-AE2-724 · kg⁻¹ · ml⁻¹) in 1% v/v Tween 80 containing PBS solution was administered by gastric tube to mice. The mice were anesthetized with isoflurane at 2, 4, 6, 10, 24, and 48 h after drug administration, and 1 to 1.5 ml of blood was taken from the inferior vena cava. The animals were then killed, and the colon was removed, dissected, and opened lengthwise. Intestinal remnants were collected from the opened colon. Colon tissues and intestinal remnants were stored at −80°C until analysis. The plasma was separated from the blood by centrifugation and stored at −80°C until analysis.

LC-MS/MS Analysis of ONO-AE2-724 and ONO-AE1-437 in Tissue and Serum Samples. The tissue samples (colonic tissue and intestinal remnants) were homogenized in acetonitrile. Then 10 μl of the tissue extract was transferred into a tube; 10 μl of the internal standard solution, 10 μl of ethanol, and 10 μl of acetonitrile were added to the tube and mixed; and 210 μl of the mixture of water and acetonitrile (50:50, v/v) containing 0.1% v/v formic acid was then added and mixed. The sample was transferred into a HPLC vial after filtration.

Then 50 μl of the plasma sample was transferred into a tube, and 10 μl of the internal standard solution and 2 ml of acetonitrile were added and mixed. The sample was centrifuged at 2080g for 5 min. The supernatant was transferred into a tube and evaporated to dryness. The dried residue was dissolved in 100 μl of water and acetonitrile (50:50, v/v) containing 0.1% v/v formic acid. The sample was transferred into a HPLC vial after filtration.

The assay was performed on a LC-MS/MS system that consisted of an API4000 MS/MS system with a TurboIonSpray ionization source (Applied Biosystems/MDS Scieix, Foster City, CA) and an Agilent 1200 HPLC system. ONO-AE1-437 and ONO-AE2-724 were separated using a YMC-Pack Pro C18 column (50 mm × 2.1 mm i.d. column, 5-μm particle size; YMC Co., Ltd., Kyoto, Japan). Mobile phases A (0.1% v/v formic acid) and B (acetonitrile containing 0.1% v/v formic acid) were used for a linear gradient elution with 25 to 80% B for 0.5 min, hold at 80% B for 10 min, and then 80 to 25% B for 0.01 min. The flow rate was 0.2 ml/min, and the injection volume was 5 μl for the tissue sample or 20 μl for the plasma sample.

MS/MS analysis was performed in multiple reaction monitoring mode using a mass transition m/z of 435.0 → 119.0 for ONO-AE1-437 and m/z 440.0 → 119.0 for the deuterium-labeled form of ONO-AE1-437 (ONO-AE2-247, internal standard) in negative ionization mode and m/z 603.2 → 185.0 for ONO-AE2-724 in positive ionization mode. The mass spectrometer parameters used for ONO-AE1-437 and were as follows: declustering potential, 65 V; entrance potential, −10 V; collision energy, −24 V; collision cell exit potential, −9 V; dwell time, 500 ms; curtain gas, 10 ps; ion spray voltage, −4500 V; temperature of heater gas, 700°C; nebulizer gas, 60 psi; turbo gas, 50 psi; and collision gas, 8 psi. For ONO-AE2-724, the mass spectrometer settings were as follows: declustering potential, 91 V; entrance potential, 10 V; collision energy, 35 V; collision cell exit potential 14 V; dwell time, 1000 ms; curtain gas, 20 ps; ion spray voltage, 5500 V; temperature of heater gas 700°C; nebulizer gas, 80 ps; turbo gas, 70 ps; and collision gas, 6 ps.

Calibration curves were generated using 1/X²-weighted linear regressions and were used to calculate the concentrations of the unknown samples. Analyst version 1.4.2 software (Applied Biosystems/ MDS Scieix) was used for data acquisition and processing.

Administration of ONO-AE2-724 and EP4-MS and Preparation of Experimental Colitis in Mice. The protective effects of EP4-MS and ONO-AE2-724 were investigated in female BALB/c mice. To evaluate the potential protective effect of ONO-AE2-724 toward experimental colitis, animals were intravenously treated with single doses of ONO-AE2-724 (1 mg/kg) 1 h before TNBS instillation. In each experiment, the mice were divided into four groups (n = 12): normal and TNBS-induced colitis groups treated with or without ONO-AE2-724. To evaluate the protective effect of EP4-MS, animals were orally treated by gavage with single doses of EP4-MS 24 h before TNBS instillation. According to the results from the entrapment association of ONO-AE2-724 and PLGA microspheres, in 1 mg EP4-MS contain 0.1 mg of ONO-AE2-724. Therefore, 0.2 ml of 10 mg · kg⁻¹ · ml⁻¹ (1 mg of ONO-AE2-724 · kg⁻¹ · ml⁻¹) in 10% Tween 80 containing PBS solution was administered by gastric tube to the mice. In each experiment, the mice were divided into eight groups (n = 12): normal and TNBS-induced colitis groups treated with or without PLGA microspheres (control vesicle) (10 mg/kg), unloaded ONO-AE2-724 (1 mg/kg), or EP4-MS (10 mg/kg). Colitis was induced with TNBS according to the modified methodology described previously (Matsuura et al., 2005). In brief, mice were lightly anesthetized with isoflurane inhalation, and then a catheter (polyvinyl chloride tube, outside diameter 1.35 mm; Atom Medical, Tokyo, Japan) was carefully inserted into the colon (4 cm proximal to the anus). To induce colitis, TNBS (2 mg in 100 μl of 50% ethanol solution) was slowly administered. To assure the distribution of TNBS within the entire colon, mice were carefully maintained at a 45° angle (head down position) for 2 min and then returned to their cages. One hour later, the animals were given free access to food and water. Throughout the experiments, mice were monitored for body weight loss. At 72 h after TNBS administration, the animals were killed, and the colon was removed, dissected and opened lengthwise.

Histological Analysis and Evaluation of Microscopic Damage. Each excised colon was immediately fixed in 10% formaldehyde solution, embedded in paraffin, sectioned at 5-μm thickness, mounted on glass slides, and then deparaffinized. For histological analysis, slices were stained using hematoxylin and eosin (H&E) standard techniques and examined in cross sections at 200× and 1000× magnification under the microscope. To observe goblet cells, periodic acid-Schiff (PAS) staining was used (PAS-Alican Blue double-staining was not chosen because of convenience for cell count). In brief, sections were incubated in 0.5% periodic acid solution for 5 min and then in Schiff reagent for 15 min. PAS-positive goblet cells were counted over the size of a grid (1-mm grid with total 100 squares) at 100× original magnification in 10 view fields randomly selected from each slide.

Immunohistological Analysis of Cell Apoptosis and Proliferation in Colon. To evaluate the cell apoptosis in the colon, sections were immunoreacted with primary antibodies against single-strand DNA (ssDNA) (1:100; Dako, Glostrup, Denmark), and colon epithelium regeneration was assessed using an antibody specific for the mouse homolog of the Ki-67 proliferation associated antigen (1:100; Dako). Immunohistological staining was performed on a paraffin-embedded colonic tissue section (5 μm) according to the methods described previously (Toda et al., 1999). After deparaffinization and antigen retrieval by either of above methods, endogenous peroxidase activity was blocked by treatment with 3% H₂O₂ in
methyl alcohol for 30 min. The glass slides were washed in PBS (6 times, 5 min each) and mounted with 1% normal serum in PBS for 30 min. Then primary antibody was applied overnight at 4°C, and slides were incubated with biotinylated anti-serum (second antibody) diluted to 1:300 in PBS for 40 min, followed by washes in PBS (6 times, 5 min each). Avidin-biotin-peroxidase complex (ABC-Elite; Vector Laboratories, Burlingame, CA) at a dilution of 1:100 in BSA was applied for 50 min. Incubation and washing procedures were performed at room temperature if not specified. After washing in PBS (6 times, 5 min each), a coloring reaction was obtained with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Dako), and nuclei were counterstained with hematoxylin.

Images were obtained by using a DP-2 digital camera connected to an AX 80 light microscope (both from Olympus, Tokyo, Japan). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section were captured, and a threshold optical density that best discriminated staining from the background was obtained using ImageJ 1.44 imaging software (National Institutes of Health, Bethesda, MD). The total pixel intensity was determined, and data were expressed as optical density, using a counting grid at 200× original magnification. Epithelial cell proliferation and apoptosis were also determined by counting Ki-67- and ssDNA-positive cells per crypt in both MS- and EP4-MS pretreated mice with colitis. At least 10 crypts were counted on each slide, and four slides were counted for each mouse.

Quantitative Real-time PCR. Total RNA was purified from colonic tissue using a RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. cDNA was generated from 1 ng of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). cDNA was analyzed for content using a SYBR Green-based, quantitative fluorescent PCR method (Applied Biosystems, Foster City, CA). Fluorescence was detected with an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). The primers summarized in Table 1 were used. The following PCR conditions were used: 95°C for 15 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for the final 10 min. GAPDH was used as a housekeeping gene. Assays were performed in duplicate. Fold induction was calculated using the Ct method.

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**TABLE 1**

Primers used in quantitative real-time PCR analysis

Characterization of EP4-MS and In Vitro Drug Release Profile. Both the EP4-MS and MS had a spherical shape with 24.289 ± 0.157 μm (EP4-MS) and 17.890 ± 0.267 μm (MS) average diameters (Fig. 2A). The entrapment efficiency of ONO-AE2-724 within the PLGA microspheres was approximately 100%, and it can be explained by the fact that ONO-AE2-724 is a highly lipophilic compound. Figure 2B illustrates the in vitro drug release profiles obtained from the formulation used later in the in vivo experiments by representing the remaining percentage of ONO-AE2-724 with respect to the amount of ONO-AE2-724 encapsulated. The drug release profile showed a sustained release of the drug over 5 weeks, resulting from the diffusion of the drug through the polymer.

Colonic Tissue, Intestinal Remnants, and Serum Distribution of ONO-AE2-724 and Its Active Form of ONO-AE1-437 after EP4-MS Oral Administration. The drug distribution of encapsulated ONO-AE2-724 and its de-esterified active form ONO-AE1-437 in mouse colonic tissue, intestinal remnant, and serum were determined at different time points after oral administration of a single dose (10 mg/kg) (Fig. 3). Although high amounts of ONO-AE2-724...
were detected in colonic tissue and intestinal remnants using EP4-MS between 2 and 48 h after its administration, only low amounts (near the limit of detection) of ONO-AE1-437 were detected in the serum even 2 or 4 h after administration. ONO-AE2-724 metabolism in vivo is rapid, and the drug concentration of unloaded ONO-AE2-724 in the colon 2 h after oral administration is under the limit of detection (data not shown). This result indicated that EP4-MS released a high concentration of ONO-AE2-724 in the colon locally, but it did not circulate systemically.

**ONO-AE2-734 Intravenous Pretreatment Prevents the Development of Colitis in Mice.** In a pilot study, because ONO-AE2-724 was a newly synthesized EP4 agonist, we initially determined its potential protective effect in TNBS-induced colitis. TNBS caused loss of body weight and severe inflammation manifested by shortened, thickened, and erythematous colons. Animals received the single dose of ONO-AE2-724 (1 mg/kg i.v.) 1 h before TNBS instillation in the colon. The loss of body weight was monitored throughout the experiment. Three days after TNBS instillation, animals were killed, and colon length and damage were analyzed. Pretreatment with ONO-AE2-724 significantly inhibited the loss of body weight at day 2 after TNBS administration compared with that in the vehicle-pretreated TNBS-induced colitis group ($p < 0.05$) (Fig. 4A). ONO-AE2-724 pretreatment also significantly restored colon length ($p < 0.05$) (Fig. 4, B and C). H&E staining revealed considerable tissue damage in the vehicle-pretreated group but not in the normal or ONO-AE2-724-pretreated groups (Fig. 4D).

**Oral Intake of EP4-MS Prevents the Development of Colitis in Mice.** To evaluate the protective effect of EP4-MS in TNBS-induced colitis, animals received MS (10 mg/kg p.o.), unloaded ONO-AE2-724 (1 mg/kg p.o.), and EP4-MS (10 mg/kg p.o.) 1 day before TNBS administration. EP4-MS pretreatment significantly decreased the loss of body weight at day 2 after TNBS administration in both MS-pretreated and ONO-AE2-724-pretreated TNBS-induced colitis groups ($p < 0.05$) (Fig. 5A). EP4-MS pretreatment significantly restored colon length ($p < 0.05$) (Fig. 5, B and C). Hematoxylin and eosin staining showed that the pretreatment with EP4-MS inhibited considerable tissue damage and inflammation in the EP4-MS-pretreated group. PAS staining revealed significant restoration of the number of goblet cells in the EP4-
MS-pretreated group compared with that in the MS-pretreated group ($p < 0.01$) (Fig. 5D).

**EP4-MS Administration Inhibited the Colonic Epithelial Cell Apoptosis and Promoted Cell Proliferation.** At the cellular level, we monitored apoptotic cells using ssDNA immunostaining. In vehicle (MS, without TNBS)- and EP4-MS-pretreated groups, only low levels of apoptosis were detected by the ssDNA antibody in contrast to the extensive apoptosis of epithelial cells in mice with TNBS-induced colitis (Fig. 6A, top panels). We also monitored proliferation of epithelial cells using Ki-67 as a marker because intestinal epithelial cells are turned over rapidly. Ki-67 is an intracellular polypeptide whose synthesis reaches its maximum during the S phase of the cell cycle. In general, Ki-67-positive cells were localized largely at the crypt epithelium of the mucosal layer and were rarely found in the submucosal, muscular, or serosa layers. EP4-MS treatment abundantly produced Ki-67-positive cells in contrast to the vehicle and TNBS administration groups (Fig. 6A, bottom panels). Approximately 19.6 ± 3.7% of crypt epithelial cells were Ki-67-positive in the EP4-MS-pretreated group, compared with 13.2 ± 2.4% in the MS-pretreated group (Fig. 6B). On the contrary, 1.0 ± 1.1% of crypt epithelial cells were ssDNA-positive in the EP4-MS-pretreated group, compared with 5.3 ± 2.7% in the MS-pretreated group (Fig. 6C). It seems that EP4-MS may accelerate the regenerative capability of epithelial cells.

**EP4-MS Administration Suppresses the Expression of Colonic Proinflammatory Cytokines and Induces CD4$^+$CD25$^+$FoxP3$^+$ Regulatory T Cells in Mice with TNBS-Induced Colitis.** We estimated the expression of mRNA levels of proinflammatory (IL-1$\beta$, IL-2, IL-6, TNF-$\alpha$, and IFN-$\gamma$) and anti-inflammatory (IL-10 and TGF-$\beta$) cytokines in the colonic tissues 3 days after TNBS-instilled mice by quantitative RT-PCR. Pretreatment of EP4-MS inhibited expression of mRNA levels of all measured proinflammatory cytokines compared with the MS-pretreated TNBS-induced colitis group ($p < 0.05$) (Fig. 7A), but a significant difference in mRNA expression levels of anti-inflammatory cytokines between the two groups was not observed. In addition, the sequential change in plasma IL-10 concentration revealed that EP4-MS administration elevated the plasma IL-10 level significantly, but the elevation after TNBS instillation were not observed ($*p < 0.05; **p < 0.01$) (Fig. 7B). The results suggested that EP4-MS pretreatment suppresses the initial process of colonic inflammatory induction by TNBS.

Finally, we investigated whether pretreatment with EP4-MS could increase CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs). We isolated LPLs from either MS-pretreated or EP4-MS-pretreated mice with colitis. The percentage of FoxP3$^+$ Treg cells within the CD4$^+$CD25$^+$ T cells of the LPLs was significantly higher than the animals without colitis and the MS-pretreated colitis groups ($p < 0.05$) (Fig. 7C).

**Discussion**

In this study, we demonstrated that a newly synthesized EP4-selective agonist, ONO-AE2-724, prevented TNBS-induced colitis. In an attempt to improve the intestinal distribution pattern of the administered EP4-selective agonist, PLGA microspheres that incorporated ONO-AE2-724 were...
designed. The microspheres increased the stability of ONO-AE2-724 in the intestine without an increase in the drug concentration in plasma, and its oral administration showed the same protective effect on TNBS-induced colitis as that of ONO-AE2-724 by intravenous administration. This activity was accompanied by modification of pro- and anti-inflammatory cytokine expression and Treg differentiation, which reflects the generation of inflammatory innate immune responses. Immunohistochemical analysis also revealed that ONO-AE2-724 suppressed colonic epithelial cells apoptosis and facilitated cell regeneration.

The current medications for IBDs are mostly based on 5-ASA, steroids, and immunosuppressive drugs such as azathioprine, 6-mercaptopurine, and cyclosporine (Sonu et al., 2010; Mowat et al., 2011; Turner et al., 2011). However, these agents display limited beneficial actions, and long-term immunosuppressive drug administration may induce more serious complications such as renal dysfunction. Therefore, many studies have been conducted to find additional therapeutic agents (Koboziev et al., 2011).

Several studies have demonstrated that EP4-selective agonists possess anti-inflammatory properties. EP4-selective agonists therefore represent a novel class of immune-modulator drugs that could be useful for the management of inflammatory diseases such as arthritis (Shibata-Nozaki et al., 2011), sepsis (Sakamoto et al., 2004), inflammatory pain (Omote et al., 2002), and colitis (Kabashima et al., 2002; Nitta et al., 2002; Jiang et al., 2007, 2010). In a human study, patients with UC refractory to 5-ASA responded favorably to treatment with \([[3\beta,[1\alpha]]-2\beta-(3S)-3-Hydroxy-4\{3-(methoxymethyl)phenyl\}-1-butenyl]-3α-\)
hydroxy-5-oxocyclopentane-1α-y[thio]propylthioacetic acid chloridin (ONO-4819CD), an EP4 agonist (Nakase et al., 2010). The authors also reported that systemic exposure of EP4 agonists caused side effects of neutropenia, headache, hot flashes, constipation, diarrhea, fever, and hypotension. These reported reactions were mild and transient, but it will be important to minimize the potential side effects of EP4 agonists for the treatment of IBDs because the demonstration of significant side effects will preclude the continued use of therapy. Moreover, their clinical trial protocol of these authors seems to be slightly complicated (patients received a 2-week consecutive infusion of EP4 agonist at a dose of 36 ng/kg b.wt. twice a day), and actually few applicants were enrolled in the trials. It would be helpful for those patients to receive the same therapeutic effect of EP4 agonists by oral administration. Therefore, we created PLGA microspheres containing the EP4 agonist, ONO-AE2-724, for targeted colonic delivery and examined its preventive effects for TNBS-induced experimental colitis in mice, which is typified by colonic transmural damage caused by hapten-induced delayed hypersensitivity and has been used as a model to study human Crohn’s disease.

Previous studies with EP4 agonists in a mouse IBD model of 3/5% dextran sodium sulfate-induced colitis revealed their ability to inhibit the innate immune response (Kabashima et al., 2002; Nitta et al., 2002), their antiapoptotic activity on intestinal epithelial cells, and a stimulating effect on mucus secretion from Goblet cells (Jiang et al., 2007, 2010). In the present TNBS-induced mice colitis model, it was found that pretreatment by the EP4 agonist significantly reduced colonic inflammation reactions and facilitated a recovery in body weight. A previous report suggested that high-dose treatment with a PGE1 analog, misoprostol (60 μg/mouse; nearly 3 mg/kg), exacerbated TNBS-induced colitis in BALB/c mice and also suggested the proinflammatory role of PGE2 in IBDs (Sheibanie et al., 2007). However, it was also reported that low-dose misoprostol treatment (2 and 20 μg/mouse) had marginal effects. Although these results could be interpreted to indicate that PGE2 signaling facilitated the inflammatory process, we hypothesized that high-dose misoprostol would significantly affect vasodilatation and blood pressure, and, in fact, treatment with misoprostol was toxic for mice after relatively high-dose TNBS (3 mg/mouse) colitis induction. Therefore, at first, we tested the pretreatment effect of a single intravenous injection of EP4 agonist (1 mg/kg) in mice with TNBS-induced colitis mice. A significant reduction in colonic injuries was similarly observed using the same drug dose as that for oral pretreatment with EP4-MS. This result suggested that pretreatment with an EP4 agonist suppressed proinflammatory cytokine mRNA expression and facilitated Treg induction, which would induce tolerance toward TNBS-induced colonic damage. Moreover, histopathological analysis revealed antiapoptotic activity and promotion of cell proliferation. Our results support the previously reported colitis prevention and treatment effects of EP4 agonists. In an autoimmune liver disease model in mice, we found previously that preactivation of PGE2-EP4 signaling diminished liver damage, and we considered that PGE2-EP4 signaling would be an important pathway to generate immunological tolerance (Okamoto et al., 2011). However, it is also true that in some disease models such as arthritis and encephalomyelitis, PGE2-EP4 signaling acts as a proinflammatory mediator (Honda et al., 2006; Yao et al., 2009; Esaki et al., 2010). As reported previously, we emphasize that more studies will be needed to determine the precise role of PGE2-EP4 signaling in colitis (Dey et al., 2006).

The reliable colonic delivery of an anti-inflammatory drug by using PLGA microspheres was reported previously. PLGA microspheres were taken up into the colonic lymphatic tis-
sues and Peyer’s patches (Tabata et al., 1996; Nakase et al., 2000), and the tissue distribution analysis suggested that PLGA microspheres were highly deposited in inflamed tissue by phagocytosis of macrophages. The pharmacokinetics of this drug delivery system showed that serum levels of ONO-AE1-437, the active form of ONO-AE2-724, in the mice treated with EP4-MS were not increased because the size of the microspheres does not allow absorption by enterocytes, and this drug delivery system seems unlikely to induce systemic side effects. On the contrary, the continuous increase of ONO-AE1-437 at the site of action was not observed, which indicates that the possibility of a systemic effect of the EP4 agonist was not precisely excluded. As another group suggested (Komaba et al., 2007), prostaglandins and those derivatives are rapidly metabolized, and high-sensitivity detection of those molecules in vivo is still technically difficult.

This would be one of the reasons that the concentration of activated ONO-AE1-437 in the colonic tissue could not be determined by LC-MS/MS analysis. Nevertheless, oral intake of ONO-AE2-724 without the PLGA carrier does not prevent TNBS-induced colitis, which suggests that this EP4 agonist needs to be delivered in a targeted manner to exert a therapeutic effect, similar to that of PGE2 and other chemically modified PGE2 analogs.

Although this initial study revealed the possibility of EP4 agonist oral treatment for IBDs, the choice of an optimal particle size and degradation time for the design of a particular carrier system remains to be resolved in the future. Several reports have focused on the advantages of nano-sized particles as drug carriers (Lamprecht et al., 2001; Meissner et al., 2006; Alves et al., 2011). However, nano-sized particles have the possibility of systemic distribution, which may cause unanticipated side effects, and further studies will be needed to determine the optimal particle size for local, espe-
cally intestinal, targeted drug delivery. In addition, in this study, LC-MS/MS analysis revealed that ONO-AE2-724 was not detected until 48 h after administration in mouse colonic tissues and intestinal remnants. Integrating these results with previous results with regard to the relation of particle size and inflamed tissue uptake will help in the design of an optimal colonic PLGA delivery system for the enhancement of the anti-inflammation activities of EP4 agonists.

In summary, the present results show for the first time that oral administration of the EP4 agonist, ONO-AE2-724, loaded into PLGA microspheres can effectively prevent TNBS-induced colitis in mice. We propose that EP4-Ms may be therapeutically useful for prevention and treatment of human IBDS, but further studies are needed to optimize the size distributions and degradation time of PLGA microspheres for the best intestinal activation of EP4 agonists. In the future, comparative studies on the therapeutic efficacy between EP4-Ms and other drugs commonly used to treat IBDS such as 5-ASA and steroids will be performed.

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Authorship Contributions

Participated in research design: Okamoto, Uemoto, and Tabata. Conducted experiments: Okamoto. Performed data analysis: Okamoto and Tabata. Wrote or contributed to the writing of the manuscript: Okamoto, Uemoto, and Tabata.

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