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Emodin protects mice against radiation-induced mortality and intestinal injury via inhibition of apoptosis and modulation of p53

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ABSTRACT

The aim of this study was to explore the protective effect of emodin, a plant-derived anthraquinone, against gamma radiation-induced mortality and intestinal injury in mice, and to investigate the radioprotective molecular mechanism. C57BL/6 male mice were pre-treated with emodin for 7 days via oral gavage before gamma radiation. We found that pretreatment with emodin prolonged mice survival time after 9 Gy total body irradiation (TBI). Mice were sacrificed at 1 week after 7 Gy TBI, we found that emodin attenuated intestinal morphological changes and increased villus height, crypt numbers, and reduced villus and crypt apoptosis as well as inhibited the expression of p53. MTT assay, flow cytometry, Hoechst 33258 staining, real-time PCR, and Western blotting indicated that emodin pretreatment can effectively increase human umbilical venous endothelial cells (HUVECs) viability and attenuate cell apoptosis; it also inhibited the expression of p53, Bax, and Caspase3 in HUVECs after irradiation. In summary, these results suggest the potential of emodin as an effective radioprotectant against radiation-induced intestinal injury.

1. Introduction

Radiotherapy is one of the most important treatment strategies for human malignancy. About half of the cancer patients are treated with radiotherapy (Ostrau et al., 2009). Although effective in the treatment of malignant tumors, radiotherapy induces normal tissue toxicity; this may involve acute radiation syndromes in the gastrointestinal tract (GI), the hematopoietic system, and cerebrovasculature (Burdelya et al., 2008; Singh et al., 2012; Ghoneum et al., 2013). Total body irradiation at high dose (≥9 Gy) will trigger an acute GI toxicity that often results in death (Williams et al., 2010; Dorr and Meineke, 2011). It has been reported that radiation can induce cell apoptosis, oxidative stress, and inflammation leading to mortality (Zhao and Robbins, 2009; Lorimore et al., 2013). The intestines are highly sensitive to radiation, and radiation causes GI epithelium and endothelial injury play an important role in the development of an acute radiation response (Cagin et al., 2015).

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a plant-derived anthraquinone, has been reported to exhibit diverse biological activities including: anti-inflammatory; antioxidant; antimicrobial; anticancer; and modulation of the immune system, of the vasomotor system, as well as metabolic processes (Muto et al., 2007; Shrimali et al., 2013; Sharma and Tiku, 2014). It has previously been reported that emodin has a protective effect regarding gamma radiation-induced toxicity by inhibiting DNA damage and oxidative stress in murine splenocytes (Sharma and Tiku, 2014). However, it remains unknown whether emodin has a radioprotective effect *in vivo*, and attenuates radiation-induced intestinal injury. Thus, the objective of our study was to carry out a further evaluation of the possible effects of emodin in protecting mice from gamma radiation-induced mortality and intestinal injury, and the potential molecular mechanism involved.

2. Materials and methods

2.1. Mice and cell lines

Male C57BL/6 mice (6–8 weeks old) were purchased from SIPPR-BK Experimental Animal Co. (Shanghai, China). All mice were managed under specific pathogen-free conditions and used according to the animal experimental guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Ani-

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mals. This study was approved by the Scientific Investigation Board of the Second Military Medical University, Shanghai. Human umbilical vein endothelial cells (HUVECs) were purchased from AllCells, LLC (Emeryville, CA, USA) and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) with penicillin and streptomycin (100 U/mL or 100 mg/mL) (Thermo Scientific, Waltham, MA, USA) in a humidity incubator with 5% CO_2 at 37 °C.

2.2. Gamma irradiation and emodin treatment

Mice were randomly divided into different groups, namely the control group, the gamma radiation group, and the emodin treatment group (15, 30 and 60 mg/kg/day, intragastrically, diluted with 0.5% CMC-Na, continuous dosing for 1 week); the control and gamma radiation groups were given 0.5% CMC-Na. Mice body weight was recorded every other day for emodin treatment schedule and after 7 Gy TBI. Emodin (purity ≥ 98%) was purchased from Dalian Meilun Biology Technology Co. (Dalian, China) Mice were placed in holders and received a total dose of 7, 8, 8.5and 9 Gy (2 Gy/min) total body ^{60}Co gamma radiation at the Irradiation Center (Faculty of Naval Medicine, Second Military Medical University, Shanghai, China). After irradiation, mice continued to be monitored.

Cells incubated at 37 °C were exposed to normoxia (5% CO_2) for 24 h. Emodin was dissolved in dimethyl sulfoxide, and was further diluted to the required concentration using cell culture media. The cells were then exposed to 10, 1, and 0.1 μM emodin under normoxia for 24 h, followed by exposure to total dose of 24, 32, 40 and 48 Gy ^{60}Co gamma-rays at a dose rate of 5.63 Gy/min at the Irradiation Center. The cells were sequentially incubated for 24 h post-irradiation.

2.3. Determination of survival time

The survival rates of mice that received 7, 8, 8.5 and 9 Gy gamma radiation were calculated using the Kaplan–Meier method.

2.4. Morphological examination

Mice were sacrificed on the seventh day after total body gamma irradiation at a dose of 7 Gy. The jejunum was dissected and fixed in 4% paraformaldehyde solution; the segments were dehydrated in serial alcohol solution. Tissues were then embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin (H&E). The sections were examined under a light microscope (Leica, Solms, Germany). The operator was blinded with respect to the various groups during the analysis. A minimum of 10 well-oriented villus per tissue section (at least 5 sections from each gut specimen) was measured and the number of crypts was counted in each circumference. The number of goblet cells density was calculated from the mouse jejunum tissue following a standard H&E staining by recording a total of 10 crypts per mouse. Five mice were used to each group.

2.5. TUNEL assay

Apoptotic jejunal cells were identified using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (In SituCell Death Detection Kit, Roche, Basle, Switzerland). For the TUNEL assay, the slides were heated to 60 °C followed by washing in xylene and rehydration through a series of concentration gradient ethanol. Tissue sections were incubated with proteinase K in working solution for 20 min. Subsequently, the slides were incubated in the TUNEL reaction mixture containing the label and enzyme solution. After rinsing the slides with phosphate-buffered saline (PBS), samples were analyzed under a microscope. Apoptosis cells were identified as those with a brown-stained nucleus. Longitudinal sections from each mouse were counted and for each section, 50 villi and 40 crypts were checked.

2.6. Western blot assay

The mouse jejunum was dissected at the seventh day after total body irradiation at a dose of 7 Gy, and extra fat tissue was cleaned in cold PBS on ice. The total protein was extracted from freshly isolated jejunal segments. Western blotting was performed to separate the protein. The blot was incubated with GAPDH and p53 primary antibodies (Cell Signaling Technology, Danvers, USA) overnight at 4 °C. The blot was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Kangchen, Shanghai, China) for 1 h at room temperature. Immunoreactive proteins were detected using ECL western blotting substrate (Thermo Scientific, Waltham, MA, USA).

The experiments were carried out as previously described. After cells were exposed to 24 Gy gamma radiation and incubated for 24 h; p53, Bax and Caspase3 expression levels in total cell lysate were detected using western blot analysis. Briefly, cells were lysed with Protein Extraction Regent (Pierce, Rockford, USA) supplemented with a protease inhibitor cocktail. The protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). Equal amounts of extracts were separated on SDS-PAGE gels; then they were transferred onto nitrocellulose membranes (Millipore, Massachusetts, USA) and blotted with the antibodies indicated. Bax, Caspase3 and β-actin primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The intensity of each protein band was measured using Quantity One.

2.7. Cell viability assay

To detect cell viability, the MTT assay was performed. This method is based on the reduction of MTT by the mitochondrial dehydrogenases of living cells into insoluble formazan crystals. The purple formazan is dissolved in dimethyl sulfoxide. The absorbance of the purple solution directly reflects the number of viable cells. The MTT assay was used to assess cell viability in 24 h after 24, 32, 40 and 48 Gy gamma radiation according to the manufacturer's protocol (Shanghai Chemical Reagent Company, Shanghai, China).

2.8. Flow cytometry assay

Apoptosis was detected using the AnnexinV-FITC Apoptosis Detection Kit according to the manufacturer's instructions (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Invitrogen, Carlsbad, USA). After a 24 h exposure to 24 Gy gamma radiation, the 1 million HUVECs were harvested and washed twice in cold PBS.Add 5 μL Alexa Fluor® 488 annexin V (Component A) and 1 μL 100 μg/mL PI working solution to each 100 μL of cell suspension, the cells were then incubated at room temperature for 15 min in darkness, and analyzed using a BD FACS Caliber flow cytometer (BD Biosciences, San Jose, USA).

2.9. Hoechst 33258 staining

Cells were stained with Hoechst 33258 (Sigma-Aldrich Co, St. Louis, USA) based on the manufacturer's protocol and examined under a Leica SP5 fluorescence microscope. The images were analyzed using LAS-AF software (Leica, Buffalo Grove, IL). The average percentage of apoptotic cells was calculated in five to seven randomly selected high-power fields.