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Received November 8, 2011; accepted December 1, 2011

ABSTRACT
Intestinal subepithelial myofibroblasts (ISMFs) are mesenchymal cells that reside in the subepithelial region throughout the intestine. When the intestine is damaged, the migratory and mitotic responses of ISMFs are crucial for wound closure. However, their mechanism of action remains unknown. We have investigated the role of cyclooxygenase (COX) and its metabolite prostaglandin E₂ (PGE₂) in the wound repair process of bovine ISMFs. The action of a mechanical scratch in a layer of ISMFs in cell culture elevated the levels of both COX-2 mRNA expression and PGE₂ secretion 1 and 6 h after the event. After 24 h ISMFs had migrated to and reduced the wounded area around the site of the scratch. Treatment with the COX-1/2 inhibitor indomethacin, the COX-2 inhibitor 3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethylisoxazole (CAY10404), or E prostanoid receptor 2 or 4 (EP2–EP4) antagonists significantly inhibited wound repair. Conversely, inhibition of wound closure by indomethacin was reversed by treatment with PGE₂ or agonists of the receptors EP2, EP3, or EP4 but not of EP1. Although EP2 to EP4 stimulation did not influence ISMF proliferation, it did stimulate ISMF migration in the transwell cell migration assay. It is noteworthy that cell migration stimulated by EP2 and EP4 was inhibited by the tyrosine kinase receptor inhibitor genistein and also by (Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid (SU6668). However, cell migration stimulated by EP3 was unaffected. Reverse transcription-polymerase chain reaction showed EP2 or EP4 stimulation elevated the level of mRNA expression for fibroblast growth factor-2, which stimulates ISMF migration. Collectively, COX-2-dependent PGE₂ secretion promotes wound healing by ISMFs. PGE₂-EP3 signaling may directly stimulate ISMF migration. PGE₂-EP2/4 signaling indirectly stimulates ISMF migration by elevating the level of growth factor secretion.

Introduction
Intestinal subepithelial myofibroblasts (ISMFs) are mesenchymal cells that form a thin layer beneath the epithelial layer (Furuya et al., 2005). Both ISMFs and the intestinal epithelial layer function as barriers against bacterial infiltration of the lumen (Furuya et al., 2005; Blikslager et al., 2007). Intestinal inflammation can lead to ISMFs being damaged and denuded (Francoeur et al., 2009), resulting in an increased susceptibility to infection. Thus, an understanding of the mechanism of wound closure by ISMFs is indispensable to the development of future treatments for intestinal barrier dysfunction.

Cyclooxygenase (COX) is one of the factors accelerating tissue regeneration (Peskar, 2005; Radì and Khan, 2005). COX converts arachidonic acid to prostaglandin H₂, a precursor of other prostaglandin species. COX has two isozforms:
COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and contributes to maintaining tissue homeostasis, whereas COX-2 is an inducible form of the enzyme that is modulated by a variety of inflammatory stimuli. Prostaglandin E2 (PGE2) is a major product of both isoforms, and its actions can result in the development of pathophysiology by activating four different E prostanoid receptors: EP1, EP2, EP3, and EP4 (Hata and Breyer, 2004).

Studies have shown that PGE2-EP4 signaling accelerates the repair of gastrointestinal ulcers in a mouse model (Jiang et al., 2009; Takeuchi et al., 2010). Another study has shown that ISMFs strongly express COX-2 in a chronic inflammation model in mice (Davids et al., 2010). Those studies suggest the importance of COX-PGE2 signaling in intestinal wound healing. However, the detailed mechanism of the signaling remains unknown. In this article, we investigated the role of COX-dependent PGE2 secretion in wound repair in primary isolated ISMFs.

Materials and Methods

Materials. The following reagents and materials were used in the experiments: fetal bovine serum (FBS) (Nichirei Biosciences Inc., Tokyo, Japan); random RT-primer, ReverTra Ace (Toyobo Engineering, Osaka, Japan); ExTaq (Takara Biomedical, Otsu, Japan); PGE2, CAY10404 [3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethyl-isoxazol], AH6809 (6-isoproxy-9-oxoanthene-2-carboxylic acid), AH23848 [(7-5x)-(1S,1a(Z)-biphenyl)-4-ylmethoxy)-2p-(4-morpholino)-3-oxyxyclopylentyl]-4-heptonic acid, calcium salt), hydrate), MK886 [1-(4-chlorophenyl)-3-[(1,1-dimethylthyl)thio]-a, a-dimethyl-5-(1-methylthyl)-1H-indole-2-propanoic acid, sodium salt], 6-ketoPGF1α, enzyme immunoassay kit, and PGE2-enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI); SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole] (Calbiochem, San Diego, CA); anticytotoxic and TRizol (Invitrogen, Carlsbad, CA); fibroblast growth factor-2 (FGF-2) (US Biological, Swampscott, MA); indomethacin, gelatin, genistein, PGI2, and LY798106 {2-[4-(3-bromo-2-methoxyphenyl)sulfonyl]-3-[2-(2-napthalenylmethyl)phenyl]-2-propenamide} (Sigma, St. Louis, MO); SU6668 [(2S)-2-[4,4-dimethyl-5-oxo-1,2-dihydro-indol-3-ylidenemethyl]-1H-pyrrol-3-yl-proionic acid} (Toei Bioscience, Ellisville, MO); and PGE2-parametric assay kit (R&D Systems, Minneapolis, MN). ONO-DI-004 [T5S-2,5-ethano-6-oxo-17,20-dimethyl prostaglandin E1], ONO-AE1-259-01 (11L15-O-dimethyl prostaglandin E2), ONO-AE2-248 (169-9-deoxy-9-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-diehyro prostanol prostaglandin F2), and ONO-AE1-329 [16-(3-methoxyethyl)phenyl-o-tetranor-3,7-dithia-prostaglandin E1] were kindly donated by the Ono Pharmaceutical Company (Osaka, Japan). The concentration of each drug used in this study is referred to in previous reports as follows: MK886 (Ikeda-Matsuo et al., 2010); CAY10404 (Lee et al., 2007); and indomethacin and SC-560 (Fornai et al., 2006).

Preparation of ISMFs. Isolation of bovine ISMFs was carried out as described previously (Mahida et al., 1997). Bovine colon was dissected, and the smooth muscle layer was removed. The mucosal layer was treated with 1 mM EDTA at 37°C for 1.5 h to detach epithelial cells. Epithelial cell-free mucosa was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% anticytotoxic-antibiotic (final concentration: 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B). After the ISMFs became confluent, the epithelial-free mucosa was removed and the cells were subcultured. Cells used in the experiments were from passages 3 to 7. All experiments were conducted after serum starvation of the cells for 24 h.

Wound Healing Assay. To investigate wound-induced ISMF migration, a wound healing assay was performed as reported previously (Eligini et al., 2009). Cells were seeded in a 60-mm round dish and cultured. After 3-h incubation with each inhibitor, the cell layer was disrupted by scratching 10 linear lines on the surface both longitudinally and transversely by using a 250-μl pipette tip. Three fields with a centrally located intersection of scratched lines were chosen and photographed by using a phase-contrast microscope with a digital camera (digital sight; Nikon, Tokyo, Japan). The medium was changed with the addition of each agent to be tested. After 24-h incubation, the same fields were photographed again. Scratched areas were quantified by National Institutes of Health (Bethesda, MD) ImageJ software. The amount of wound healing was expressed as a percentage according to the following formula: (initial scratched area, agents added) − (resulting scratched area, agents added)/ (initial scratched area, no agents added) − (resulting scratched area, no agents added).

Reverse-Transcription Polymerase Chain Reaction. RT-PCR was performed as follows: the first strand of cDNA was synthesized by using a random RT primer and ReverTra Ace at 37°C for 10 min, 42°C for 1 h, 99°C for 5 min, and 4°C for 5 min. PCR amplification was performed by using ExTaq DNA polymerase and the synthetic gene-specific primers shown in Table 1. Amplification was done by using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedical, Shiga, Japan). PCR products were separated by electrophoresis on a 2% agarose gel containing 0.2 μg/ml ethidium bromide. The bands were visualized by using an ultraviolet transilluminator (Toyobo Life Science, Tokyo, Japan). Band densities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 24 cycles, COX-1 at 24 cycles, COX-2 at 24 cycles, FGF-2 at 24 cycles, vascular endothelial growth factor (VEGF)-A at 24 cycles, and hepatocyte growth factor (HGF) at 27 cycles were quantified by using the ImageJ software.

### Table 1: Primer sequences for RT-PCR

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<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Cycle Number</th>
<th>Annealing Temperature</th>
<th>Size</th>
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Table 1: Primer sequences for RT-PCR.
Measurement of PGE2 in Supernatants. An enzyme-linked immunosorbent assay was performed to measure PGE2 levels in ISMF supernatants. ISMFs were grown in six-well plates. After preincubation with each inhibitor for 3 h, cell layers were scratched as described above. The medium was changed again and followed by the addition of each inhibitor. Supernatants were harvested after 3-, 6-, and 12-h incubation, and indomethacin (30 μM) was added immediately to prevent COX activity. PGE2 levels in the supernatants were assayed following the manufacturer’s protocol. After harvesting the layer of ISMFs, the cells were homogenized in phosphate-buffered saline with 1% Triton, and the protein concentration was determined by the Lowry method (Lowry et al., 1951).

Proliferation Assay. To verify mitotic activity of ISMFs, proliferation assay was performed. ISMFs (1.0 × 10^5 cells) were seeded overnight in DMEM containing 10% FBS and allowed to attach onto each well of a six-well plate. Cells were cultured in DMEM for 24 h and treated with the agents to be tested. After 24- and 48-h incubation, the number of cells was counted. Data are expressed as a relative value to the number of cells at 0 h.

Migration Assay. The migration assay was performed as described previously (Li et al., 1999). ISMFs (1.0 × 10^5 cells/300 μl of DMEM) were seeded in the 24-well upper chamber of cell culture chambers. After 6- or 12-h incubation, membranes were fixed with 10% formaldehyde neutral buffer solution and stained with 5% Giemsa solution for 1.5 h at room temperature. In four randomly selected fields, the number of cells that migrated to the reverse side of the membrane was counted under a light microscope at 200-fold magnification.

Statistical Methods. Results are presented as the mean ± S.E.M. and were evaluated for statistical significance by using one-way analysis of variance, followed by Tukey’s test. A value of P < 0.05 was regarded as significant.

Results

COX-2-Dependent PGE2 Is Involved in Monolayer Repair of ISMFs. By 24 h after the scratches were made, ISMFs had migrated to and reduced the size of the wounded area (Fig. 1A). A COX-1/2 inhibitor, indomethacin, showed ~40% inhibition of the wound repair (1 μM indomethacin: 57.2 ± 2.6%; Fig. 1; P < 0.001; n = 6). Treatment with a COX-2 inhibitor, CAY10404 (10 μM), exhibited the same degree of inhibition (Fig. 1B; P < 0.001; n = 4), whereas treatment with a COX-1 inhibitor, SC560 (100 nM), did not inhibit wound healing. A microsomal PGE synthase-1 (mPGES-1) inhibitor, MK886 (1 μM), also suppressed wound healing (Fig. 1B; P < 0.001; n = 6). Additive treatment with 1 to 10 μM PGE2 (Fig. 1B; P < 0.05; n = 4) but not with 1 μM PGD2 or 1 μM PGL2 (data not shown; n = 4 each) reversed the inhibition by indomethacin in a dose-dependent manner (Fig. 1B; P < 0.01; n = 4–6). The data indicate that COX-2-derived PGE2 is responsible for closure of the injury site in the layer of ISMFs.

Scratch Induces Elevation of COX-2 Expression and PGE2 Secretion in ISMFs. Using RT-PCR, we initially confirmed that unscratched ISMFs constitutively express COX-1/2 and three types of PGES, mPGES-1/2 and cytosolic PGES synthase (cPGES). The mRNA expression level for COX-2 was elevated after 1 h and reached a peak at 2 h after the scratch was made (Fig. 2B; P < 0.05; n = 5). In contrast, the level of mRNA expression for COX-1 did not change throughout the experimental period (0–6 h after the scratch was made) (Fig. 2C; n = 5–6).

That unscratched ISMFs secrete PGE2 constitutively into culture supernatant was shown by enzyme-linked immunosorbent assay. The presence of indomethacin (1 μM) or CAY10404 (10 μM) eliminated this secretion (Fig. 2D; P < 0.001; n = 4 each). As shown in Fig. 2E, PGE2 secretion increased 6 to 12 h after the injury (Fig. 2E; P < 0.01; n = 4). Treatment with indomethacin (1 μM) strongly inhibited PGE2 secretion from the injured cells (Fig. 2F; P < 0.001; n = 4). Other than PGE2, the scratch did not alter the level of secretion of other types of PGs (PGD2 and PGL2; data not shown; n = 4 each).

PGE2 Accelerates Wound Closure of ISMFs via EP2, EP3, and EP4. Figure 3A represents the expression pattern of EPs in ISMFs (top) and bovine colon (bottom; positive control). ISMFs express all subtypes of EPs (EP1-EP4). We next investigated the involvement of EPs in wound repair of ISMFs. Treatment with an EP2 antagonist (AH6809, 10 μM), an EP3 antagonist, (L798106, 1 μM), and an EP4 antagonist (AH23848, 10 μM) showed ~25, ~25, and ~15% inhibition of wound repair, respectively (Fig. 3B; n = 6; P < 0.05).

Fig. 1. COX-2-dependent PGE2 is involved in wound-induced intestinal subepithelial myofibroblast migration. A, typical pictures of wound repair 24 h after the mechanical scratch (40-fold magnification; left, nontreated, right, 1 μM indomethacin). The black lines indicate the edges of the wounded area. B, quantitative analysis of the wound repair 24 h after making the scratch. Wound healing area of nontreated cells is defined as 100%. Analytical data are presented as the mean ± S.E.M. of four to six separate experiments. ***, P < 0.001 versus nontreated controls. #, P < 0.05; ###, P < 0.001 versus 1 μM indomethacin-treated cells. SC560, COX-1 inhibitor, 100 nM; CAY10404, COX-2 inhibitor, 10 μM; MK886, mPGES-1 inhibitor, 1 μM.
Fig. 2. Scratch enhanced COX-2 expression and PGE₂ secretion in ISMFs. A, mRNA expression of COX-1, COX-2, mPGES-1, m-PGES-2, cPGES, and GAPDH in ISMFs. Shown are gel electrophoresis results of the PCR products. B and C, quantitative analysis of mRNA expression of COX-2 (B) and COX-1 (C). Total RNA was extracted from ISMFs 0.25 to 6 h after making the scratch. The results are expressed as the ratio of the optical density of each PCR product to that of GAPDH. Analytical data are presented as the mean ± S.E.M. of five to six separate experiments. *, $P < 0.05$; **, $P < 0.01$ versus ratio at 0 h. D and E, the level of PGE₂ in supernatant of ISMFs. After changing medium (D) or making the scratch (E), supernatant was harvested at each time (0–12 h). The results are expressed as nanogram per milligram of protein. Analytical data are presented as the mean ± S.E.M. of four separate experiments. ***, $P < 0.01$; ****, $P < 0.001$ versus unscratched cells. Indo- methacin (1 μM) and CAY10404 (10 μM) were used in these experiments.

Fig. 3. PGE₂ induced wound repair of ISMFs via EP2, EP3, and EP4. A, mRNA expression of PGE₂ receptor subtypes in ISMFs (top) and bovine whole colon tissue (bottom). Figures represent gel electrophoresis results of PCR products. B and C, quantitative analysis of the wound repair 24 h after the scratch. Wound repair area of nontreated cells is defined as 100%. Analytical data are presented as the mean ± S.E.M. of four separate experiments. **, $P < 0.01$; ###, $P < 0.001$ versus nontreated cells. ***, $P < 0.001$ versus 1 μM indomethacin-treated cells. Drug concentrations were: PGE₂, 1 μM; EP2 antagonist, 10 μM; EP3 antagonist, 1 μM; EP4 antagonist, 10 μM; EP1 to EP4 agonists, 1 μM.
sistent, additive treatment with agonists for EP2 (1 μM ONO-AE1-259-01), EP3 (1 μM ONO-AE-248), and EP4 (1 μM ONO-AE1-329) restored the wound healing inhibited by indomethacin (Fig. 3C; \( P < 0.001; n = 5–7 \)). However, treatment with an EP1 agonist (1 μM ONO-DI-004) did not restore this healing. These results suggest the importance of PGE\(_2\)-EP2-4 signaling in ISMF wound closure.

**The Effect of PGE\(_2\) on ISMF Proliferation and Migration.** Wound repair is a consequence of cell proliferation and/or migration. As shown in Fig. 4A, treatment with 1 μM indomethacin and additive treatment with 1 μM PGE\(_2\) did not influence ISMF proliferation (0–48 h; \( n = 4–6 \)), whereas 10% FBS alone stimulated cell proliferation (positive control; \( P < 0.001; n = 6 \)).

In the transwell cell migration assay, treatment with indomethacin (1 μM) suppressed ISMF migration (Fig. 4B; 12 h; \( P < 0.05; n = 4 \)). In the following experiments, we added indomethacin (1 μM) into both the upper and lower chambers to inhibit endogenous PGE\(_2\) production (Fig. 4, C, E, and F). In the presence of indomethacin, additive treatment with PGE\(_2\) or agonists for EP2, EP3, or EP4 potentiated ISMF migration at 1 μM (Fig. 4, B and C; \( P < 0.05; n = 4 \) each). When EP1 was used no potentiation was seen.

**Tyrosine Kinase Inhibitors Suppress EP2- and EP4-Mediated ISMF Migration.** Previous reports showed that PGE\(_2\) induces the secretion of growth factors in ISMFs, and these growth factors stimulate epithelial cell migration by attachment to the tyrosine kinase receptors (Shao et al., 2006). Using the wound healing assay, treatment with genistein (10 μM) or SU6668 (10 μM) was shown to inhibit wound repair by ~50% (Fig. 4D; \( P < 0.05; n = 4 \) each).

Using the transwell cell migration assay, it was shown that sole treatment with either of the tyrosine kinase receptor inhibitors genistein (10 μM, for 12 h) or SU6668 (10 μM, for 12 h) did not influence ISMF migration (Fig. 4E; \( n = 4 \) each). However, these inhibitors did attenuate EP2 and EP4 agonist-induced migration (Fig. 4E; \( P < 0.05; n = 4 \) each). It was interesting to note that EP3 agonist-induced ISMF migration was not affected by either of these tyrosine kinase inhibitors (Fig. 4F; \( n = 4 \) each). These results suggest that PGE\(_2\)-EP2/4 signaling indirectly stimulates ISMF migration by elevating the level of growth factor secretion, whereas PGE\(_2\)-EP3 signaling can directly stimulate ISMF migration.

**EP2 and EP4 Activation Potentiates mRNA Expression of FGF-2 in ISMFs.** Previous reports showed that PGE\(_2\) increases the expression of growth factors including PGE\(_2\)-EP2/4 signaling can directly stimulate ISMF migration.

**Fig. 4.** The effect of PGE\(_2\) on ISMF proliferation and/or migration. A, growth curves of ISMFs (0–48 h). Data are presented as the mean ± S.E.M. of four to six separate experiments. 

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**A.**

- Non-treated
- FBS (10%)
- Indomethacin
- Indomethacin + PGE\(_2\)

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**B.**

- Indomethacin
- PGE\(_2\)
- Indomethacin + PGE\(_2\)

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**C.**

- Non-treated
- PGE\(_2\)
- EP1 agonist
- EP2 agonist
- EP3 agonist
- EP4 agonist

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**D.**

- Non-treated
- Genistein
- SU6668

---

**E.**

- EP2 agonist
- EP4 agonist
- Genistein
- SU6668

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**F.**

- EP3 agonist
- Genistein
- SU6668
FGF-2 (Battersby et al., 2007), VEGF-A (Bradbury et al., 2005), and HGF (Zhang et al., 2000). Using RT-PCR we next assessed the level of mRNA expression of these genes. A mechanical scratch on the surface of the layer of ISMFs elevated the level of mRNA expression for FGF-2 (6 h after the scratch; Fig. 5A; \( P < 0.05; n = 4 \)), but not VEGF-A or HGF (data not shown; \( n = 4 \) each). Treatment with indomethacin (1 \( \mu M \)) tended to decrease mRNA expression for FGF-2 in unscratched ISMFs, and its presence significantly attenuated the scratch-induced elevation of FGF-2 expression (Fig. 5B; \( P < 0.05; n = 5–7 \)). Additive treatment with PGE\(_2\) (1 \( \mu M \)) or agonists (1 \( \mu M \)) for EP2 or EP4, but not EP1 or EP3, restored the level of FGF-2 expression that had been inhibited by indomethacin (Fig. 5B; \( P < 0.05; n = 5–7 \)).

**FGF-2 Stimulates ISMF Migration.** We next examined whether FGF-2 induces ISMF migration. As shown in Fig. 5C, treatment with FGF-2 (10 ng/ml) for 6 h enhanced ISMF migration that had been inhibited by 10 \( \mu M \) genistein or 10 \( \mu M \) SU6668 (\( P < 0.001; n = 4 \) each).

**Discussion**

In the present study, we have shown that a mechanical scratch in a layer of ISMFs growing in cell culture induces COX-2-dependent PGE\(_2\) secretion and secreted PGE\(_2\) stimulates ISMF wound healing in an autocrine fashion via EP activation.

Rat or mouse ISMFs are widely used to investigate the characteristics of ISMFs. In this study, however, we used bovine ISMFs for reasons described previously (Iwanaga et al., 2010). First, it is easier to isolate a larger number of bovine ISMFs from the animal, whereas the number of ISMFs isolated from one rodent is limited because of their smaller size. Second, bovine ISMFs maintain their physiological properties after multiple passages better than do rodent ISMFs. Thus, bovine ISMFs are a more convenient tool for investigating the physiological and pathophysiological roles of ISMFs.

Inhibitors of COX-2 suppressed wound-induced migration by ISMFs, but subsequent additive treatment with PGE\(_2\) reversed this inhibition (Fig. 1B), restoring the initial level of wound-induced ISMF migration. Using the dextran sulfate-induced colitis model in mice, inhibition of COX-2 inhibits wound healing. PGE\(_2\) reverses this inhibition (Tanaka et al., 2009). This result is in agreement with those from our in vitro study. Although a previous study showed that other PGs such as PGI\(_2\) also stimulate the wound-induced migration of human fibroblasts (Hatane et al., 1998), we could not detect any effects of PGI\(_2\) or PGD\(_2\) on ISMF migration (\( n = 4 \); data not shown).

Although treatment with indomethacin inhibited ISMF wound repair, the inhibition was only partial (~40%). Endothelin-1 and tissue inhibitor of metalloproteinase 1 are reported to promote wound healing in human ISMFs (Kerno-chnan et al., 2002; Di Sabatino et al., 2007). Mediators other than PGs are assumed to be involved in their method of action.

As shown in Fig. 2E, the concentration of secreted PGE\(_2\) 12 h after making the scratch was approximately 0.42 \( \mu M \) (1252.1 ± 48.0 ng/mg protein). In contrast, a significantly higher concentration of PGE\(_2\) (10 \( \mu M \)) was required to overcome the inhibition of wound healing caused by indomethacin (Fig. 2B). It is possible that injured ISMFs may significantly

**Fig. 5.** PGE\(_2\), stimulated FGF-2 expression in ISMFs via EP2 and EP4, which may result in promoting wound closure of ISMFs. A and B, quantitative analysis of mRNA expression of FGF-2 in ISMFs. Total RNA was extracted from ISMFs 2 to 12 h (A) and 6 h (B) after making the scratch. The results are expressed as the ratio of the optical density of each PCR product to that of GAPDH. Analytical data are presented as the mean ± S.E.M. of four to seven separate experiments. *, \( P < 0.05 \) versus ratio at 0 h; **, \( P < 0.01 \) versus ratio of unscratched cells. #, \( P < 0.05 \) versus scratched cells. †††, \( P < 0.001 \) versus nontreated cells. \( \bullet \), \( P < 0.05 \) versus scratched cells. ††, \( P < 0.001 \) versus PGE\(_2\) (10 ng/ml)-treated cells. Dosage of the drugs was: PGE\(_2\), 1 \( \mu M \); EP1 to EP4 agonist, 1 \( \mu M \); genistein, 10 \( \mu M \); SU6668, 10 \( \mu M \).
increase the secretion of PGE$_2$ to promote wound healing and the concentration at the edge of the wound may reach more than 0.42 $\mu$M. We have focused here on the role of ISMF-derived PGE$_2$. However, epithelial cells or immune cells infiltrating into the lamina propria during inflammation also secrete PGE$_2$ (Bowman and Bost, 2004; Degagné et al., 2009). PGE$_2$ released from these cells may also contribute to ISMF wound healing in vivo.

Unscratched ISMFs express an inducible type of COX, COX-2, as well as COX-1 (Fig. 2A). A previous study also showed that isolated human ISMFs express COX-2 even if unstimulated (Mahida et al., 1997). Physical stimulation during isolation and/or cell culture in ISMFs may also induce COX-2 expression in the cells. We did not investigate the mechanism of COX-2 elevation induced by a mechanical scratch. It has been reported that a reactive oxygen species produced by physical stimulation during the course of an action such as making a wound elevates COX-2 expression in vascular endothelial cells (Eligini et al., 2009). ATP is also known to induce COX-2 expression in many types of cells such as intestinal epithelial cells and monocytes (Degagné et al., 2009; Gavala et al., 2010). These mediators may also be involved in the scratch-induced elevation of COX-2 levels in ISMFs.

Our results showed that at the same concentration (1 $\mu$M) each synthetic EP2 to EP4 agonist tested exhibited a stronger effect than PGE$_2$ during ISMF wound recovery (Fig. 3B). This may be caused by their increased stability with and affinity to the receptor compared with PGE$_2$.

FGF-2 acted as an agonist on EP2 and EP4, increasing the degree of induced ISMF migration (Fig. 5, B and C). EP2 and EP4 coupled with Gs protein led to the stimulation of cAMP and protein kinase A levels (Regan, 2003). In both human fibroblasts and rat Müller cells, PGE$_2$ augmented FGF-2 expression, which was accompanied in turn by an increase in intracellular cAMP levels (Cheng et al., 1998; Sakai et al., 2001). These signal pathways may mediate FGF-2 production in ISMFs.

EP3-mediated migration was not sensitive to receptor tyrosine kinase inhibitors (Fig. 4E). A previous in vitro study showed that EP3 activation directly stimulates human lung fibroblast migration in a Rho kinase-dependent manner (Li et al., 2011). Indeed, EP3-mediated migration was observed earlier ($n = 4$; 8 h; data not shown) compared with EP2- and EP4-mediated migration (12 h; Fig. 4C). EP3 activation may directly stimulate ISMF migration.

In Fig. 4D, tyrosine kinase receptor inhibitors exhibited ~50% inhibition of wound healing, whereas indomethacin showed weaker (~40%) inhibition (Fig. 1B). Although we revealed that wound induces growth factor secretion in ISMFs via the COX-2-PGE$_2$-EP2/4 pathway, other pathways, such as purinergic signaling, may also be involved in growth factor elevation (Hill et al., 2010). Tyrosine kinase receptor inhibitors may inhibit the effects of growth factors that is produced via both COX-2-PGE$_2$-EP2/4-dependent and -independent pathways.

PGE$_2$ did not increase proliferation activity in bovine ISMFs (Fig. 4A). However, a previous study showed that PGE$_2$ stimulated proliferation of rat epithelial cells (Tanaka et al., 2009). Consistently, we also detected the mitotic effect of 1 $\mu$M PGE$_2$ on a rat epithelial cell line, IEC-06 (data not shown; $n = 4$). ISMFs proliferate much more slowly than epithelial cells. Difference in the basal proliferation rate may reflect these conflicting observations. Further investigation is needed to clarify this point.

In summary, we found that the act of making a scratch in a layer of cultured ISMFs induces COX-2-dependent PGE$_2$ secretion (Fig. 6). PGE$_2$-EP3 signaling may directly stimu-

![Fig. 6. Summary of this study. A, wound stimuli increased PGE$_2$ secretion in ISMFs in a COX-2-dependent manner. B, secreted PGE$_2$ may directly stimulate ISMF migration via EP3. C, PGE$_2$ also induces FGF-2 expression via EP2 and EP4, which may lead to stimulating ISMF migration.](image-url)
late ISMF migration. PGE<sub>2</sub>-EP2 and PGE<sub>2</sub>-EP4 signaling indirectly stimulates ISMF migration by increasing the degree of growth factor secretion including that of FGF-2. Promoting ISMF closure facilitates restoration of those parts of the intestinal wall that serve as a barrier to harmful foreign agents. Therefore, our findings will be useful for the development of novel treatments for impaired intestinal barrier function found during intestinal inflammation.

Acknowledgments

We thank the Otsuka Pharmaceutical Co., Ltd., for supplying ONO-DI-004, ONO-AE1-259, ONO-AE2-245, and ONO-AE1-329.

Authorship Contributions

Participated in research design: Iwanaga, Okada, and Murata.
Conducted experiments: Iwanaga and Okada.
Performed data analysis: Iwanaga and Okada.
Wrote or contributed to the writing of the manuscript: Iwanaga, Murata, Hori, and Ozaki.

References


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