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ARTUR JAVMEN

AUSRA NEMEIKAITE-CENIENE

SAULIUS GRIGISKIS

IRENA JONAUSKIENE

MARK RUDENKOV

See next page for additional authors

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S. cerevisiae β-glucan reduced viability of mouse hepatoma cells in vitro

Artur JAVMEN[1,2,*], Aušra NEMEIKAITĖ-ČĖNIENĖ[2], Saulius GRIGIŠKIS[1], Irena JONAUSKIENĖ[2], Mark RUDENKOV[1], Darius KAČIANAUSKAS[1], Mykolas MAURICAS[2]

[1]JSC "Biocentras", Vilnius, Lithuania

[2]Department of Immunology, State Scientific Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

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Abstract: β-Glucan is a natural polymer, which is widely studied due to its multiple immunomodulatory properties. In addition, recent findings indicate potent antitumor properties of β-glucan. *Saccharomyces cerevisiae*, baker's yeast, is one of the commonly used sources of β-1,3-glucan. The aim of this work was to investigate *S. cerevisiae* β-glucan immunomodulatory activity against cancer cells. In our experiments, BALB/c mice were fed with insoluble whole β-glucan particles, and then their blood was collected for experiments. MH-22a hepatoma cells were treated with the blood of mice fed with β-glucan, and tumor cell viability was investigated after the treatment. The obtained results demonstrated that leukocytes in vivo primed with whole glucan particles, in combination with soluble β-glucan, decreased MH-22a hepatoma cell viability in vitro. Our study has indicated that β-glucan obtained from *S. cerevisiae* potentially primes mouse whole blood leukocytes to induce cell death of mouse hepatoma cells.

Key words: β-Glucan, yeast, cancer, mice

1. Introduction

β-Glucan is a natural polymer made of glucose monomers linked by β-glycosidic bonds. It is produced by various organisms including bacteria, fungi, and plants. β-Glucan polymer modifications in a variety of organisms result in differences in their glycosidic linkage position, structure, and complexity, e.g., bacterial β-1-3-glucan curdlan has a linear structure and is insoluble in water, whereas cereal β-glucans often are β-1-3,1-4-glucans and the majority of them are water soluble (Basic et al., 2008). β-Glucans are widely studied due to their multiple immunomodulatory properties (Mantovani et al., 2008). β-1,3-Glucans, in particular, have demonstrated a significant physiological effect on the mammalian immune system with their ability to activate leukocytes and trigger the immune response (Chen et al., 2008; Novak and Vetvicka, 2008; Petravić-Tominac et al., 2010; Marakalala et al., 2013). β-Glucans can be used in the treatment of fungal, bacterial, viral, and protozoal infections and to increase immune system cytotoxicity against tumor cells (Chen et al., 2008; Novak and Vetvicka, 2008; Chan et al., 2009; Javmen et al., 2013a, 2013b). It is important to note that highly purified β-glucans are characterized by very low toxicity (e.g., mouse lentinan has LD_{50} > 1600 mg/kg) (Novak and Vetvicka, 2009).

One of the commonly used sources of β-1,3-glucan is *Saccharomyces cerevisiae*, baker's yeast (Hunter et al., 2002; Pelizon et al., 2005; Shokri et al., 2008; Novak and Vetvicka, 2009; Vetvicka, 2011; Javmen et al., 2012; Javmen et al., 2013b). β-1,3-Glucan is a major component (50%–55%) of *S. cerevisiae* cell walls (Lee et al., 2000; Shokri et al., 2008). Yeast glucan has been shown to help maintain the rigidity and shape of the cell (Petravić-Tominac et al., 2010). Recent studies have also indicated that yeast cell wall β-glucan, either soluble or particulate, can exhibit antimicrobial and antitumor effects (Shokri et al., 2008; Chan et al., 2009). It is thought that β-glucan can be used as a safe, effective, therapeutic, and/or prophylactic agent, either alone or as adjuvant, to enhance the immune response in mammals with normal or decreased immunological function (Shokri et al., 2008).

As mentioned previously, recent findings indicate potent antitumor properties of β-glucan (Chan et al., 2009; Vetvicka, 2011). On the other hand, the molecular mechanisms that underlie these responses are not completely understood (Budak et al., 2008; Chan et al., 2009). The above data were obtained using both soluble and insoluble β-glucan (Li et al., 2006). The similarities in results obtained using different β-glucans might be explained by the fact that orally administrated β-glucans (animal data)

* Correspondence: arturas.biocentras@gmail.com

enter the proximal small intestine and are "captured" by the macrophages. Subsequently, macrophages internalize and hydrolyze β-glucan molecules into small fragments, which are later carried to the marrow, and thus immune response is elicited (Chan et al., 2009).

The results of our current study indicate that β-glucan obtained from *S. cerevisiae* can prime mouse blood leukocytes for cytotoxicity against MH-22a mouse hepatoma cells. In these experiments, BALB/c mice were fed with insoluble whole β-glucan particles for 1 week and their blood was collected for experiments. MH-22a cells were grown in 24-well plates for 24 h and treated with the blood of mice fed with β-glucan (diluted in cell culture medium). Soluble β-glucan was also added to the cell culture medium. The obtained results demonstrate that leukocytes in vivo primed with whole glucan particles, in combination with soluble β-glucan, decrease MH-22a hepatoma cell viability in vitro.

2. Materials and methods

2.1. Chemicals

All experiments were performed using reagent-grade materials. Insoluble *S. cerevisiae* cell wall whole β-glucan particles were extracted according to the method published by Javmen et al. (2012) with some modifications. Briefly, 3 g of dry baker's yeast was enzymatically lysed by *Streptomyces rutgersensis* yeast lysing complex and washed with distilled water. Yeast cell walls were isolated by centrifugation and resuspended in NaOH solution (1 M) using a 1 g:5 mL ratio. The mixture was heated for 2 h at 100 °C with stirring. Whole glucan particles were harvested by centrifugation and washed with distilled water until neutral pH was achieved. Soluble β-glucan (molecules of about 0.7–23 nm) was produced by enzymatic whole glucan particle hydrolysis using *S. rutgersensis* β-glucanase (Javmen et al., 2013a).

2.2. Experimental animals

Mice (8-week-old males, with a body weight of 20–24 g) were kindly donated by the animal facility of the State Research Institute Centre for Innovative Medicine. Experimental conditions were in compliance with regulatory laboratory practices and followed the Law of the Republic of Lithuania on the Care, Maintenance, and Use of Animals as well as secondary legislation, the Order of the State Food and Veterinary Service of the Republic of Lithuania "On Veterinary Regulations on Breeding, Handling, and Transportation of Laboratory Animals" and "On the Use of Laboratory Animals in Scientific Experiments" (Law of the Care, Welfare, and Use of Animals, 2002). The protocol was approved by the State Food and Veterinary Service/Ethical Committee for Animal Research (protocol No. 0202, 2009). The animals were acclimatized for 1 week prior to the study. They were housed maintaining and monitoring the temperature, humidity, and light/dark cycle (12 h/12 h). A commercial pellet diet and fresh drinking water were provided ad libitum.

Experimental mice (n = 12) were orally fed 0.1 mL water containing 0.1 mg of insoluble β-glucan particles daily for a period of 2 weeks. The above animals were sacrificed, and 100 μL of blood was taken from Jung's vein after administration of β-glucan. The blood samples (0.1 mL) were mixed with 0.9 mL of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and 50 μg of the soluble β-glucan was added. These solutions were used for in vitro experimentation. The remaining 12 mice were fed a regular diet as a control.

2.3. Tumor cell line

The cell line of the murine hepatoma MH-22a was obtained from the Institute of Cytology, Russian Federation. The cultivation of the hepatoma cells was carried out under standard conditions: cells were grown in monolayer in the cell culture flasks in DMEM containing 10% fetal bovine serum and antibiotics at 37 °C (Shvenberger and Alexandrova, 2000; Grellier et al., 2008; Obakan et al., 2014).

2.4. Whole mouse blood leukocytes

Whole mouse blood was stained with acridine orange in conjunction with ethidium bromide on microscopic glass slides (Nemeikaitė-Čėnienė et al., 2005). The leukocyte count and type (morphological appearance) were determined using a fluorescent microscope (Leica DMLB). The experiment was repeated 12 times using different mice (1 blood smear from each).

2.5. Hepatoma cell treatment

MH-22a cells were grown in a 24-well plate (1×10^5/mL) and incubated in DMEM for 24 h. Subsequently, MH-22a cells were treated with 1 mL of DMEM containing one of the following:

1) 50 μg of soluble β-glucan and 0.1% (v/v) whole blood obtained from mice fed with β-glucan,
2) 0.1% (v/v) whole blood obtained from mice fed with β-glucan,
3) 50 μg of the soluble β-glucan and 0.1% (v/v) whole blood obtained from control mice (normal diet), or
4) 50 μg of the soluble β-glucan.

As a control, MH-22a cells without any treatment or treated with 1 mL of DMEM containing 0.1% (v/v) whole blood obtained from mice fed a normal diet were used. The viability of the cells was examined after 24 h of incubation after the treatment (Nemeikaitė-Čėnienė et al., 2005). All experiments were repeated 6 times.

2.6. Tumor cell viability

Acridine orange in conjunction with ethidium bromide staining of MH-22a cells on glass slides was used to

differentiate between viable and dead cells (Nemeikaitė-Čėnienė et al., 2005; Prabhu et al., 2014). The number of viable and dead cells was determined using fluorescent microscopy (Leica DMLB).

2.7. Statistical analysis

The significance of differences between means was assessed using Student's t-test.

3. Results

Insoluble β-glucan from *Saccharomyces cerevisiae* (baker's yeast) was extracted and hydrolyzed using β-glucanase from *Streptomyces rutgersensis* as previously described by Javmen et al. (2013a) (Figure 1).

Next, mice were fed with the insoluble β-glucan for 1 week. Subsequently, mouse whole blood was collected, and the leukocyte count of mice fed with β-glucan was analyzed and compared to the blood leukocyte count of the control group.

As shown in Figure 2, feeding mice with insoluble β-glucan induced an increase in the neutrophil blood count and a concomitant decrease in lymphocyte count, while the numbers of basophilic, eosinophilic, and monocytic leukocytes were unchanged.

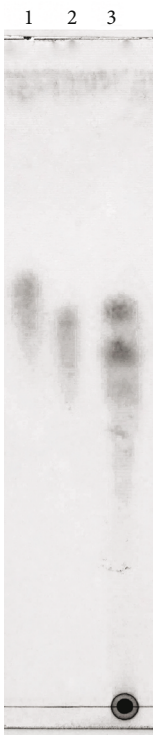

Figure 1. Thick layer chromatography of reaction products after *S. cerevisiae* cell wall β-glucan hydrolysis. 1) Glucose (20 µg), 2) sucrose (20 µg), 3) insoluble β-glucan hydrolysis products (100 µg). Solvent system: ethyl acetate-methanol-water, 52:36:13 by volume; staining: 10% sulfuric acid solution diluted with ethanol (Javmen et al., 2013b).

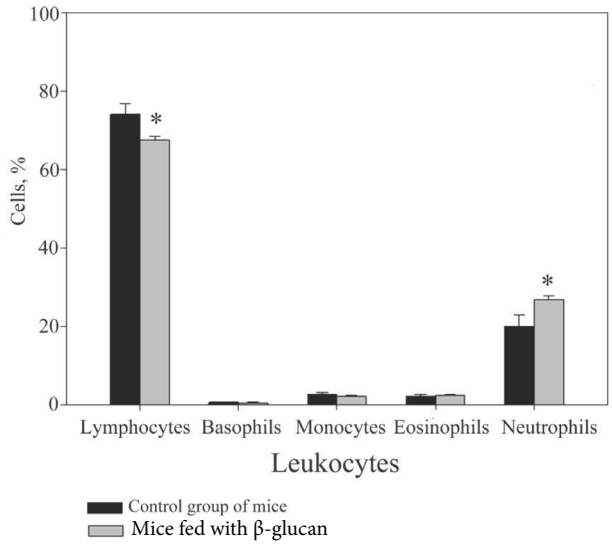

Figure 2. Blood leukocyte count of the control mice and mice treated with the insoluble β-glucan preparation. Each value represents the middle point of 12 replicates with the standard margin of error. *: $P < 0.05$.

In the next series of experiments, MH-22a hepatoma cells were incubated under the various experimental conditions described in Section 2 and MH-22a cell viability was analyzed 24 h later.

The results of our experiments indicate that the number of dead MH-22a cells is higher after treatment with the blood of mice fed with β-glucan in combination with soluble β-glucan as opposed to all other conditions, including the control (Figure 3). The dead cell count was between 2% and 4% in all of the experiments, except when MH-22a cells were treated with the blood obtained from mice fed with β-glucan in combination with soluble β-glucan (>10% dead cells) (Figure 3).

The increase in cell death (Figure 3) was correlated with a concomitant decrease in the number of adherent cells under the experimental conditions employed (Figure 4). Furthermore, it was determined that the total adherent cell count was the lowest after treatment with the blood of mice fed with β-glucan in combination with treatment with soluble β-glucan (Figure 4). Adherent cell count in the latter was about 63.5%, in comparison to the untreated cell count. In all other experiments, the adherent cell count was about 75%–85%. In the experiments with soluble β-glucan only, adherent cell count was about 97.6% compared to the untreated cells (Figure 4).

4. Discussion

β-Glucan in *Saccharomyces cerevisiae* (baker's yeast) is considered a mammalian immune system stimulator, and, as mentioned previously, can be helpful in treating infectious diseases and cancer (Vetvicka, 2011).

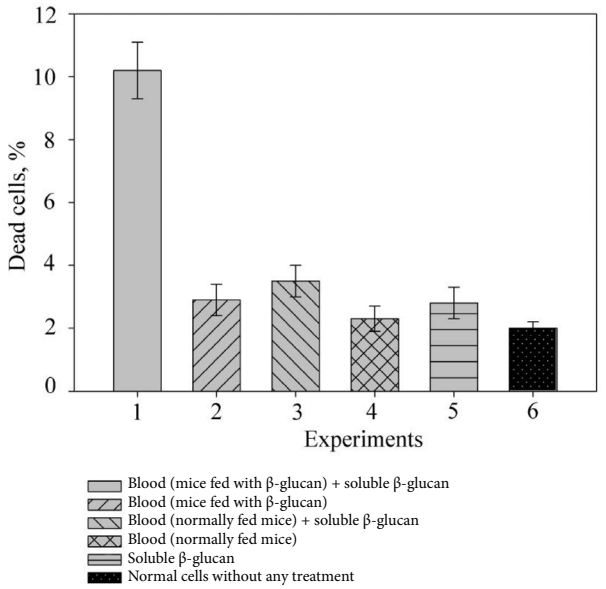

Figure 3. MH-22a cell viability. MH-22a cells were challenged under various conditions described in Section 2 and assessed for cell viability using acridine/ethidium bromide staining. The results are presented as percentage of dead cells. Each value represents the middle point of 6 replicates with the standard margin of error.

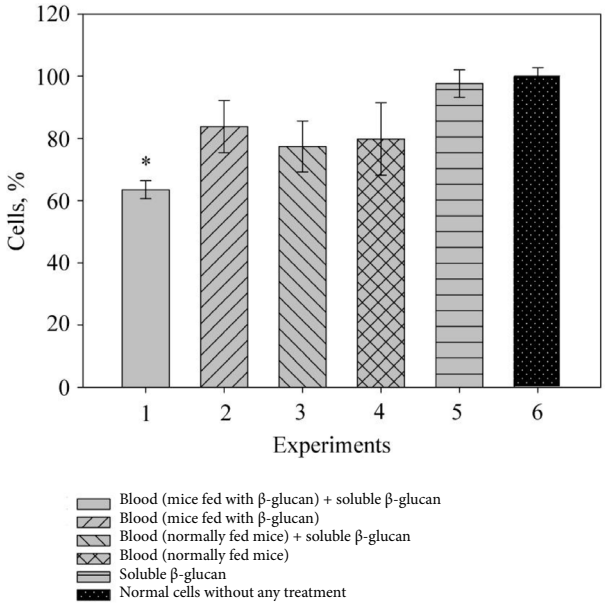

Figure 4. MH-22a cell viability. Total number of MH-22a cells after different treatments. Each value represents the middle point of 6 replicates with the standard error. *: $P < 0.05$ represents a significant difference between control mice and mice treated with β-glucan preparations.

In the current study, we investigated whether *Saccharomyces cerevisiae* β-glucan primes mouse blood leukocytes for cytotoxicity against MH-22a mouse hepatoma cells. Our results indicate that hepatoma cell treatment with 0.1% (v/v) blood of mice fed with β-glucan particles, in combination with soluble β-glucan, induces MH-22a cell death in vitro. In addition, oral β-glucan administration changes blood leukocyte composition in BALB/c mice, i.e. it increases the neutrophil count and decreases the number of lymphocytes.

Although the antitumor properties of β-glucan are well established, the mechanisms involving its impact on cancer cells have not been fully investigated (Budak et al., 2008; Chan et al., 2009; Vetvicka, 2011). β-Glucans per se have no direct effect on tumor cells (Li et al., 2006; Chan et al., 2009). One hypothesis is that β-glucan induces the effector mechanism of complement receptor 3 (CR3)-dependent cellular cytotoxicity (DCC). According to this mechanism, leukocyte CR3 receptors do not trigger the death of tumor cells coated with their ligands, C3b and iC3b. However, there has been much evidence suggesting that leukocyte CR3 receptors can be manipulated to trigger cytotoxicity of cells coated with iC3b. The CR3-DCC mechanism is normally used by an organism to eradicate yeast and fungi cells. The induction of CR3-DCC requires dual ligation of CR3 to iC3b and β-glucan (the main cell wall component in yeast and fungi). Since cancer cells do not contain β-glucan on their surface, they cannot trigger the CR3-DCC mechanism, even though they are coated with iC3b. Therefore, our findings suggest that tumor cells can potentially be eradicated through the CR3-DCC mechanism when soluble β-glucan is added to the medium. To this end, β-glucan primes the CR3 receptor to trigger cytotoxicity of cells coated with iC3b but lacking β-glucan (Gelderman et al., 2004; Li et al., 2007). It is also hypothesized that the CR3-DCC effectors mentioned above are actually neutrophil cells (Li et al., 2007; Driscoll et al., 2009).

Our data also confirm the previous finding that β-glucan, per se, has no effect on cell viability as assessed by the MH-22a viability assay (Figures 3 and 4). On the contrary, incubation of cells with mouse blood decreased MH-22a cell viability, although the observed changes did not reach statistical significance (Figure 3). On the other hand, all MH-22a cell treatments with mouse blood decreased the total number of adherent cells in the culture dish (Figure 4). The latter can be interpreted as follows: cells die and detach, and therefore the total adherent cell count decreases.

These findings are in agreement with existing experimental results, demonstrating that soluble β-glucan is necessary to prime leukocytes for cancer cell eradication (Li et al., 2007; Driscoll et al., 2009). However, a single MH-22a treatment with soluble β-glucan and mouse

blood was not enough to induce hepatoma cell death. We also needed mouse immune system activation with the particulate β-glucan. This fact can be interpreted as follows: the composition of the blood leukocytes changed after administration of β-glucan particles to the mice; as a result, we observed the increment of neutrophils, which are effector cells in the CR3-DCC mechanism.

In summary, the data obtained in this study demonstrate that β-glucan in *Saccharomyces cerevisiae* has no direct effect on MH-22a hepatoma tumor cells, but it potentially primes mouse whole blood leukocytes (neutrophils) to induce MH-22a death. The exact molecular mechanisms of the observed phenomenon remain to be determined.

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