Glucan Supplementation Has Strong Anti-melanoma Effects: Role of NK Cells

VACLAV VETVICKA and JANA VETVICKOVA

University of Louisville, Department of Pathology, Louisville, KY, U.S.A.

Abstract. *β-Glucan is a natural immunomodulator consisting of glucose molecules. It is a well-established modifier with significant beneficial properties in infectious diseases and cancer therapy. Glucan effects on melanoma are relatively less studied, despite the increasing incidence of this type of cancer. In the current study, we focused on possible effects of insoluble yeast-derived β-glucan on the growth of melanoma cells. We found that glucan supplementation had a strong-positive effect in both reducing tumor weight, lung colonies and overall survival rate of tested animals. In addition, glucan inhibited the damage to blood cells and potentiated the effects of regular chemotherapy. By using depletion of natural killer (NK) cells, we showed that these effects are, at least partly, mediated by direct action of glucan on NK cells.*

β-1,3 Glucans represent highly conserved structural components of cell walls in yeast, fungi, barley and seaweed. Natural β-1,3 glucans from yeast and mushrooms are well-established biological-response modifiers (1-3). Numerous *in vitro* and *in vivo* studies have shown that both particulate and soluble glucans exert their biological functions through priming complement receptor 2 (CR3; CD11b/CD18, Mac-1) to kill tumor cells (4). Subsequent studies showed that dual ligation of the CR3 receptor by iC3b (to the *N*-terminal I-domain) and glucan (to the C-terminal lectin domain) primes the receptor for cytotoxic effects (5). Additional experiments showed that glucan injections resulted in strong tumor regression and long-term survival regardless of tumor type (6). When not-complement activating IgG_1 antibodies are present or in mice lacking either C3 or CR3, no glucan-induced tumor suppression occurred (7). A combination of glucan with anti-tumor monoclonal antibodies has significant therapeutic efficacy and is currently used in numerous clinical trials (8, 9).

From the commercial point of view, two glucans successfully moved from laboratories to hospitals – in Japan, lentinan and schizophyllan have been approved drugs since 1983. Lentinan (from shiitake, *Lentinula edodes*) is a (1-3)-glucan with five 1,3 residues and two (1-6)-β-glucopyranoside branches in side-chains, with molecular weight approximately 400-800,000 Da. Schizophyllan has a similar structure and triple helix configuration with a molecular weight of 450,000 Da (10). Lentinan is mostly used in conjunction with several types of traditional chemotherapy, including cisplatin and paclitaxel (11).

The mechanisms of glucan action in cancer inhibition are not completely established and it is possible that glucan acts differently in different types of cancers. In colitis-associated colonic carcinogenesis, the treatment of mice with mushroom-derived glucan resulted in anti-proliferative effects on intestinal cells. In addition, modulation of expression of B cell lymphoma (BCL2), BLC-2-associated X protein (BAX), and cytochrome *c* genes, and complete inhibition of tumor necrosis factor alpha (TNFα)-induced inhibitor of nuclear factor BCL2-associated X protein degradation and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) nuclear translocation were observed (12). In prostate cancer, glucan most probably stimulates dendritic cells *via* the dectin-1 receptor. As a result, strong secretion of interleukins (IL) IL23, IL1 and IL6 was observed, as well as differentiation of naïve $CD4^+$ cells into T helper (Th17) and Th1 cells (13). In addition, the glucan-stimulated dendritic cells subsequently primed T-lymphocytes to stimulate B-cells to secrete IgG and IgA antibodies. In melanoma, glucans were suggested to act *via* activation of macrophages and natural killer (NK) cells (14), induction of apoptosis (15), *via* activation of B lymphocytes (16), or through inhibition of tumor-induced angiogenesis (17).

In the current study, we decided to evaluate the potential anti-melanoma effects of yeast-derived Glucan #300, which was consistently shown to be the most active glucan (18). This glucan had strong anti-tumor potential in a mouse

Correspondence to: Vaclav Vetvicka, University of Louisville, Department of Pathology 511 S. Floyd, Louisville, KY 40404, U.S.A. Tel: +1 5028521612, Fax: +1 5028521177, e-mail: Vaclav.vetvicka@louisville.edu

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model of breast and lung cancer (19), clinical trials showed positive effects in prostate cancer (20).

Materials and Methods

Animals. Female, 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was carried-out according to the University of Louisville Institutional Animal Care and Use Committee (IACUC) protocol. Animals were sacrificed by CO_2 asphyxiation at the end of the experiment.

Materials. Yeast-derived insoluble Glucan #300 was purchased from Transfer Point (Columbia, SC, USA); its purity was over 85%. RPMI-1640 medium, glutamine, antibiotics, doxorubicin, and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was procured from Hyclone Laboratories (Logan, UT, USA).

Cell lines. Murine melanoma B16 cell line (American Type Culture Collection, Manassas, VA, USA) and YAC-1 cell line were maintained in culture in RPMI-640 medium supplemented with 10% FCS at 37˚C in a humidified atmosphere supplemented with 5% CO_2.

Proliferation. Cytotoxic drugs 5-fluorouracil, doxorubicin, and actinomycin D were diluted to 1 mg/ml in phosphate buffered saline (PBS). Cells were harvested and washed six times in RPMI-1640 medium supplemented with 10% FCS. The cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/ml (150 μl/well) in the presence or absence of different concentrations of tested substances in triplicate. After five days in culture, the proliferation was evaluated using a Biotrak cell proliferation ELISA system version 2 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the instructions given by the manufacturer.

Challenge with tumor cells. 2×10^6 B16 melanoma cells were inoculated subcutaneously into shaved lateral flanks of the mice. The mice were treated orally with different doses of glucan or PBS once daily for 14 days. The sizes of tumors were determined using calipers. Tumor volume was calculated using the formula $V=(A\times B^2)/2$, where V is the volume (mm^3), A is the long diameter (mm) and B is the short diameter (mm) of the tumor.

In the study of survival, the mice were evaluated daily and the experiment was stopped at day 30. Blood was collected from the heart, and blood cell analyses were performed using a blood cell counter. Hemoglobin was evaluated using a Hemoglobin Mouse ELISA kit (Abcan, Cambridge, MA, USA) according to the manufacturer's instructions.

Lung colonization. Mice were injected *i.v.* with a single dose of B16 cells (1×10^5 cells per mouse) suspended in RPMI-1640 medium. Mice were randomly divided into control and glucan-supplemented groups (glucan was used for 15 days). All animals were killed 15 days after the injection. Lungs were fixed in Bouin's solution and scored for the number of metastatic nodules on the lung surface (21).

NK cell depletion. Mice were injected *i.p.* with antibodies to asialoGM1 (200 μg) or control IgG (200 μg) once every week from day 2 before inoculation with B16 cells (22).

NK cell assay. Splenic cells were isolated from the spleen of mice by standard methods. A cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-sec incubation in distilled water, and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6/ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with 50 μl of target cell line YAC-1. After spinning the plates at 250 × *g* for 5 min, the plates were incubated for 4 h at 37˚C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 10 μl of lysis solution was added into appropriate control wells 45 min before the end of incubation. The plates were spun at 250 × *g* for 5 min, followed by transferring 50 μl of the supernatant into flat-bottomed, 96-well microplates. After 50 μl of reconstituted substrate was added into each well, plates were covered and incubated for 30 min at room temperature in the dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC, USA) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity)=100× [(OD_{492}*experimental* − OD_{492} *spontaneous*)/(OD_{492}*maximum*−OD_{492}*spontaneous*)] as described in the manufacturer's instructions, where spontaneous release was that from target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

Results

Firstly, we evaluated the effects of glucan supplementation on blood cells. As shown in Table I, in mice challenged with melanoma, numbers of peripheral blood cells and hemoglobin levels completely changed compared to PBS-treated mice. Glucan supplementation returned these to normal, but only at the two higher doses.

Administration of melanoma cells resulted in strong increase of the weight of individual organs such as liver, lung and spleen (Table II). A low (50 μg) dose of glucan significantly lowered only the weight of liver, a higher dose (100 μg) acted on liver and spleen, and the highest dose (200 μg) returned the organ weight to normal. In addition, we found a dose-dependent lowering of tumor weight (14% by low dose, 36% by medium dose and 51% by high dose).

In the next experiments, we focused on the effects of glucan on lung colonization. Feeding mice with glucan significantly reduced the incidence of lung metastases (by 50% at low dose, 71% at medium dose and 87% at high dose), compared to the number of metastatic nodules found in untreated mice (Figure 1).

Comparable results were observed in experiments evaluating reduction of mortality. At 30 days, when 100% mortality had occurred in the control (PBS) group, 20%, 40% and 50% survival was recorded for glucan doses (50, 100 and 200 μg/mouse, resp.) (Figure 2).

Table I. *Effects of glucan administration on blood cells from mice challenged with B16 melanoma cells.*

Group	WBC (10^3/mm^3)	RBC (10^3/mm^3)	Hb (g/dl)	Platelets (10^4/mm^3)
Control	3.2±0.5	6.0±0.3	9.9±0.6	51.6±9.8
PBS	2.3±0.4	9.8±0.3	15.9±0.5	77.8±12.6
Glucan 50	2.8±0.5	7.4±0.4*	11.9±1.7*	71.2±9.9
Glucan 100	3.2±0.3*	6.8±0.5*	10.8±0.4*	62.2±8.8
Glucan 200	3.4±0.5*	5.9±0.9*	10.2±0.3*	52.3±7.5*

Data represent the mean±SD values. WBC: White blood cells; RBC: erythrocytes; Hb: hemoglobin; Control: untreated control mice; PBS: melanoma-challenged mice treated with Phosphate buffered saline (PBS). *Significant against PBS-treated group at $p<0.05$ level.

Table II. *Effects of glucan administration on weights of some organs and primary tumors of melanoma-treated mice.*

	Weight (g)			
Group	Liver	Lung	Spleen	Tumor
Control	1.11±0.05	0.15±0.01	0.11±0.01	N/A
PBS	1.87±0.08	0.23±0.03	0.39±0.05	0.59±0.12
Glucan 50	1.33±0.04*	0.20±0.03	0.35±0.07	0.51±0.15
Glucan 100	1.21±0.05*	0.18±0.02	0.29±0.08*	0.38±0.10*
Glucan 200	1.15±0.04*	0.17±0.03*	0.15±0.03*	0.29±0.11*

Data represent the mean±SD values. Control: untreated control mice; PBS: melanoma-challenged mice treated with PBS. *Significant against PBS-treated group at $p<0.05$ level.

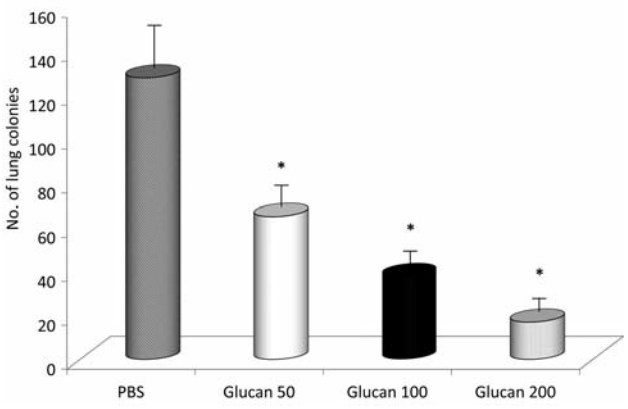

Figure 1. *Effects of glucan supplementation on the number of lung colonies. Results represent the mean from three experiments±SD. *Significant difference from the control at p<0.05 level.*

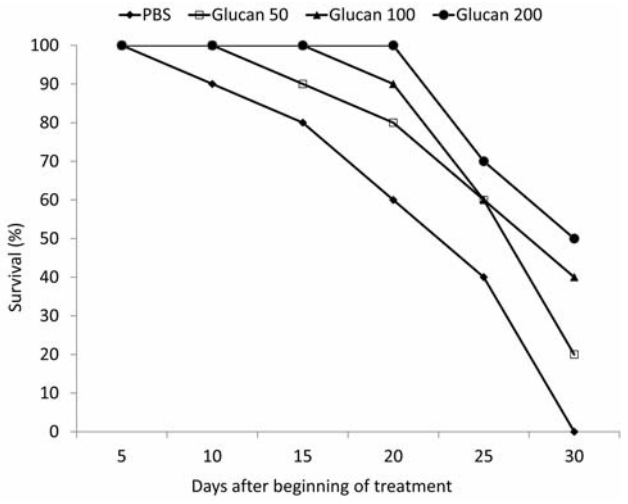

Figure 2. *Effects of glucan supplementation on survival rate of mice with melanoma. Results represent the mean from three experiments.*

To identify the effector cells for anti-tumor activity observed in previous experiments, splenic cells from mice supplemented with different doses of glucan and inoculated with melanoma cells were cultivated with YAC-1 cells. In experiments summarized in Figure 3, it is clearly shown that glucan offers strong and dose-dependent activation of NK cell activity.

To further demonstrate that the anti-melanoma effects of glucan are mediated by NK cells, we depleted the asialoGM1-positive cells by *in vivo* administration of monoclonal antibodies to asialoGM1 once every week from day 2 before inoculation with melanoma cells. We confirmed by Fluorescence-activated cell sorting (FACS) analysis that NK cells were almost completely depleted in the spleen (data not shown). In animals treated with anti-asialoGM1, we observed a fast grow of tumor, but control antibodies of the same isotype did not influence the tumor growth (Figure 4).

In the last part of our study, we focused on the potential cooperation between glucan and chemotherapeutic drugs. All three tested drugs showed dose-dependent inhibition of the growth of melanoma cells (Figure 5). When we combined individual drugs with glucan, the growth inhibition was significantly improved. Glucan alone had no effects (Figure 6).

Discussion

Biological immunomodulators commonly offer systemic effects without clearly defined mechanisms. In this investigation, we focused on the hypothesis that orally-supplemented glucan inhibits melanoma growth *via* NK cell activation. Immune therapies for solid malignancies have often focused on stimulation of the immune system. However,

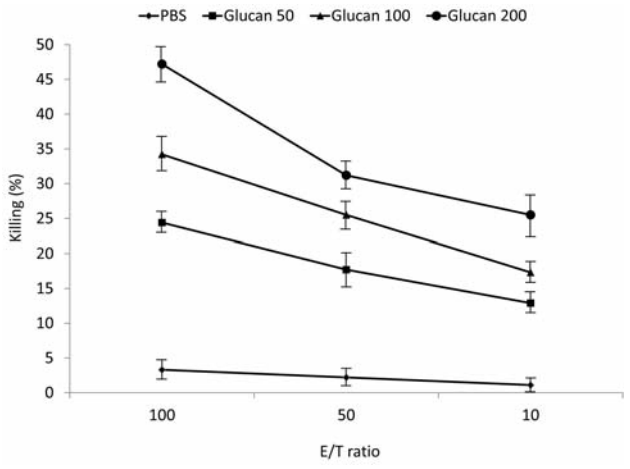

Figure 3. *Effects of glucan supplementation on the activity of natural killer cells (NK) cells at three different effector:target cell ratios. Results represent the mean from three experiments±SD. All observed killing was significantly higher than that of the control at p<0.05 level.*

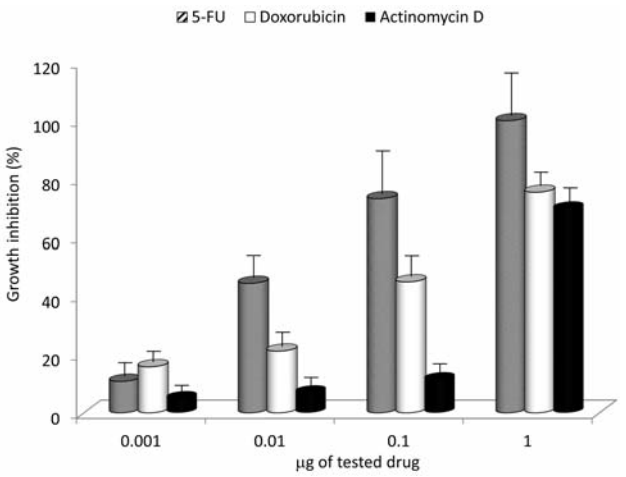

Figure 5. *The action of doxorubicin, 5-fluorouracil (5-FU), and actinomycin D on melanoma cell growth. Results represent the mean of three experiments±SD.*

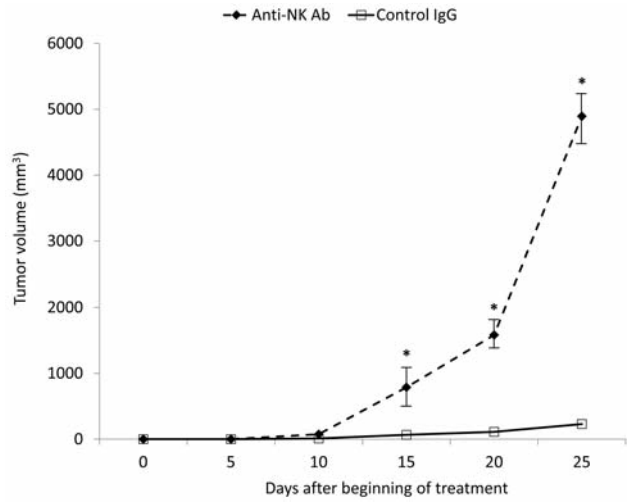

Figure 4. *Effects of depletion of natural killer cells (NK) on tumor volume. Control antibodies were of the same isotype as anti-NK cell antibodies. Results represent the mean from three experiments±SD. All observed killing was significantly higher than that of the control at p<0.05 level.*

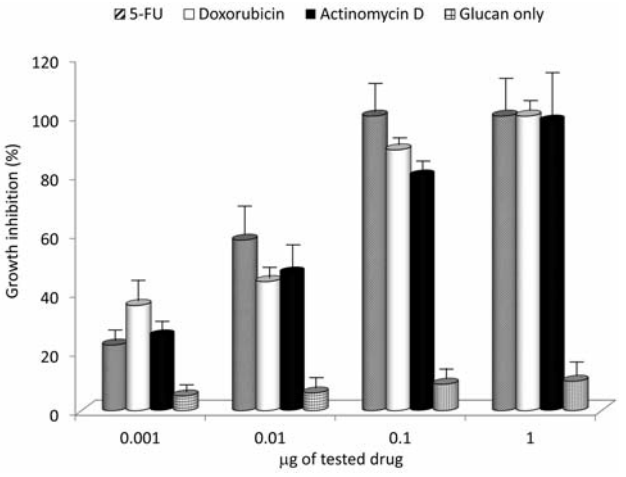

Figure 6. *The effects of treatment of glucan combined with doxorubicin, 5-fluorouracil (5-FU) or actinomycin D on melanoma cell growth. Results represent the mean from three experiments±SD.*

stimulation with either interferons or IL2 was not very successful, mostly due to considerable toxicity (23). Glucan, with a well-established ability to activate immune reactions and almost no demonstrable toxicity (24), seems to be a good candidate for immunity-related treatments.

Since the pioneering investigations, numerous animal studies, as well as human trials, have shown remarkable antitumor activity of glucan against a wide variety of different tumor types, including breast, lung, and gastrointestinal cancer. However, melanoma was studied only rarely.

Older studies demonstrated that glucan administration together with bi-specific antibodies can inhibit pulmonary metastasis of melanoma cells *via* T-cell activation (25,26) and these effects might be improved by converting glucan into the sulfated form (27). Oat-derived glucan exhibited direct cytotoxicity and pro-apoptotic properties (15).

Suggested mechanisms involved reduction of intracellular ATP levels and collapse of mitochondrial membrane potential (15).

Since lipopolysaccharide (LPS) contamination might mask the effects of any biological response modifier, we checked the LPS contamination of our samples using a 10-μg/ml solution of polymyxin B. We prepared LPS-free oligosaccharides and tested them on phagocytosis. The results were identical to those using regular samples (data not shown) therefore, we concluded that LPS is not responsible for data recorded in this study.

Our previous studies have indicated that yeast-derived β-glucans prime neutrophils or NK cells for cytotoxicity against iC3b-opsonized tumors as a result of complement activation by anti-tumor monoclonal antibodies or natural antibodies (7, 28). Oral administration of yeast-derived glucan has shown direct antitumor effects in animal studies (for review see 29) and indirect effects in human trials (20, 30).

The analysis of blood cells in the solid tumor model showed that glucan did not have any hematopoietic toxicity compared to common chemotherapeutic drugs such as 5-fluorouracil (31). On the contrary, the higher glucan doses managed to abrogate the suppression and return levels to the normal state, which corresponds with the known palliative effects of glucan on hematological damage (32). These data confirmed that glucan can alleviate hematopoietic toxicity and we hypothesize that this activity of glucan might be one of the steps in cancer suppression.

Our data show that glucan supplementation significantly reduced the number and weight of primary tumors and the number of lung colonies. Activated macrophages and NK cells mediate natural immunity against tumors, and glucan can induce secretion of important cytokines such as IL1, IL2 and interferon (IFN) which subsequently play a role in of activation T-lymphocytes (33). In addition, activated macrophages, stimulated either directly by glucan or indirectly by cytokines, can kill tumor cells directly or by release of nitric oxide (18). The effect of NK cell depletion which significantly enhanced the frequency of metastasis was in agreement with the previous report (14).

The effects of glucan against melanoma hour been related the inhibition of heparanase activity (34), but the real reason might be the use of sulfated glucan. In our studies, we focused on immunological aspects only. Other studies have found that glucan supplementation resulted in both MHC class I-positive and -negative malignant melanoma (22), again *via* stimulation of NK cell activity. Overall effects and survival rates in our study were comparable to those described by Di Luzio *et al*. (35).

When we compared the effects of glucan on cytostatic activity of the chemotherapeutic drugs doxorubicin and actinomycin D on tumor cells, we found that glucan has the capacity to up-regulate their effects on proliferation of melanoma cells, similarly to the effects previously described in the HEp-2 model (36).

In conclusion, our results showed that oral administration of insoluble β-glucan purified from *Saccharomyces cerevisiae* has strong, dose-dependent effects in reducing melanoma growth. Detailed studies suggested that glucan acts *via* activation of NK cells. In addition, glucan can up-regulate the direct effects of chemotherapeutic drugs with simultaneous protection of hematopoiesis. Our study provides preliminary insights into perspectives of developing β-glucan as an adjuvant in cancer treatment.

Conflicts of Interests

No conflicts of interest exist for the authors.

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