ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 12, pp. 1312-1315. © Pleiades Publishing, Inc., 2006.
Published in Russian in Biokhimiya, 2006, Vol. 71, No. 12, pp. 1613-1617.

Cytotoxicity Effect of Algal Polysaccharides on HL60 Cells

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Received April 7, 2006
Revision received June 30, 2006

Abstract—The present study shows the cytotoxic effect of three different classes of algal polysaccharides on HL60 cells. Three galactofucans, fucoidan, and glucan were the polysaccharides utilized in this analysis. The parameters used for evaluating cell viability were [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) reduction, protein content, and phosphatase activity. We demonstrated stimulation of phosphatase activity, MTT reduction, and protein content in relation to three types of galactofucans (1, 2, and 3) with different molecular weights (1600, 1200, and 360 kD). However, when HL60 cells were treated with galactofucan type 3, the total protein remained unchanged. Under the same experimental conditions, an expressed increase in the phosphatase activity was detected when galactofucan 3 was utilized. In relation to the mitochondrial function, the stimulation was higher in cells treated with galactofucan type 1. Fucoidan did not have a significant effect on MTT reduction, but protein content was decreased (IC_{50} around 30 μg/ml). Glucan also activated all the parameters that were analyzed, and this effect was more expressed in the phosphatase activity and in the protein content. This study provides new insights into the cytotoxic action of polysaccharides on HL60 cells and suggests for the first time the possible involvement of phosphatases in this process.

DOI: 10.1134/S0006297906120042

Key words: fucan, glucan, HL60 cells, cytotoxicity, algae

Bioactive algal polysaccharides have generated considerable interest due to their biological properties, such as anticoagulant, antithrombotic, antiinflammatory, antiviral, immunostimulant, and antitumor activities [1-3]. Polysaccharides are molecules with a high degree of biocompatibility and low toxicity. Sulfated polysaccharides such as fucoidan or fucans have been cited because they bind in diverse proteins from extracellular matrix and cellular structures due to their polyanionic character. These polysaccharides, consisting mainly of sulfated L-fucose, are easily extracted from the cell wall of brown algae (i.e., Phaeophyceae) and can represent more than 40% of the dry weight of isolated cell walls [3, 4]. The search for new drugs has recently raised increased interest in sulfated fucans. Their structures contain large amounts of sugars other than fucose, such as galactose, mannose, xylose, or uronic acid, and sometimes even proteins [5, 6]. However, they could be classified as homofucans (fucoidan) and heterofucans. Fucans have a wide spectrum of activity in biological systems and are present in echinoderms [7]. These compounds interfere in several cellular processes such as migration, adhesion, proliferation, and apoptosis [3, 8].

The β-glucans consist of linear or slightly branched polysaccharides built up mainly of (1→3)-linked β-D-glucopyranose units. The neutral glucan-like polymers can act as biological response modifiers. Receptors present in lymphocytes and macrophages, denominated Dectin-1 and CR3, recognize this class of polysaccharides [9].

The HL60 cell line was established in 1977 from a patient with acute myeloid leukemia. Cells of line HL60 are used as a model in research and have played a pivotal role in studies on differentiation and growth of human leukemias [10]. These cells largely resemble promyelocytes, which proliferate continuously in suspension culture and can be induced to differentiate terminally *in*

Abbreviations: MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; pNPP) *p*-nitrophenylphosphate; pNP) *p*-nitrophenol.

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vitro. HL60 cells are a well-established model for studying the effect of different physiological and pharmacological agents on cell growth and maturation [11-13].

Numerous assays have recently been developed as alternatives for evaluating *in vitro* toxicity of several compounds. Thus, the therapeutic and toxicological effects of a compound are important parameters in the verification of its applicability in pharmacology. The aim of this study was to evaluate the cytotoxicity of algal polysaccharides in HL60 cells using three different parameters: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) reduction, protein content, and phosphatase activity.

MATERIALS AND METHODS

Materials. Fucoidan, a homofucan, from *Fucus vesiculosus* was purchased from Sigma (USA). A glucan and galactofucans from seaweed *Lobophora variegata* were isolated and fractionated by gel filtration using Sepharose CL-4B. Polymers with 1600 (galactofucan 1), 1200 (galactofucan 2), and 360 kD (galactofucan 3) were obtained as previously standardized by [14]. The chemical analyses were made as described by Silva et al. [15].

Cell culture. HL60 cells were purchased from the American Type Culture Collection (ATCC) and routinely grown in suspension in RPMI 1640 medium containing 2 mM L-glutamine and antibiotics (100 IU penicillin/ml, 100 μg streptomycin/ml) and supplemented with 10% heat-inactivated fetal bovine serum, in a 5% CO_2 humidified atmosphere at 37°C. The cells were plated in 24-well tissue culture plates at a density of $3 \cdot 10^5$ cells/ml and after 72 h treated with polysaccharides for 24 h. The polymer concentration was from 5 to 50 μg/ml.

Cytotoxicity assays. MTT reduction: the medium containing polysaccharides was removed and 1 ml of MTT solution (0.5 mg MTT/ml of culture medium) was added to each well. After incubation for 4 h at 37°C, the medium was removed and the formazan crystals formed were solubilized in 1 ml of ethanol. The plate was shaken for 5 min on a plate shaker and absorbance measured at 570 nm [16].

Protein phosphatase assay. The enzyme was obtained after cell lysis with 0.1 M acetate buffer, pH 5.5. The reaction mixture (final volume, 1 ml) contained 0.1 M acetate buffer, pH 5.5, 5 mM *p*-nitrophenylphosphate (pNPP), and cell extract enzyme. The amount of *p*-nitrophenol (pNP) produced was measured at 405 nm [17].

Protein quantification. Protein concentration was determined by a modification of Lowry's method [18].

Statistical evaluation. All experiments were performed in triplicate. The results shown represent the mean and standard deviation determined by ANOVA, followed by *t*-test.

RESULTS AND DISCUSSION

The chemical composition and molecular weight of the polymers used are shown in the table. The relative proportions of sugars vary among the various fractions. Thus, galactose is the main sugar present in the polymers named galactofucans, and fucose is the sugar found in greater amounts in all fractions with exception of glucans. The three galactofucans have different molecular weights (1600, 1200, and 360 kD) determined by gel-permeation chromatography on Sepharose CL-4B. These fractions were not contaminated with laminarans (a group of the reserve β-D-glucans found in brown algae) because glucose was not detected. In these fractions, neither alginic acid nor protein was found (results not shown). These facts are indicative of the purification of these polysaccharides. The composition of sugar reported in this table indicates the occurrence of a sulfated polysaccharide in galactofucans 1, 2, and 3 with level of sulfate of 23.3, 30.4, and 35.5%, respectively (see the table). Another polysaccharide, fucoidan, is a sulfated homofucan containing high level of sulfated fucose. Its structure was previously studied by Patankar et al. [1]. The results of the chemical analysis of this polymer obtained in the present paper are in agreement with these authors.

The present study shows preliminary results on the cytotoxic effect of algal glucans on HL60 cells. Glucans are glucose polymers found in the cell walls of plants, fungi, and bacteria and have a long scientific history and

Chemical composition and molecular weight of the algal polysaccharides

Sample	Polysaccharides, %	Molecular weight, kD	Fucose	Galactose	Glucose	SO_3Na
Galactofucan 1	45.7	1600	1	3	—	1.50
Galactofucan 2	62	1200	1	3	—	2.10
Galactofucan 3	55	360	1	3	—	2.30
Fucoidan	64	170	1		—	1.70
Glucan	77.5	11	—	—	1	—

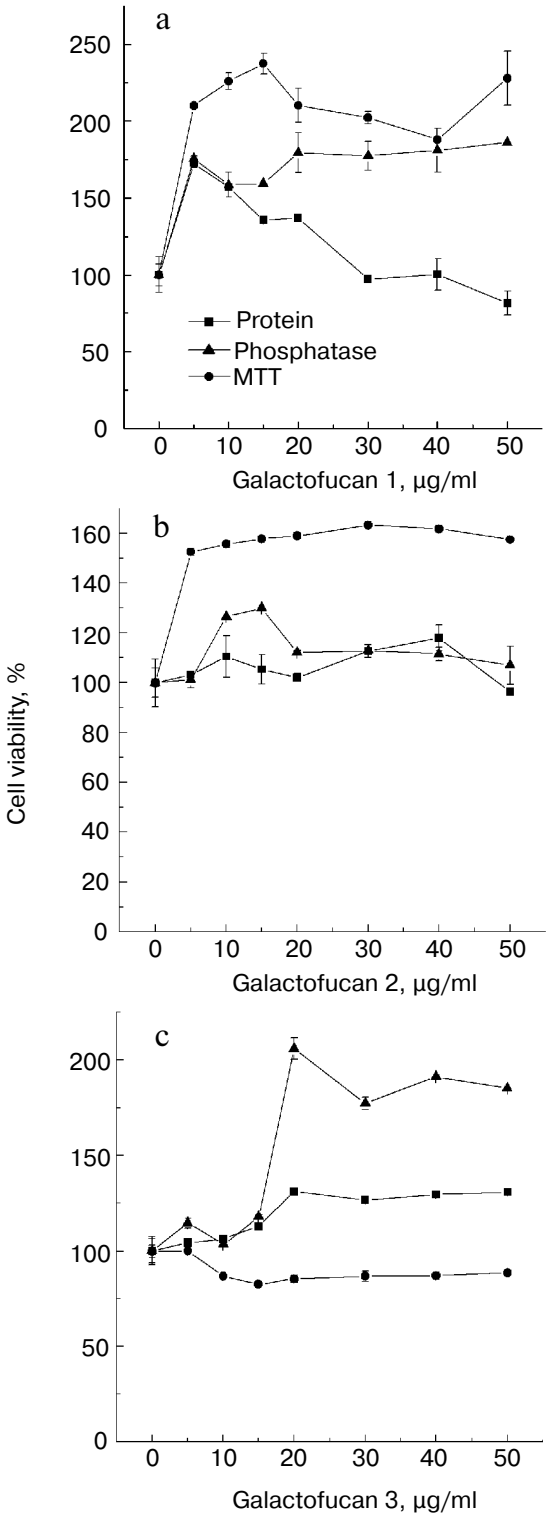

Fig. 1. Cytotoxicity evaluation of the effect of galactofucans on HL60 leukemic cell line. The HL60 cells were treated with different concentrations of galactofucan type 1 (a), type 2 (b), and type 3 (c) for 24 h. In the absence of these compounds, MTT reduction was considered as being 100%. The experiment was performed in a 24-well plate. Results represent the means ± SD of three experiments run in triplicate ($p < 0.05$).

a reference list including hundreds of papers. The glucan used in this work was also chemically characterized. It is soluble in water and has only glucose as constituent and a molecular weight of 11 kD. This polysaccharide was not contaminated with protein and other sugars (table).

In this work, the cytotoxicity of compounds was evaluated using parameters based on the different cellular metabolic conditions. The MTT reduction provides information about mitochondrial function. The protein content represents a cell number index, and phosphatase activity can indicate cellular metabolic function related to phosphate metabolism pathways [19, 20].

Phosphatases are related to several signal pathways. Thus, they are an important parameter, since these enzymes can regulate positively or negatively the cell proliferation and apoptosis [10]. The activity of these enzymes can be also used to evaluate cellular viability [16]. Protein phosphatases may also be involved in the process of HL60 cell differentiation [11].

In relation to galactofucans 1, 2, and 3, we demonstrated stimulation of phosphatase activity, MTT reduction, and protein content (Figs. 1a, 1b, and 1c). However, when the HL60 cells were treated with galactofucan type 3, total protein remained unchanged. Under the same experimental conditions, an expressed increase in phosphatase activity was detected when galactofucan 3 was

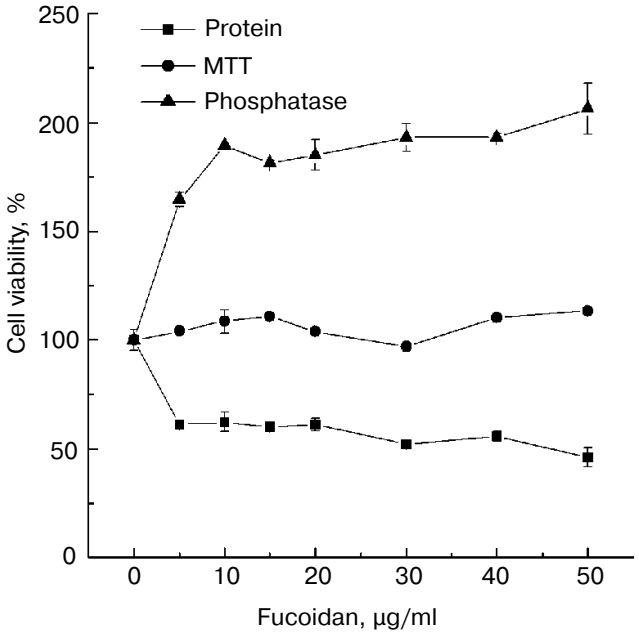

Fig. 2. Cytotoxicity evaluation of the effect of fucoidan on HL60 leukemic cell line. The HL60 cells were treated with different concentrations of fucoidan for 24 h. In the absence of compounds, MTT reduction was considered as being 100%. The experiment was performed in a 24-well plate. Results represent the means ± SD of three experiments run in triplicate ($p < 0.05$).

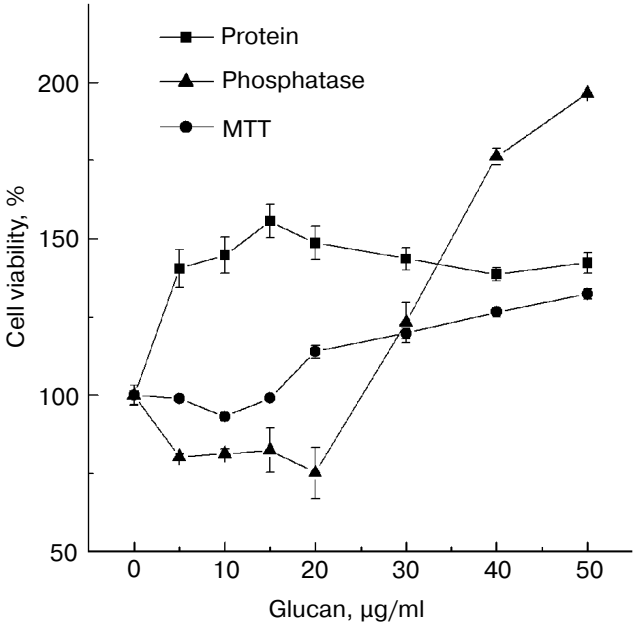

Fig. 3. Effect of glucan on HL60 leukemic cells. The HL60 cells were treated with different concentrations of glucan for 24 h. In the absence of the compounds, MTT reduction was considered as being 100%. The experiment was performed in a 24-well plate. Results represent the means ± SD of three experiments run in triplicate ($p < 0.05$).

used. In relation to mitochondrial function, the stimulation was higher in cells treated with galactofucan type 1. Figure 2 shows the effect of fucoidan, a homopolymer of fucose and sulfate, in the parameters mentioned above. MTT reduction was not affected by this compound. Protein content was decreased, and according to the graph, the IC_{50} value was around 30 μg/ml.

HL60 cells were also incubated with glucan from *L. variegata*. We observed high level of proteins at 15 μg/ml of this polysaccharide. The results of phosphatase activity with this polymer were dose dependent with high activity to these enzymes at 30-50 μg/ml. At the same concentrations, an increase in the cellular viability was also observed. In conclusion, glucan, a neutral polymer, caused activation of all parameters analyzed and did not show cytotoxicity (Fig. 3).

Collectively, our findings provide new preliminary insights into the cytotoxic action of polysaccharides on HL60 cells. The molecular mechanism of the cytotoxic effect of these polymers is not clear, but this investigation suggests for the first time the possible involvement of phosphatases in this process.

This work was supported by CAPES and Conselho Nacional de Tecnologia e Pesquisa, No. 475867/03-3.

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