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Histidine deficiency attenuates cell viability in rat intestinal epithelial cells by apoptosis via mitochondrial dysfunction

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ABSTRACT

Objective: To reveal the importance of histidine in intestinal epithelial cells, we investigated whether reduction in histidine concentration affects cell viability and apoptosis in rat intestinal epithelial cells (IEC-6).

Methods: Culture cells were incubated in DMEM with or without histidine. Cell viability was measured by the MTT assay. The expression of caspase-3, -8, -9, and -12 was evaluated by western blot analysis. Activated caspase-3 was used as an apoptosis index. To determine the apoptosis pathway, activated caspase-8, -9, and -12 were also examined. Mitochondrial dysfunction was evaluated by the mitochondrial membrane potential assay. Some experiments were also performed on other cells (gastric mucosal cells or kidney cells), to compare with the results of IEC-6 cells.

Results: Histidine deficiency significantly reduced cell viability after 6 h and induced caspase-3-dependent apoptosis after 9 h, in IEC-6 cells. Also, histidine deficiency decreased the mitochondrial membrane potential after 6 h. Therefore, we speculated that apoptosis was induced by mitochondrial dysfunction. Additionally, histidine at concentrations higher than 10 μM prevented the decrease in cell viability and the facilitation of apoptosis in IEC-6 cells. In rat gastric mucosal cells, similar results to the IEC-6 histidine deficiency results were obtained, but not in rat kidney cells.

Conclusions: This is the first report showing that histidine deficiency reduced cell viability and induced apoptosis in IEC-6 cells, and that a small amount of histidine supplementation prevented and improved the IEC-6 cell injury. This is a potential new clinical treatment against intestinal and/or gastric cell injury that would improve the patient's quality of life.

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1. Introduction

The small intestine is an important organ for absorbing nutrients and its injury exerts severe damage to biological functions. Therefore, amelioration of intestinal injury can enhance the patient's quality of life (QOL).

Intestinal injury is a major problem in clinical medicine, as it has not yet been fully defined. It has been thought that intestinal injury occurs as a complication of other diseases or induced by drug administration and stress. Recently, injury, especially in the small intestine, has been noted in clinical medicine, because development of endoscopic techniques has enabled detection of bleeding and ulcers in the small intestine, which could not be found with conventional methods [1].

Amino acids are important as nutrients in the body. Most people in developed countries get enough amino acids from proteins in the diet [2]. However, in pathological conditions, the balance of amino acids is disrupted by various factors. The blood levels of amino acids in some diseases have been reported [3–6]. Some diseases, such as pancreatic cancer and lung cancer [7,8], reduce the blood concentration of histidine, among other amino acids. Histidine is an amino acid that has various biological functions such as supplying materials for protein synthesis, being a precursor of physiologically active substances, and regulating hormone secretion and cell turnover. Histidine deficiency induced by administration of a

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histidine-free diet causes a negative nitrogen balance, skin lesions, and a decrease in blood albumin, hemoglobin and hematocrit [9,10]. In physical stress such as traumatic shock and burn injury, the blood histidine concentration decreases, causing aggravation of complicating pathological conditions [11]. Furthermore, in chronic kidney disease (CKD), reduction in the blood histidine concentration causes collapse of proteins, induction of inflammation and elevation in oxidative stress, which exacerbate the prognosis [12]. Moreover, gastrointestinal bleeding and intractable ulceration have been observed in severe CKD patients who needed dialysis [12]. Furthermore, in inflammatory bowel disease (IBD), reduction in the blood histidine concentration has been described [13–15].

Although there are many reports about histidine deficiency, little is known about the effect of histidine on intestinal injury.

Based on these reports, we hypothesized that there are correlations between the decrease in the histidine concentration and intestinal injury, especially small intestinal epithelial damage. We investigated whether the decrease in the histidine concentration affects cell viability and apoptosis in rat intestinal epithelial cells (IEC-6).

2. Material and methods

2.1. Cell culture

Rat intestinal epithelial cells (IEC-6, RIKEN BioResourse Center, Tsukuba, Japan), rat gastric mucosal cells (RGM1, RIKEN BioResourse Center, Tsukuba, Japan), and rat kidney cells (NRK, RIKEN BioResourse Center, Tsukuba, Japan) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing antibiotic solution (100 U/ml of Penicillin and 100 μg/ml of Streptomycin, Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS; Equitech-Bio Inc., Kerrville, TX, USA, or GE Healthcare, Little Chalfon, UK) [16]. The other media used were FBS-free DMEM without histidine (ΔHis; Cell Science & Technology Institute, Inc., Miyagi, Japan), DMEM [negative control: Full medium = DMEM, ΔHis medium (Cell Science & Technology Institute, Inc.) with 200 μM L-histidine (Wako pure chemical industries, Ltd., Osaka, Japan)], and amino acid-free DMEM (positive control: Zero medium, Cell Science & Technology Institute, Inc.). All cells were cultured in a 5% CO_2 incubator at 37 °C.

2.2. Cell viability assay

Cells were precultured for 24 h in 96-well plates (10,000 cells per well) in DMEM with 10% FBS. Then, the medium was replaced with Full medium, Zero medium or ΔHis medium, and cultured for 3, 6, 9, 12 or 18 h. Also, histidine concentration media (final 1, 5, 10, 50, 200 μM: ΔHis medium supplemented with each histidine concentration) were used. Cell viability was evaluated with Cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions [17]. Absorbance was read at 450 nm using the SpectraMax 190 Microplate Reader (Molecular devices, Tokyo, Japan). References included readings at 650 nm. The values for each treatment group were averaged per experiment.

2.3. Evaluation of cellular apoptosis

Western blot analysis was used to determine activated caspase-3 levels for each treatment group. Activated caspase-3 was used as an apoptosis index. Also, to determine the apoptosis pathway, activated caspase-8, -9 and -12 were examined. Cells were precultured for 24 h in 6 cm dishes (600,000 cells per well) in DMEM with 10% FBS. Thereafter, the medium was replaced with Full medium, Zero medium or ΔHis medium, and cultured for 3, 6, 9 or 12 h. Furthermore, the histidine concentration media (final 1, 5, 10, 50, 200 μM: ΔHis medium supplemented with each histidine concentration) were used. Cells were collected by a scraper and homogenized in RIPA buffer containing Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). The protein concentration was measured with the BCA Protein Assay Kit (Thermo Fisher Scientific). Adequate amounts of protein (5–10 μg) were electrophoresed in 10–15% SDS polyacrylamide gels, and then electrotransferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, membranes were blocked at room temperature with blocking buffer followed by incubation with the following primary antibodies: anti-caspase-3 polyclonal antibody (Cell Signaling, Danvers, MA, USA; 1:5000), anti-caspase-8 monoclonal antibody (Abcam, Cambridge, UK; 1:2000), anti-caspase-9 monoclonal antibody (Cell Signaling; 1:1000), anti-caspase-12 polyclonal antibody (Abnova, Taipei, Taiwan; 1:1000) and monoclonal anti-β-actin antibody (Sigma-Aldrich; 1:5000). β-Actin was used as a loading control. The antibodies were diluted with Can Get Signal immunoreaction Enhancer Solution (Toyobo, Osaka, Japan). After several washes in Tris-buffered saline with Tween 20, membranes were incubated with the following secondary antibodies: polyclonal goat anti-rabbit or anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark; 1:5000). The antibodies were diluted with Can Get Signal immunoreaction Enhancer Solution (Toyobo). Finally, membranes were washed, and bands were detected by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescent HRP Substrate (Millipore). ECL signals were measured by Image Quant Las 4000 mini (GE healthcare Japan, Tokyo, Japan) and were analyzed by ImageJ (NIH, Bethesda, MD, USA).

Additionally, fluorescence microscopy with FITC-Annexin V staining was used to observe the apoptosis phenomena. Cells were precultured in DMEM with 10% FBS in slide chambers (20,000 cells per well) for 24 h. Then, the medium was replaced with Full medium or ΔHis medium, and cultured for 6 or 12 h. Apoptosis was evaluated with the Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell, Heidelberg, Germany) following the manufacturer's instructions. The excitation/emission wavelengths were 492 nm/514 nm, respectively. The detection was performed by a TCS SP5 MP fluorescence microscope (Leica microsystems, Wetzlar, Germany).

2.4. Evaluation of mitochondrial membrane potential

Cells were precultured for 24 h in 96-well plates (10,000 cells per well) in DMEM with 10% FBS. Then, the medium was replaced with Full medium, Zero medium or ΔHis medium, and cultured for 3, 6, 9 or 12 h. The mitochondrial membrane potential was evaluated with the Mito-ID Membrane Potential Cytotoxicity Kit (Enzo Life Sciences, New York, NY, USA) following the manufacturer's instructions. The excitation/emission wavelengths were 490 nm/590 nm, respectively. The detection was performed by a Wallac 1420 ARVO MX plate reader (Perkin Elmer, Waltham, MA, USA).

2.5. Statistics

Data were analyzed by the independent samples *t*-test using IBM SPSS statistics ver. 23. Values are mean ± SD. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of histidine concentration on cell viability

To examine the change in cell viability by His deficiency, IEC-6 cells were cultured for 18 h in ΔHis medium and cell viability was

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