Cancer Chemother Pharmacol (2003) 51: 216–220
DOI 10.1007/s00280-003-0576-1

ORIGINAL ARTICLE

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Stimulation of intestinal epithelial restitution by prostaglandin E_1 analogue

Received: 28 May 2002 / Accepted: 11 December 2002 / Published online: 26 February 2003

Abstract *Background*: 5-Fluorouracil (5-FU) causes intestinal mucosal damage and malabsorption. We have recently reported that coadministration of 17*S*,20-dimethyl-*trans*-Δ^2-prostaglandin E_1 (OP-1206), a stable synthetic analogue of prostaglandin E_1, with 5-FU to rats protects the small intestine from 5-FU-induced damage. Enterocyte proliferation would contribute to the restitution of the wounded intestinal mucosa. Thus, we investigated the effect of OP-1206 on the proliferation of rat jejunal crypt cells (IEC-6 cells) treated with 5-FU. *Methods*: Proliferation of IEC-6 cells was evaluated in terms of [^{3}H]-thymidine incorporation and using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Mucosal healing was assessed by measuring the speed of resealing across the denuded area of an IEC-6 cell monolayer. *Results*: OP-1206 stimulated [^{3}H]-thymidine incorporation into subconfluent IEC-6 cells pretreated with 5-FU and increased the number of IEC-6 cells. AH23848B, an EP4 prostaglandin receptor antagonist, blocked the OP-1206-stimulated [^{3}H]-thymidine incorporation into IEC-6 cells. The speed of resealing across the denuded area of a wounded IEC-6 cell monolayer was found to increase following treatment with OP-1206. *Conclusions*: OP-1206 stimulated the proliferation of IEC-6 cells treated with 5-FU, indicating a possible mechanism for the protective effect of OP-1206 against 5-FU-induced damage to the small intestine. OP-1206 was shown to be active in intestinal mucosal healing.

Keywords Prostaglandin E_1 · 5-Fluorouracil · IEC-6 cells · Mucosal healing · Enterocyte proliferation · Crypt cells

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Introduction

The intestinal epithelium contributes to the protection of the living body from many luminal irritants and pathogens. Intestinal epithelial dysfunction, as for example in inflammatory bowel disease (Crohn's disease, ulcerative colitis etc.), perturbs the barrier function [1, 2, 3]. Some antitumor drugs such as 5-FU are known to cause serious gastrointestinal toxicity. Excess permeation of large molecules and malabsorption of D-glucose, D-xylose, L-leucine, L-tryptophan and sulfanilamide have been observed in patients and experimental animals that had received 5-FU [4, 5, 6, 7, 8, 9].

The intestinal mucosa is composed of a dynamic epithelial cell population. The intestinal crypt cells proliferate and migrate to the villus tip, which maintains mucosal homeostasis physiologically. Antitumor drugs such as 5-FU block intestinal epithelial proliferation and perturb its homeostasis [10].

Administration of a synthetic analogue of prostaglandin (PG) E_1, OP-1206 (17*S*,20-dimethyl-*trans*-Δ^2-PGE_1), to rats has been shown to protect the small intestine from damage induced by methotrexate (MTX) [11, 12, 13]. OP-1206 suppresses the generation of reactive oxygen species which provoke the injury in the MTX-induced damage to the small intestine [14] and decreases DNA, RNA, proteins and polyamines (spermine and spermidine) in the jejunal mucosa of rats caused by MTX administration [15]. We have found that OP-1206 protects the small intestine from 5-FU-induced damage (hyperpermeability, leukocyte infiltration etc.) [16], although the mechanism remains to be clarified.

The IEC-6 cell line was derived from the crypt cells of the jejunum of normal rats. This cell line is often used as a model of the mucosal healing of the small intestine [17]. In this study, we investigated the effect of OP-1206 on the proliferation of IEC-6 cells exposed to 5-FU, intending to elucidate the mechanism for its protection against 5-FU-induced damage and malabsorption in small intestine.

Materials and methods

Materials

The IEC-6 rat jejunal crypt cell line was purchased from American Type Culture Collection (Rockville, Md.). Dulbecco's modified Eagle's medium (DMEM) and gentamicin sulfate solution were obtained from Gibco (Rockville, Md.). Fetal bovine serum (FBS) was from ICN Biomedicals (Costa Mesa, Calif.). OP-1206 was a kind gift from Ono Pharmaceutical Company (Osaka, Japan). AH23848B ([1α(*Z*),2β,5α]-(+)-7-[5-[1,1′-(biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl)-4-heptenoic acid) was a kind gift from GlaxoSmithKline Medicines Research Centre (Greenford, UK). All cell culture apparatus was purchased from Asahi Techno Glass (Chiba, Japan). 5-FU was from Wako Pure Chemicals (Tokyo, Japan). 3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Chemicals (St. Louis, Mo.). Other reagents were of analytical grade. 5-FU was dissolved in dimethyl sulfoxide (final concentration of dimethyl sulfoxide in culture medium 0.5%). OP-1206 was dissolved in ethanol (final concentration of ethanol in culture medium 0.1%). Dimethyl sulfoxide and ethanol did not affect the results obtained under these conditions.

Cell culture conditions

IEC-6 cells in DMEM containing 10% FBS and 10 μg/ml gentamicin sulfate were routinely cultured in a water-saturated atmosphere containing 5% CO_2 at 37°C. FBS was used after heating to inactivate the complement system (56°C, 30 min).

[^{3}H]-Thymidine incorporation into IEC-6 cells

IEC-6 cells were plated on a 24-well multiplate. After the cells had attached to the bottom of the multiplate, the cells were incubated in DMEM containing 0.1% FBS for 18–20 h. The subconfluent cells were incubated in DMEM containing 0.1% FBS and 1 m*M* 5-FU for 1 h, and then washed twice with fresh DMEM containing 0.1% FBS. The cells were preincubated with or without 10 μ*M* AH23848B for 10 min, and were then incubated with DMEM containing 10% FBS and OP-1206 (0–100 ng/ml). After a 20-h incubation, the cells were incubated with 0.2 μCi [^{3}H]-thymidine for 4 h at 37°C. The cells were washed three times with ice-cold phosphate-buffered saline, and were then solubilized with 2% sodium dodecyl sulfate solution. The radioactivity of the incorporated [^{3}H]-thymidine was determined using a liquid scintillation counter (LSC-3500; Aloka Company, Tokyo, Japan).

MTT assay of IEC-6 cells

The viability of IEC-6 cells was determined by the MTT assay. Briefly, IEC-6 cells were plated on a 96-well multiplate at 2×10^3 cells/well. After the cells had attached to the bottom of the multiplate, the cells were incubated in DMEM containing 0.1% FBS for 18–20 h. The subconfluent cells were incubated in DMEM containing 0.1% FBS and 1 m*M* 5-FU for 1 h, and then washed twice with fresh DMEM containing 0.1% FBS. The cells were incubated with OP-1206 (0, 20, 40, 60 and 100 ng/ml) for 24 or 48 h. MTT solution (0.5 mg/well) was added to each well and 4 h later, the produced formazan was solubilized with acid-isopropanol solution (0.04 *N* HCl/isopropanol). The absorbance at 540 nm (reference at 620 nm) was determined by a microplate reader (MPR A4; Tosoh Company, Tokyo, Japan).

Wounding assay of IEC-6 cell monolayers

IEC-6 cells were plated on a type I collagen-coated 24-well multiplate at 1×10^5 cells/well. Cells were cultured for 3 days after growing to confluency. The cell monolayer was incubated in DMEM containing 0.1% FBS and 1 m*M* 5-FU for 1 h, and then washed twice with fresh DMEM containing 0.1% FBS. The center of the cell monolayer was aspirated for 10 s using an aspirator with a Pasteur pipette (inner diameter 1 mm), and the wounding of the cell monolayer was created (time 0). The wounded cell monolayer was then incubated with OP-1206 (0, 20, 40, 60 and 100 ng/ml) for 24 h. The speed of resealing was calculated as follows: (diameter across the denuded area at 0 h incubation)−(diameter across the area after 24 h incubation).

Results

The effect of OP-1206 on the proliferation of IEC-6 cells treated with 5-FU was determined in terms of [^{3}H]-thymidine incorporation and by the MTT assay. Subconfluent IEC-6 cells were pretreated with 1 m*M* 5-FU for 1 h, and then incubated in culture medium containing 10% FBS and OP-1206 (20, 40 and 100 ng/ml) for 24 h. Incorporation of [^{3}H]-thymidine into the subconfluent IEC-6 cells was suppressed significantly by treatment with 5-FU (Fig. 1). OP-1206 alone (20, 40 and 100 ng/ml) did not affect [^{3}H]-thymidine incorporation into the subconfluent IEC-6 cells (data not shown). [^{3}H]-Thymidine incorporation into IEC-6 cells treated with 5-FU was increased significantly by incubation in medium containing 10% FBS and OP-1206 (20, 40 and 100 ng/ml), compared with incorporation in medium without OP-1206 (Fig. 1a). On the other hand, the OP-1206-enhanced [^{3}H]-thymidine incorporation into IEC-6 cells pretreated with 5-FU disappeared in the presence of the EP4 antagonist, AH23848B (Fig. 1b). AH23848B itself did not affect [^{3}H]-thymidine incorporation into IEC-6 cells under these experimental conditions. Subconfluent IEC-6 cells were pretreated with 1 m*M* 5-FU for 1 h and then incubated in medium containing 10% FBS and OP-1206 (20, 40, 60 and 100 ng/ml) for 24 and 48 h. The number of IEC-6 cells was suppressed significantly by treatment with 5-FU (Fig. 2). When incubated with OP-1206 for 24 h, the number of IEC-6 cells treated with 5-FU increased to a level similar to those without 5-FU (Fig. 2a). Incubation with OP-1206 for 48 h induced about a 1.5-fold increase in the number of IEC-6 cells (Fig. 2b). OP-1206 alone (20, 40, 60 and 100 ng/ml) did not affect the number of IEC-6 cells (data not shown).

The effect of OP-1206 on the speed of resealing across the denuded area of an IEC-6 cell monolayer was determined as a measure of mucosal healing. The speed of resealing of an IEC-6 cell monolayer treated with 5-FU and OP-1206 in the presence of 10% FBS in the culture medium was determined. The IEC-6 cell monolayer was pretreated with 1 m*M* 5-FU for 1 h and then incubated with various concentrations of OP-1206 for 24 h. 5-FU treatment resulted in a significant decrease in the speed of resealing of IEC-6 cells (Fig. 3). However, OP-1206

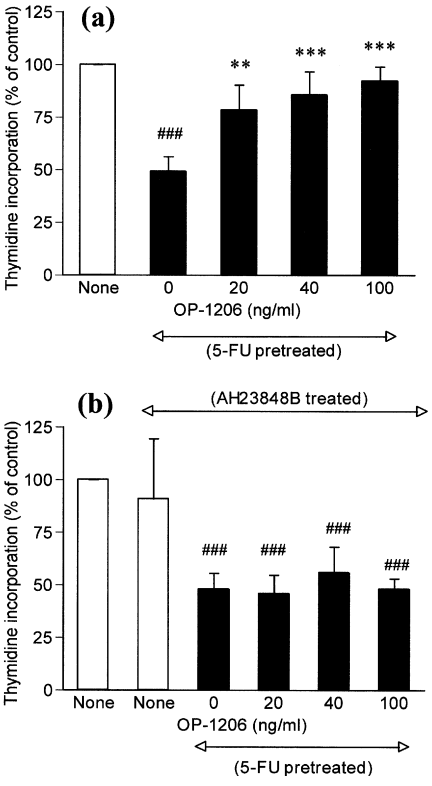

Fig. 1a, b [^{3}H]-Thymidine incorporation into IEC-6 cells treated with or without 5-FU and OP-1206. IEC-6 cells treated with 1 m*M* 5-FU for 1 h were incubated without the EP4 antagonist, AH23848B (**a**) or with 10 μ*M* AH23848B for 10 min (**b**) and then incubated with OP-1206 (0, 20, 40 and 100 ng/ml). The data are expressed as the percent of [^{3}H]-thymidine incorporation into the treated IEC-6 cells in relation to that of control cells and are the means ± SEM of four independent experiments with triplicate determinations. *Open columns* cultures without 5-FU and OP-1206 treatment. $^{###}P < 0.001$ vs control (*None*); $^{**}P < 0.01$, $^{***}P < 0.001$ vs with 5-FU (without OP-1206)

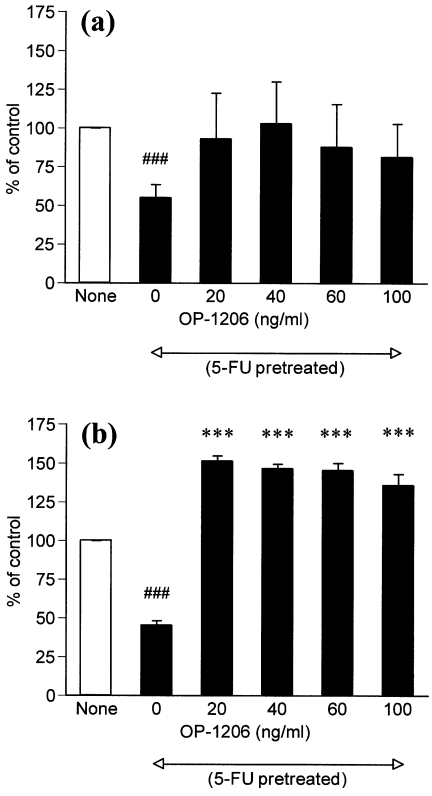

Fig. 2a, b Effect of OP-1206 on IEC-6 cells treated with 5-FU. IEC-6 cells treated with 1 m*M* 5-FU for 1 h were incubated with OP-1206 (0, 20, 40, 60 and 100 ng/ml) for 24 h (**a**) and 48 h (**b**). Viable cells were determined by the MTT assay. The data are expressed as the percent of viable cells treated with 5-FU and OP-1206 in relation to that of control cells treated with dimethyl sulfoxide (a solvent used to dissolve 5-FU) and ethanol (a solvent used to dissolve OP-1206) and are the means ± SEM of four independent experiments with quadruplicate determinations. *Open columns* cells without 5-FU and OP-1206 (control). $^{###}P < 0.001$ vs control (*None*); $^{***}P < 0.001$ vs with 5-FU (without OP-1206)

(20, 40, 60 and 100 ng/ml) enhanced the speed of resealing of IEC-6 cells treated with 5-FU. OP-1206 alone (20, 40, 60 and 100 ng/ml) resulted in about a 1.6- to 1.7-fold increase in the speed of resealing of IEC-6 cells (control; data not shown).

Discussion

5-FU is metabolized to 5-fluorouridine-5′-triphosphate or 5-fluoro-2′- deoxyuridine-5′-monophosphate. Thymidylate synthase is a target molecule of 5-fluoro-2′-deoxyuridine-5′-monophosphate. Inhibition of thymidylate synthase causes DNA dysfunction. 5-Fluorouridine-5′-triphosphate is incorporated into RNA and disturbs RNA metabolism. The toxicity of 5-FU in the gut is related to 5-fluorouridine-5′-triphosphate incorporation into RNA on enterocytes [10]. OP-1206 increased the [^{3}H]-thymidine incorporation into subconfluent IEC-6 cells (Fig. 1). Moreover, treatment of IEC-6 cells with OP-1206 increased the number of cells as determined by

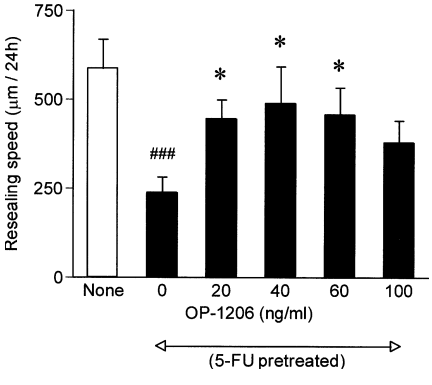

Fig. 3 Stimulatory effect of OP-1206 on the migration of IEC-6 cells treated with 5-FU. After wounding the cell monolayer by 1 m*M* 5-FU treatment for 1 h, the monolayer was incubated in culture medium containing 10% FBS and OP-1206 (0, 20, 40, 60 and 100 ng/ml) for 24 h. *Open column* without 5-FU and OP-1206 treatment. The data represent the means ± SEM of four independent experiments with quadruplicate determinations. $^{###}P < 0.001$ vs control (*None*); $^{*}P < 0.05$ vs without OP-1206

the MTT assay (Fig. 2). Thus, OP-1206 stimulated the proliferation of 5-FU-treated IEC-6 cells.

Prostaglandin endoperoxidase H synthase-1 and -2 are present in the nuclear envelope [18]. 16,16-Dimethyl PGE_2 alleviates the decrease in crypt cells of mouse intestine caused by γ-radiation, indomethacin and dextran sodium sulfate administration, and cyclooxygenase-1 expression in the intestinal epithelium is downregulated by such treatment [19, 20]. These observations suggest that PGE_2 produced through cyclooxygenase-1 promotes crypt cell proliferation. PGE_1 binds to PGE receptors EP3 and EP4 with high affinity (K_i 1.1 and 2.1 n*M*, respectively) [21]. EP4 mRNA is expressed in the jejunum of mice [22]. In recent studies in which the roles of prostanoids in dextran sodium sulfate-induced colitis were examined using mice deficient in each of the eight types and subtypes of prostanoid receptors, it was found that EP4 maintains intestinal homeostasis by maintaining mucosal integrity and downregulating the immune response [23]. Thus, the OP-1206-stimulated proliferation of IEC-6 cells exposed to 5-FU (Fig. 1) may occur via the EP4 receptor. A receptor antagonist would be very useful to clarify the contribution of the receptor to the proliferation. AH23848B is known as an EP4 antagonist [24, 25, 26, 27] and was used here to study the contribution of the EP4 receptor to the proliferation. Interestingly, OP-1206-stimulated [^{3}H]-thymidine incorporation into IEC-6 cells disappeared in the presence of the antagonist (Fig. 1b). These results suggest that the action of OP-1206 is mediated by EP4 in IEC-6 cells. It has recently been shown that EP3 and EP4 are localized in the nuclear envelope and that 16,16-dimethyl PGE_2 and M&B 28767 (an EP3 agonist) promote inducible nitric oxide synthase (iNOS) gene transcription and nuclear calcium transients through nuclear EP receptors [28]. These observations suggest that prostanoid modulates gene transcription. In addition, Uribe et al. [29] have reported that 15*R*,15-methyl-PGE_2 promotes crypt cell growth in rat small intestine.

Epithelial migration across the denuded area of intestinal mucosa is a rapid process. Measurement of the speed of resealing across the denuded area of IEC-6 cells is very useful for assessing both migration and proliferation of cells. As shown in Fig. 3, OP-1206 stimulated the resealing speed of IEC-6 cells. Some prostanoids have been shown to have a cytoprotective effect in the intestinal mucosa against various cytotoxic agents [30]. OP-41483 (5*E*-6,9-deoxa-6,9-methylene-15-cyclopentyl-16,17,18,19,20-pentanor-PGI_2), a stable analogue of prostacyclin, has been shown to be able to restore the piroxicam-induced suppression of IEC-6 cell migration [31]. PGE_1 and its analogue have been shown to have a potent protective effect against hepatic damage [32, 33]. Misoprostol, a PGE_1 analogue, protects the gastrointestinal tract of rats from experimental colitis [34, 35]. The mechanism of misoprostol-mediated protection has not been clarified, but it seems that it promotes intestinal mucosal healing. On the other hand, the synthetic PGE_1 analogue OP-1206, which was developed as a compound having strong antithrombotic and antiplatelet activities [36, 37], has been reported to suppress diabetic neuropathy, thermal hyperesthesia, and retinal damage [38, 39, 40]. In the present study OP-1206 was shown to prevent 5-FU-induced suppression of proliferation of IEC-6 rat crypt cells. OP-1206 was further shown to promote intestinal mucosal healing. These effects may possibly be related to the protection of the small intestine from 5-FU-induced damage and the resulting malabsorption.

In summary, IEC-6 cells exposed to 5-FU decreased [^{3}H]-thymidine incorporation into the cells and the number of cells. The cells increased upon incubation with OP-1206. OP-1206 significantly enhanced the speed of resealing of the denuded area of an IEC-6 cell monolayer, suggesting promotion of intestinal mucosal healing. Thus, the stable PGE_1 analogue, OP-1206, stimulated proliferation of IEC-6 cells treated with 5-FU, indicating a possible mechanism for the protective effect of OP-1206 against 5-FU-induced damage to the small intestine.

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