〈Regular Article〉

A potential protective effect of 5-aminolevulinic acid against anticancer drug-induced damage to intestinal mucosa

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ABSTRACT An effective anti-cancer chemotherapy regimen can cause adverse events such as antineoplastic drug-induced oxidative stress in tissues. When hemeoxygenase-1 (HO-1) is induced in tissues under oxidative stress, a tissue protective effect is observed. The non-proteinogenic amino acid 5-aminolevulinic acid (5-ALA) induces HO-1 in normal tissue and is hypothesized to provide an intestinal epithelial protective effect against drug-induced gastrointestinal mucosal disorders such as diarrhea and stomatitis. To validate this hypothesis, we introduced organoid culture from mouse intestinal epithelium. Using this organoid culture system, SN-38, the active metabolite of the antineoplastic drug irinotecan which is known to induce gastrointestinal mucosal disorders, was administered with and without 5-ALA to investigate the cytotoxic and protective effects of HO-1 suppression and expression. In normal intestinal epithelial cells, HO-1 induction by 5-ALA was observed. HO-1 expression was suppressed by the administration of SN-38 in the absence of 5-ALA, but was maintained by the co-administration of 5-ALA. In the mouse intestinal organoids, the same administration of 5-ALA induced HO-1 expression. When SN-38 was administered to the organoids, a cell death signal was expressed with an oxidative stress response, but the co-administration of 5-ALA induced HO-1 expression and cell death was decreased. The induction of HO-1 expression by 5-ALA administration suppresses intestinal epithelial cell death mediated by oxidative stress caused by antineoplastic drugs and is a potential new intestinal epithelial protective therapy against drug-induced gastrointestinal mucosal injury. doi：10.11482/KMJ-E202147047 *(Accepted on February 15, 2021)*
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INTRODUCTION

Recent progress of systemic chemotherapy has been developing multimodal treatments for gastrointestinal cancers, with improved disease control rates[1]. For clinical oncologists, it is further important to provide an effective chemotherapy regimen to each case and also to adequately handle several adverse events including digestive symptoms, caused by anti-cancer drugs[2].

It has been shown that anti-cancer treatment induces oxidative stress in some organs, and subsequent tissue damages causes chemotherapy-related symptoms[3]. Under the induction of oxidative stress, several relevant molecules are involved in this process, one of which is hemeoxygenese-1 (HO-1). When HO-1 is induced in tissues under oxidative stress, a tissue protective effect is observed[4, 5], thus maintaining the expression of HO-1 can be expected as a favorable strategy to reduce chemotherapy-related adverse events.

5-aminolevulinic acid (5-ALA), which is a non-proteinogenic amino acid and is known to be capable of upregulating HO-1[6-8], is the first product of the porphyrin synthesis pathway and is the source of heme synthesis required for survival in normal cells[9]. In cancer cells, 5-ALA is not converted to heme, but is instead converted to protoporphyrin IX (PpIX) in the mitochondria[10]. This metabolism is utilized for photodynamic diagnosis of cancer that applies the principles of light emission due to the photosensitivity of PpIX[10-12] and is also used in the clinical application of photodynamic therapy (PDT) which induces apoptosis through an accumulation of reactive oxygen species[10-12]. It has been reported that 5-ALA has a protective effect against a major anticancer agent cisplatin-induced acute kidney injury through a reduction of oxidative stress by HO-1[6]. Following this idea, we hypothesized that 5-ALA also provides a protective effect against drug-induced gastrointestinal mucosal damage, which can represent certain digestive disorders such as diarrhea and stomatitis.

To validate this hypothesis, an intestinal epithelial environment model was prepared using organoids separated and cultured from murine small intestinal epithelium[13, 14]. SN-38, the active metabolite of another major antineoplastic drug irinotecan, which is known to induce gastrointestinal mucosal disorders[15-17], was administered separately and together with 5-ALA to investigate the cytotoxic and protective effects of HO-1 suppression and expression in mouse intestinal organoids.

MATERIALS AND METHODS

Cells and reagents

IEC6 and IEC18, both of which are rat intestinal epithelial cells, were obtained from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan) and grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). The colon carcinoma cell lines DLD-1 and HT-29 were also cultured in RPMI-1640 medium and McCoy's 5a medium, respectively, and supplemented with 10% FBS (Gemini Biologicals, West Sacramento, CA, USA), 100 units/ml of penicillin G sodium, 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained in a monolayer culture at 37℃ in humidified air with 5% CO_2. These cells lines were treated with reagents for subsequent western blot experiments.

5-ALA was kindly provided by SBI Pharmaceuticals Co. Ltd. (Tokyo, Japan) and SN-38 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

Cells treated with 5-ALA and SN-38 were washed twice with cold PBS and protein lysis buffer (M-PER mammalian protein extraction reagent, Thermo Fisher Scientific, Rockford, IL, USA) was added to the plate. The plate was gently shaken at

room temperature for 5 min, then the cells were scraped with a cell scraper and the cell lysates were transferred to a centrifuge tube. The cell lysates were clarified by centrifugation (15 min at 15,000× *g* at 4℃) and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels. The separated proteins were then transferred onto Hybond PVDF transfer membranes (Millipore, Bedford, MT, USA) and incubated with primary antibodies at 4℃ overnight, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 hour. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used for signal detection. The following antibodies were used for western blot analysis: HO-1 (P249) polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-actin (AC-15) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and secondary horseradish peroxidase-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Each experiment was repeated at least twice and the representative data is displayed.

Organoid culture

An intestinal epithelial environment model was prepared using organoids isolated and cultured from rat small intestinal epithelium. Intestinal organoids were established using IntestiCult Organoid Growth Medium (STEMCELL Technologies Inc., Vancouver, Canada) according to the manufacturer's protocol. Briefly, harvested mouse small intestine was minced and washed by centrifugation. Pelleted intestinal crypts were resuspended with growth medium and Matrigel, and the suspension was placed in the center of each well in a 12-well plate. Solidified Matrigel with intestinal crypts were incubated with liquid growth medium in a humidified incubator and the medium was exchanged three times per week until the crypts budded enough for passage. When the density reached 150 organoids per well, they were passaged for experiments.

Immunohistochemistry

Organoid tissues treated with reagents were fixed with 10% formalin and paraffinized for sectioning. Embedded 5 μm sections on slides were subjected to immunohistochemical staining. Briefly, slides were deparaffinized, rinsed and microwaved in a 10 mmol/L citrate buffer solution (pH 6.0) for 15 min for antigen retrieval. After cooling, deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 15 min to block endogenous peroxide activity. The primary antibody was diluted in Dako antibody diluent with background reducing components (Agilent Technologies, Inc., Santa Clara, CA, USA) at a dilution of 1:500. Overnight incubation with the primary antibody at 4℃was followed by immune-bridging with Avidin DH-biotinylated horseradish peroxide complex (Nichirei Biosciences, Inc., Tokyo, Japan). Color development was achieved using a DAB/H_2O_2 solution (Histofine DAB substrate kit; Nichirei Biosciences, Inc.) and Mayer's hematoxylin as counterstaining. Some sections were subjected to normal serum blocking and omission of the primary antibody as a negative control. The primary antibody used in this study was anti-HO-1 rabbit polyclonal antibody (ENZO Life Sciences, Inc., Farmindale, NY, USA). Each experiment was repeated at least twice and the representative data is displayed.

Cell death signal detection assay

Cytotoxicity by SN-38 on the rat intestinal organoids was assayed by Apoptotic/Necrotic/ Healthy Cell Detection Kit (PromoCell GmbH,

Heidelberg, Germany) according to the manufacturer's instructions. Briefly, the mouse intestinal organoids were cultured and treated with 5-ALA and SN-38 on glass slides. After 24-hour treatment, the organoids were further incubated with staining solution containing FITC-Annexin V, Ethidium Homodimer III, and Hoechst 33342 for 15 minutes at room temperature, under protection from light. Stained organoids were classified by each fluorescent color, green (FITC-Annexin V, represents apoptotic), red (Ethidium Homodimer III, represents necrotic) or blue (Hoechst 33342, represents healthy) under a fluorescent microscope. This experiment was repeated twice to confirm that the similar results were obtained. The representative data was used to display as figure panels.

RESULTS

5-ALA maintains HO-1 expression in normal intestinal cells

First, we examined the expression status of HO-1 in normal intestinal epithelial cells treated with the antineoplastic reagent SN-38, both with and without 5-ALA. When the rat intestinal epithelial cells IEC-6 and IEC-18 were treated with 5-ALA, the oxidative stress-related protein HO-1 was induced. This confirmed that HO-1 expression was suppressed by the administration of SN-38 in the absence of 5-ALA, but was maintained by the co-administration of 5-ALA in both IEC-6 and IEC-18 cells (Fig. 1A and B). Interestingly, the induction of HO-1 by 5-ALA was not observed in the colon cancer cells (Fig. 1C and D).

The effect of 5-ALA on HO-1 expression in an organoid culture

Next, we tried to reproduce a similar effect in an organoid culture, which mimics the physiological structure of intestinal tissues. When 5-ALA was administered in the same way to the rat small intestinal epithelium organoids, HO-1 expression was induced in a dose-dependent manner (Fig. 2).

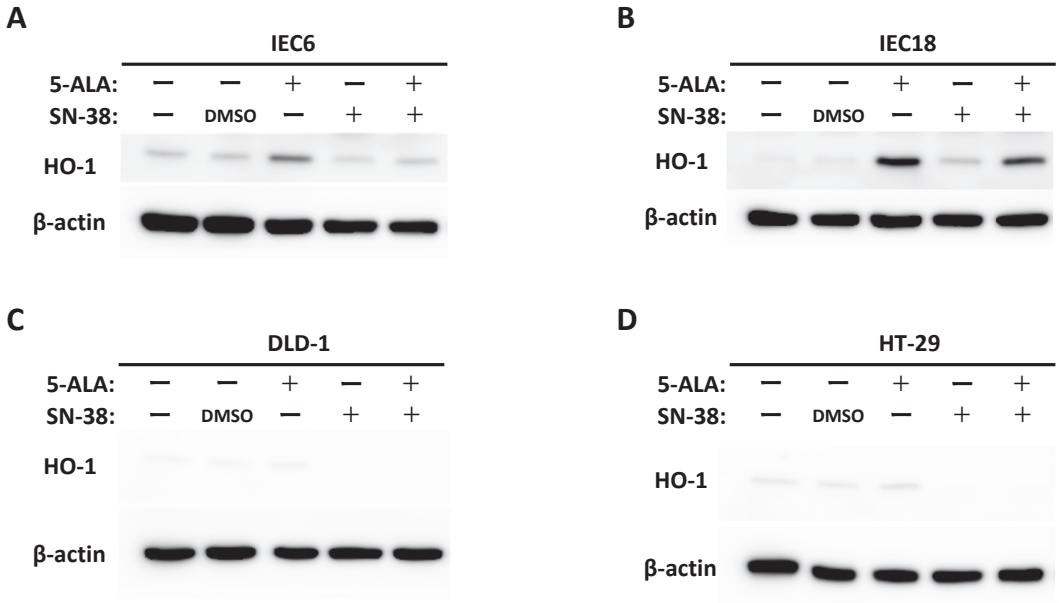

Fig. 1. 5-ALA maintains HO-1 expression in normal intestinal cells but not in cancer cells
Rat intestinal epithelial cells (A: IEC6, B: IEC18) were cultured in a 5-ALA-containing medium and were treated both with and without SN-38, a derivative of irinotecan, to examine the expression change of HO-1 by western blot. Similar experiments were performed using colorectal cancer cell lines (C: DLD-1, D: HT-29). β-actin served as an internal control.

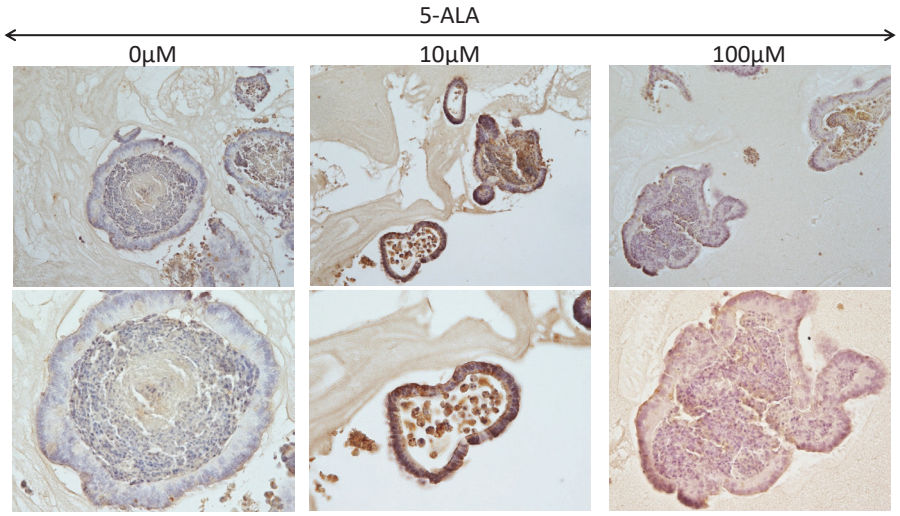

Fig. 2. The effect of 5-ALA on HO-1 expression in an organoid culture
Mouse intestinal organoids were established and cultured with different concentrations of 5-ALA to observe the expression status of HO-1 by immunohistochemistry. The upper panels represent microscopic images at a lower magnification (×100), and the lower panels were more highly magnified images (×200). Brownish color by DAB in cytoplasm of organoid-constituent cells represents positive staining of HO-1.

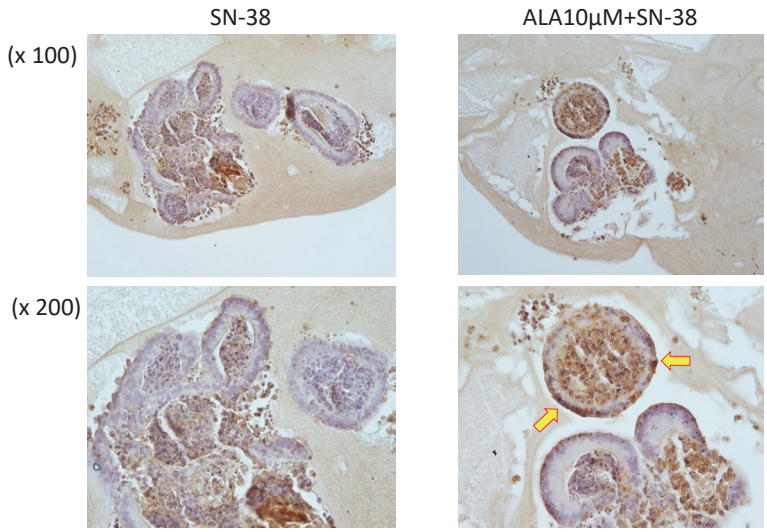

Fig. 3. 5-ALA maintains HO-1 expression in organoids under SN-38 treatment
Mouse intestinal organoids were treated with SN-38 and/or 5-ALA to determine HO-1 expression by immunohistochemistry. Arrows show the HO-1 expression in epithelial cells that mimic the intestinal mucosa structure in the organoids. The upper panels represent microscopic images at a lower magnification (×100), and the lower panels were more highly magnified images (×200).

5-ALA maintains HO-1 expression in organoids under SN-38 treatment

In the mouse intestinal epithelial organoid cells, HO-1 expression was unclear under SN-38 treatment, whereas the co-administration of 5-ALA maintained HO-1 expression as observed in 2-dimentional cultured intestinal cells (Fig. 3).

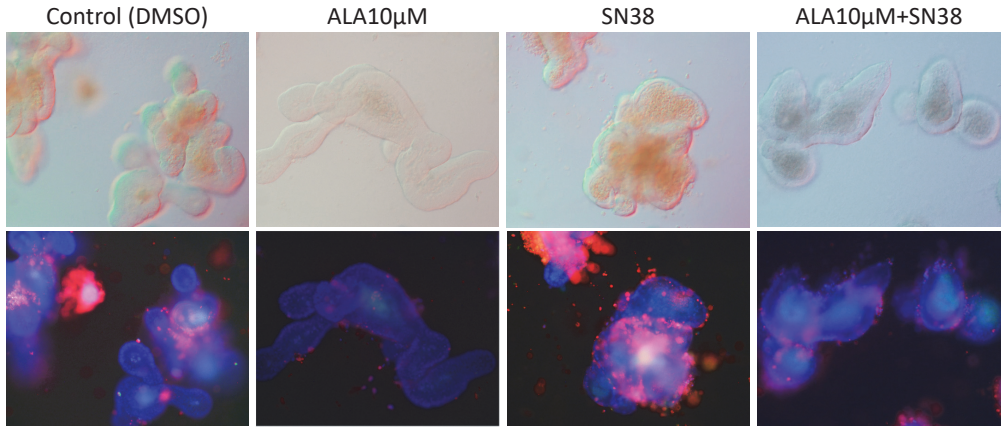

Fig. 4. 5-ALA reduces SN38-induced cell death in intestinal organoids
Mouse intestinal organoids were treated with SN-38 and/or 5-ALA and were examined with fluorescent cell death markers for anti-cancer drug-induced cell death and the potential cell protective effect of 5-ALA. The upper panels are optical microscopic views and the lower panels are fluorescent images (Blue: viable cells, Red: Necrotic cells, Green: Apoptotic cells).

5-ALA reduces SN38-induced cell death in intestinal organoids

Finally, we determined whether 5-ALA overcomes the cytotoxicity of SN-38. A cell death signal was expressed when SN-38 was administered to the organoid, but cell death was reduced when 5-ALA was co-administered. This outcome suggests that the 5-ALA induced HO-1 expression suppresses intestinal epithelial cell death caused by antineoplastic agents (Fig. 4).

DISCUSSION

The induction of HO-1 and its cytoprotective effect mediated by 5-ALA, which was observed in this study, appeared to follow a report by Terada *et al*. about a protective effect of 5-ALA on acute kidney injury, which is a common adverse event when cisplatin is used to treat gastrointestinal cancer[6]. Recently, several researchers have also reported that 5-ALA induced HO-1 in other types of tissue and cells[7, 8, 18, 19]. As shown in this study, this inducible effect was not observed in cancer cells, HO-1 induction must be a critical molecule for normal cells to maintain their homeostasis.

In this study, we utilized an organoid culture system to mimic the intestinal epithelial environment, which has an advantage in the more similar biological setting of intestinal mucosal structure than 2-dimensional cell culture. Another advantage of using organoids is to easily reproduce the same experimental setting as organoids can be passaged and frozen-stocked[20–22]. However, organoids still remain artificial compared to real physical circumstances, we should consider to examine a protective effect of 5-ALA on SN-38-mediated intestinal mucosal damage in a certain animal model, or in a 3-dimensional culture model such as organoids mimicking human intestinal epithelia in the future.

5-ALA is known to be required for cell survival[9], thus its existence is expected to energize normal cells including gastrointestinal epithelial cells. On the other hand, addition of 5-ALA to cancer cells rather reacts opposite by accumulating PpIX in mitochondria, which can induce apoptosis[10–12]. This is potentially intriguing to excess 5-ALA treatment for a cancer-bearing model in combination of chemotherapy with photodynamic therapy. This

combination therapy might create a certain synergic anticancer effect with less gastrointestinal damages, expecting that excess amount of 5-ALA can distribute to both normal cells and cancer cells, the former would survive due to reduced drug-induced oxidative stress by HO-1 and the latter would undergo potent apoptosis by both chemotherapy and PDT with accumulating PpIX. Though, it should be careful that excess amount of 5-ALA may introduce unfavorable effect on normal tissues, as there is a report that 5-ALA induced apoptosis in normal gastric epithelial cells, by stimulating oxidative stress[23]. In this report, the activity of HO-1 was not described, presumably the event might occur independent of HO-1, or the condition of 5-ALA administration such as concentration and duration could affect the activity status of oxidative stress differently. Since it is quite advanced enough yet to establish preventable treatments against adverse events, further determination of 5-ALA effectiveness is waiting to be explored.

In conclusion, the 5-ALA-mediated induction of HO-1 expression suppresses the upregulation of oxidative stress in the intestinal epithelia, which could be a potential approach to prevent intestinal mucosal damage caused by chemotherapy. This report suggests a possible novel potency of 5-ALA for a future protective therapy against drug-induced gastrointestinal mucosal injury.

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CONFLICT OF INTEREST

None of authors listed above have any conflict of interest related to this article.

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