International Journal of Biological Macromolecules 77 (2015) 214–221

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Modulation of gene expression and cell cycle by botryosphaeran, a (1→3)(1→6)-β-d-glucan in human lymphocytes

Maressa Malini[a], Marilesia Ferreira de Souza[a], Marcelo Tempesta de Oliveira[a], Lusânia Maria Greggi Antunes[b], Suely Gomes de Figueiredo[c], Aneli M. Barbosa[d,e], Robert F.H. Dekker[e], Ilce Mara de Syllos Cólus[a,*]

[a] Departmento de Biologia Geral, Centro de Ciências Biológicas – Universidade Estadual de Londrina, Londrina – PR, Brazil
[b] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto – SP, Brazil
[c] Departamento de Ciências Biológicas, Centro de Ciências Fisiológicas – Universidade Federal do Espírito Santo, Vitória – ES, Brazil
[d] Departmento de Química, Centro de Ciências Exatas – Universidade Estadual de Londrina, Londrina – PR, Brazil
[e] Biorefining Research Institute, Lakehead University, Thunder Bay, Ontario, Canada P7B 5E1

ARTICLE INFO

Article history:
Received 6 February 2015
Received in revised form 2 March 2015
Accepted 10 March 2015
Available online 18 March 2015

Keywords:
Fungal β-D-glucan
Human lymphocytes
Doxorubicin

ABSTRACT

There is growing interest in the anticancer and immunomodulatory potential of fungal β-D-glucans. In the present study, the modulation of gene expression via RT-qPCR and cell cycle kinetics via flow cytometry were assessed in human normal and tumor (Jurkat) lymphocytes after treatment with botryosphaeran (a fungal (1→3)(1→6)-β-D-glucan) from *Botryosphaeria rhodina* MAMB-05. Cell cultures were treated with botryosphaeran either alone, or in combination with doxorubicin (DXR), in a post-treatment protocol. The expression of genes involved in immunomodulatory processes, apoptosis and cell cycle control, as well as β-D-glucans cell receptors were assessed. Flow cytometry analysis identified tetraploid Jurkat cells in G_1 phase when treated with botryosphaeran combined with DXR. This antiproliferative effect in G_1 may be associated with down-regulation of the expression of genes involved in the G_1 checkpoint. The repression of the *CCR5* gene following botryosphaeran treatment, either alone or in combination with DXR, in tumor lymphocytes indicates a possible affinity of this particular (1→3)(1→6)-β-D-glucan for the receptor CCR5. Therefore, botryosphaeran action appears to be involved in the repression of genes related to the G_1 phase of the cell cycle and possibly in the interaction of the botryosphaeran, either alone, or in combination with DXR, with the CCR5 receptor.

1. Introduction

Exopolysaccharides (EPSs) are exocellular-secreted carbohydrate biopolymers produced during metabolic processes in microorganisms such as bacteria and fungi [1]. They are generally associated with protecting cells against the external environment and may act as carbon and energy reserves during periods of environmental stress [2].

According to Roupas et al. [3], *in vitro* and *in vivo* studies have described the benefits of the exopolysaccharides for health, including immunomodulatory, antitumor, antimicrobial and hypocholesterolemic effects. The effectiveness of EPSs as immunomodulators, especially the group of β-D-glucans, allowed validation and approval of some of these molecules as adjuvants for the treatment of cancer in Japan, such as compound formulations of lenthionine (a cyclic organ sulfur compound) and the proteoglycan Krestin (PSK) [4].

The antitumor activity of β-D-glucans is due to their ability to act as biological response modifiers (BRMs) [5], which involves binding of these molecules to specific host cell receptors, such as the complement receptor CR3, and dectin-1 [6,7]. This leads to the production of cytokines and, consequently, stimulation of the immune system through their effects on natural killer (NK) cells, macrophages and T lymphocytes [6].

Recently, Zong et al. [8] proposed that the antitumor action of β-D-glucans may occur through prevention of the development of cancer due to consumption of these bioactive molecules in the diet; through direct anticancer action, such as the induction of

* Corresponding author at: Departamento de Biologia Geral – CCB, Universidade Estadual de Londrina – UEL, Rodovia Celso Garcia Cid, PR (445), Km 380, CEP 86057-970 Londrina, Paraná, Brazil. Tel.: +55 43 3371 4417; fax: +55 43 3371 4207.
E-mail addresses: ilcecolus@gmail.com, colus@uel.br (I.M.d.S. Cólus).

http://dx.doi.org/10.1016/j.ijbiomac.2015.03.010
0141-8130/

apoptosis in tumor cells; through immunopotentiation of anti-tumor activity in combination with chemotherapy; and through inhibition of tumor metastasis. The number of studies addressing direct interventions involving the consumption of fungal polysaccharides in humans is small, and the existing research suggests few or no side-effects [3].

Botryosphaeran, an EPS ((1→3)(1→6)-β-D-glucan) secreted by the ascomycetes fungus *Botryosphaeria rhodina* MAMB-05, is composed of a linear main chain of D-glucose residues bound by β-type glucosidic bonds (1→3), to which D-glucose (D-Glcp) and gentiobiose (β-D-Glcp-(1→6)-β-D-Glcp) residues are bound by (1→6)-β-glucosidic linkages [9].

Studies conducted by our research group have revealed a lack of mutagenicity and an antimutagenic effect of botryosphaeran in the peripheral blood and bone marrow cells of mice *in vivo*. This protective effect was dose dependent, and significant antimutagenic activity was detected, even at low doses of botryosphaeran [10]. Miranda-Nantes et al. [11] furthermore demonstrated that botryosphaeran exhibited hypoglycaemic and hypocholesterolaemic properties in rats with diabetes and hyperlipidaemia.

Given the biological effects described thus far for botryosphaeran and its potential therapeutic and biotechnological applications, *in vitro* assays using normal and tumor cells represent an important analytical tool for evaluating its selectivity as a therapeutic agent. Molecules showing selective activity for tumor cells decrease systemic toxicity and exert a more effective action [12].

Gene expression analyses associated with the versatility of flow cytometry assays, which provide multiple and simultaneous targets to assess cell death and cell kinetics in selected cell populations, were employed in this study. Thus, the present study investigated the effect of treatment with botryosphaeran, either alone or in combination with the chemotherapeutic agent doxorubicin (DXR) on normal and tumor lymphocytes to evaluate its effects on the modulation of the expression of genes associated with the recognition of β-D-glucan, immunomodulation, apoptosis and cell cycle regulation. In addition, the effect of this treatment on cell cycle kinetics was also evaluated.

2. Materials and methods

2.1. Microorganism and cultivation

B. rhodina (MAMB-05 isolate) was grown by submerged fermentation on sucrose as sole carbon source for 72 h at 28 °C under agitation conditions (180 rpm) as described by Steluti et al. [13].

2.2. Botryosphaeran production

Following fermentation, the fungal mycelium was removed, and the supernatant was recovered by centrifugation (1250 × *g*/15 min), and subsequently dialyzed for 48 h. The dialyzed solution was precipitated through the addition of three volumes of isopropanol and stored overnight at 4 °C. Then, the precipitate (EPS) was recovered by centrifugation, dissolved in distilled water and dialyzed with frequent changes of water over 48 h, and the EPS solution lyophilized and stored at −20 °C until used.

2.3. Preparation of botryosphaeran solutions

For use in treating of the cell lines under study, botryosphaeran was solubilized in sterile water to produce a stock solution of 3.0 g/L. A sample of this solution was utilized for the determination of reducing sugars by the cupro-arsenate method [14], and total sugars by the phenol-sulfuric method [15]. The concentration of approximately 3.0 g/L was confirmed through these measurements, and this stock solution was then used throughout the experiments.

2.4. Cell culture

The biological effects of botryosphaeran were investigated in normal and leukaemic (Jurkat cells) lymphocyte cell cultures. The latter were kindly provided by the Blood Center of the Clinical Hospital of Ribeirão Preto Medical School – University of São Paulo (Brazil), and were kept under liquid nitrogen until use.

Normal human lymphocytes were obtained through the collection of 10 mL volumes of peripheral blood from four healthy males, aged 18–35 years old, who were non-smokers and non-alcoholic, with no recent history of disease or exposure to radiation, pesticides or medications. All volunteers signed an informed consent form approved by the Ethics Committee for Research with Human Beings of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil. The collected blood was diluted in PBS at 1:1 ratio (10 mL blood: 10 mL PBS), homogenized by inversion and subjected to the density gradient separation technique using *Ficoll Histopaque*® 1077 (Sigma–Aldrich, St. Louis, MO, USA) to obtain isolated lymphocytes.

2.5. Culture conditions

Normal and tumor lymphocytes were cultured in sterile 15 mL conical tubes (25 cm^2, Corning, Lowell, MA, USA) containing RPMI 1640 culture medium (Gibco, Carlsbad, CA, USA) supplemented with 20% foetal bovine serum (Gibco), 0.01 mg/mL streptomycin (Sigma–Aldrich, St Louis, MO, USA), 0.005 mg/mL penicillin (Sigma–Aldrich) and 2.38 mg/mL HEPES (Sigma–Aldrich). The conical tubes were kept at 37 °C under an atmosphere of 5% CO_2 at an inclination of approximately 45°, and were carefully inverted three times daily.

2.6. Experimental design

To evaluate the effect of botryosphaeran (30 μg/mL), either alone or in combination with the chemotherapeutic agent doxorubicin (DXR; 0.20 μg/mL) on the cell cycle kinetics of normal and tumor lymphocytes, approximately 2×10^5 cells were seeded in sterile 15 mL conical tubes, incubated for a cell cycle (period of 24 h) in complete RPMI 1640 culture medium, and then subjected to one of the following treatments: (i) PBS for 24 h (negative control); (ii) DXR for 2 h (positive control); (iii) botryosphaeran for 24 h; (iv) combination of botryosphaeran + DXR for 2 h (simultaneous treatment); (v) botryosphaeran for 22 h, with washing of tubes, changing of the culture medium and the addition of DXR for 2 h (pre-treatment); (vi) DXR for 2 h with washing of tubes, changing of the culture medium and the addition of botryosphaeran for 22 h (post-treatment).

For gene expression assays, the same botryosphaeran concentration was maintained (30 μg/mL), but only the post-treatment protocol was evaluated.

All assays involving Jurkat cells comprised three independent experiments, each of which included an experimental triplicate.

2.7. Analysis of cell cycle kinetics by flow cytometry

Samples for analysis by flow cytometry were prepared using the *Cycle Test Plus DNA* kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Readings were obtained in a BD FACSCantoTM II Flow Cytometer (Becton,