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Effects of barley β-glucan on radiation damage in the human hepatoma cell line HepG2

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ABSTRACT

Damage to normal tissue is an obstacle to radiotherapy of cancer. We have tested whether barley β-glucan can enhance radioprotection in the human hepatoma cell line HepG2. The cytotoxicity of β-glucan was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. A clonogenic assay was used to study the sensitivity of cells to β-glucan, ionizing radiation (2–8 Gy), and the combination of both treatments. Acridine Orange/ethidium bromide staining was used to examine induction of apoptosis by β-glucan, radiation (6 Gy), and the combination. DNA strand breaks were assessed by the comet assay. The MTT assay showed that treatment with β-glucan was not cytotoxic. Indeed, a slight increase in cell viability was observed. Pre-treatment with β-glucan, 1 μg/ml, for 72 h protected HepG2 cells against radiation, as indicated by increased surviving fraction, reduced apoptosis, and fewer DNA strand breaks. These results show that barley β-glucan is a radioprotective agent.

1. Introduction

Ionizing radiation is a powerful treatment modality against tumor cells, but it is not tumor specific and may affect neighboring normal cell as well [1–4]. Various polysaccharides, including glucan, mannan, and glucomannan have anti-genotoxic, antioxidative, immunomodulatory, anti-infective, and anti-tumoral properties [5]. Barley β-glucan is a linear polysaccharide of β(1→3) and β(1→4) linked D-glucose molecules. The chain conformation of β-glucan is altered at different concentrations [6–10], which significantly affects its bioactivity [11–13]. β-Glucan is known to reduce blood lipid levels, including cholesterol and triglycerides [14–16]. Polysaccharides have been used for decades to stimulate the immune system and immune modulation by β-glucan is beneficial for cancer prevention and hematopoietic recovery [17–22]. The biological activity of β-glucan is mediated by pattern recognition receptors located on immune and hematopoietic cells. Thus, the radio-protective properties of glucans are closely related to hematopoietic recovery [23–26]. However, β-glucan's cellular and molecular effects on other tissues are still unknown.

Liver injury during right chest radiotherapy has been reported widely. The liver is located under the right ribs, just beneath the right lung, and its protection from ionizing radiation is essential. In this study, we used the human hepatoma cell line (HepG2), widely used as a model for in vitro cytotoxicity experiments [27–32], to determine whether β-glucan could enhance DNA repair and exert beneficial cyto- and radio-protective effects after treatment with X-rays.

2. Materials and methods

2.1. Drugs and reagents

The following chemicals were used in this study. β-Glucan (G6513) was purchased from Sigma Aldrich (USA). Low-melting-point agarose (LMP), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and trypan blue were obtained from Sigma Chemical (Germany). RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCO (UK). Dimethylsulfoxide (DMSO), agarose, trypsin, Triplex 3, buffer salts, methyl hydrobenzoate, methyl green, and acetic acid were obtained from Merck (Germany). Acridine orange and ethidium bromide dyes were purchased from Hopkins & Williams Ltd. (Chadwell Health, UK) and Merck (Germany), respectively.

Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LMP, low-melting-point agarose; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; SCGE, single cell gel electrophoresis; SEM, standard error of mean; AO/EB, acridine orange/ethidium bromide; LPS, lipopolysaccharide; TLR, Toll-Like Receptor; NF_kB, Nuclear Factor Kappa B; MMS, methyl methane sulfonate.

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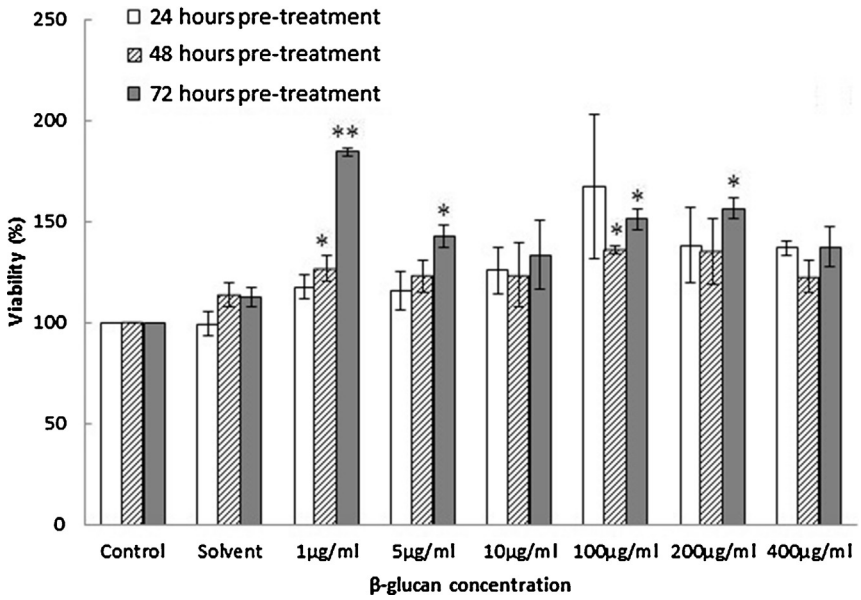

Fig. 1. 2000 cells/cm^2 were seeded into 24 well plates and treated with different concentrations of β-glucan (1, 5, 10, 100, 200, 400 μg/ml) for 24 h (open bar), 48 h (dotted bars) and 72 h (solid bars). Bars indicate means ± standard deviation of three independent repetitions.

2.2. Cell culture

The human hepatoma cell line HepG2 was obtained from the National Cell Bank of Iran (Pasteur Institute, Iran) and routinely sub-cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 μg/ml streptomycin (Jaberebn-Hayan, Iran) and 500 μg/ml penicillin (Sigma, USA) at 37 °C in a humidified atmosphere and 5% CO_7. Cells were maintained as monolayer cultures, and subculturing was routinely performed when cells reached 80–90% confluency.

2.3. Growth curve

Cells were plated at 2×10^4 cells/cm^2 in T25 culture flasks (Nunc, Denmark) and incubated overnight. β-Glucan was added (0, 1, or 100 μg/ml) and cells were incubated for 72 h. Cells were then thoroughly washed and plated at 2×10^4 cell/cm^2 in a 24-well plate (Nunc, Denmark) and allowed to adhere overnight. Three wells were counted every 24 h for nine days. Media were changed every two days. The assay was performed in triplicate.

2.4. MTT assay

Briefly, 2×10^4 cells/cm^2 were seeded into 96-well plates (Nunc, Denmark) and incubated overnight to ensure attachment of cells before β-glucan was added. The following day, cells were treated with 1–400 μg/ml β-glucan and incubated for 24, 48, or 72 h. After indicated times, cells were washed with PBS and MTT solution (0.5 mg/ml, in PBS) was added to each well and incubated for another 4 h at 37 °C [33]. Formazan crystals were dissolved in 100 μl DMSO, and the absorbance was determined at 570 nm using an Asys High-tech Eliza reader spectrophotometer. The number of viable cells was then calculated as follows:

$$\text{number of viable cells}\,(\%) = \frac{\text{Abs of sample} \times 100}{\text{Abs of control}}$$

The assay was performed in triplicate.

2.5. Fluorescence microscope analysis of apoptosis and necrosis

Apoptosis and necrosis were analyzed by the differential uptake of fluorescent DNA binding stains ethidium bromide and acridine orange (AO/EtBr). Acridine orange is a vital dye that stains both live and dead cells, whereas ethidium bromide only stains those cells that have lost their membrane integrity. Briefly, both adherent and floating cells were collected and stained with a mixture of AO (0.5 mg/ml) and EtBr (0.5 mg/ml) solution (1:1, v/v). The stained cells were visualized by Axoscope 2 plus fluorescence microscope (Zeiss, Germany) using 20× magnification. 200 cells were analyzed to calculate the fraction of apoptotic and necrotic cells [34]. The assay was performed in triplicate.

2.6. Irradiation

X-Irradiation was carried out at room temperature using Siemens PRIMUS linear accelerator (Germany) at Pars Hospital (Tehran, Iran). 72 h after β-glucan (1 μg/ml) treatment, cell culture flasks were replenished with 25 ml fresh medium. Cells were irradiated with doses of 2, 4, 6, or 8 Gy at a dose rate of 2 Gy/min. Control flasks were sham-irradiated at the same time. β-Glucan was removed from cell culture before radiation exposure by replenishing cell culture completely. The assay was performed in triplicate.

2.7. Clonogenic survival assay

Control and pre-treated cells with 1 μg/ml β-glucan were trypsinized 30 min after irradiation and appropriate numbers of cells were plated into 60 mm Petri dishes (Nunc, Denmark) for survival analysis using clonogenic assay [35,36]. Cells were incubated for 14–16 days to form colonies, fixed with formaldehyde (Merck), and stained with 2% crystal violet solution [37]. The plating efficiency and surviving fraction at each dose of irradiation was determined. All data points were the means of three experiments.

$$\text{Plating efficiency}\,(\text{PE}) = \frac{\text{Number of colonies counted}}{\text{Number of cells seeded}} \times 100$$

$$\text{Surviving fraction}\,(\text{SF}) = \frac{\text{Number of colonies counted}}{\text{Number of cells seeded} \times (\text{PE}/100)}$$

2.8. Comet assay

The alkaline phosphate comet assay was performed immediately after exposure to ionizing radiation [38–41]. For each experiment, a set of four slides was processed simultaneously, including: negative or solvent control (C), positive control, which was pretreated with 1 μg/ml β-glucan for 72 h (B), negative control exposed to 6 Gy ionizing radiation (R6), and positive control exposed to 6 Gy ionizing radiation and β-glucan (1 μg/ml) (B6). The assay was performed 0, 30, 60, 120 min and 24 h after radiation exposure. After irradiation, cells were trypsinized and suspended in 70 ml pre-warmed LMP agarose (0.5% in PBS) and 65 ml of the suspension was deposited on a fully frosted slide which was pre-coated with 80 ml 1% normal agarose in PBS. The agarose was allowed to set at 4 °C for 10 min. The slides were then put into a tank filled with lysis buffer solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris/HCl adjusted to pH 10, 1% Triton X-100 freshly added) for 1 h at 4 °C. To allow DNA unwinding, the slides were incubated in fresh electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13.6) for 30 min at 4 °C. The slides were then placed into a horizontal electrophoresis tank and electrophoresis was performed at 16 V (1 V/cm, 300 mA) for 30 min at room temperature. After electrophoresis, the slides were placed in fresh neutralization buffer (0.4 M Tris/HCl adjusted to pH 7.5) before staining with 50 μl ethidium bromide solution (20 μg/ml) and were observed at 20× magnification using a Zeiss Axoscope 2 fluorescence microscope (Germany) [42]. 150 randomly selected cells per slide were visually scored and analyzed using image analysis software (Tri Tek Comet Score 1.5). Data represent three independent experiments.