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RESEARCH ARTICLE

β(1-3)-D-glucan affects adipogenesis, wound healing and inflammation

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Abstract Numerous types of β(1-3)-D-glucans have been isolated from almost every species of yeast, grain, and fungi. These products have been extensively studied for their immunological and pharmacological effects. In this paper we evaluated the possibility whether individual β(1-3)-D-glucans will have an activity in less studied areas such as adipogenesis and inflammation. Our results showed that of the tested β(1-3)-D-glucans, yeast-derived insoluble Glucan #300, strongly inhibited adipogenic differentiation, supported wound healing and significantly lowered skin irritation. The remaining β(1-3)-D-glucans were significantly less active. Taken together, our study showed that with respect to natural β(1-3)-D-glucans, there is a clear yes-or-no effect suggesting that highly purified and highly active β(1-3)-D-glucans will have pleiotropic biological impact, whereas poorly isolated and/or less active β(1-3)-D-glucans will have only mediocre properties.

Keywords β(1-3)-D-glucan · Adipocytes · Inflammation · Wound healing · Immunity

Introduction

Natural products, useful in preventing and/or treating various diseases, have been sought after throughout civilization. Documented history of polysaccharides as immunomodulators goes back to the 40s of the last century when Shear and Turner (1943) described a polysaccharide substance, again from *Serratia marcescens* cultures, that caused necrosis of tumors. About the same time, Pillemer and Ecker (1941) isolated an insoluble fraction from fresh baker's yeast (*Saccharomyces cerevisiae*) which can specifically inactivate the third component of complement; this preparation was named zymin, Ecker's fraction, and lately zymosan. Pillemer and his coworkers (1955) demonstrated that the essential activity of zymosan consists in a β(1-3)-D-glucans-rich fraction. Further research on biological properties of β(1-3)-D-glucans was pioneered by DiLuzio et al. (1970). Subsequent studies demonstrated that β(1-3)-D-glucan administration caused significant phagocytic stimulation of the reticuloendothelial system, enhanced host defense mechanisms, and resistance to experimental tumors (for review see Novak and Vetvicka 2008, 2009).

Despite long-term interest and research, the mechanisms of how β(1-3)-D-glucan affects our health remained a mystery for a long time. Only in the last decade, extensive research by numerous scientific groups has helped to reveal the extraordinary effects that β(1-3)-D-glucan has on the immune system. Experiments first looked for the receptors of β(1-3)-D-glucan and soon focused on two particular receptors—one called complement receptor type 3 (CR3 receptor) (Vetvicka et al. 1996) and the other Dectin-1 (Willment et al. 2001) as promising targets of β(1-3)-D-glucan. These experiments helped to establish the mechanisms of β(1-3)-D-glucan action. Traditionally, β(1-3)-D-glucan is considered to be a stimulator of the cellular immunity. Binding of β(1-3)-D-glucan to a specific receptor activates macrophages. The activation consists of several interconnected processes including increased chemokinesis, chemotaxis, migration of macrophages to particles to be internalized, degranulation that leads to increased expression of adhesive molecules on the macrophage surface, adhesion

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to the endothelium, and migration of macrophages to tissues. In addition, β(1-3)-D-glucan binding also triggers intracellular processes, characterized by the respiratory burst after phagocytosis of invading bacteria thus increasing the content and activity of hydrolytic and metabolic enzymes, as well as the signaling processes leading to activation of other phagocytes plus the secretion of cytokines and other substances initiating inflammation reactions. For an excellent review regarding interaction of β(1-3)-D-glucans with macrophages see (Schepetkin and Quinn 2006).

In addition to the stimulation of macrophages, β(1–3)-D-glucan has been found to significantly stimulate anti-infection immunity (for review see Vetvicka and Novak 2011) and potentiate anti-cancer immunity (Nakao et al. 1983) and holds wound-healing healing activities (Wei et al. 2002). Added to the effects of β-glucan oriented toward the immune system, β(1-3)-D-glucans were also shown to reduce the total and LDL cholesterol levels of hypercholesterolemic animals and patients (Tietyen et al. 1990). Our later study is not only made a direct comparison of the cholesterol-lowering activity of two different yeast-derived β(1-3)-D-glucans, but also is the first to compare normal animals and mice with experimentally-induced cholesterolemia (Vetvicka and Vetvickova 2009). In addition, we showed that the type of branching is probably not responsible for these effects.

In addition to the effects mentioned above, β(1-3)-D-glucan has been shown to have other numerous biological effects. An interesting study used β(1-3)-D-glucan for the treatment of patients with allergic rhinitis. The results of the study showed that levels of IL-4 and IL-5, which are responsible for the allergic inflammatory response, were decreased with β(1-3)-D-glucan treatment, while the levels of IL-12 were increased. Moreover, the eosinophils, which are important effector cells of the inflammatory response, were decreased. In summary, β(1-3)-D-glucan might have a role as an adjunct to the standard treatment of patients with allergic rhinitis (Kirmaz et al. 2005). β(1-3)-D-Glucan was recently found to have an additional use—the regulation of stress. We measured the effects of various types of β(1-3)-D-glucan on the levels of stress-induced corticosterone. As experimentally induced stress, we used either restraint or cold. Our results (Vetvicka and Vancikova 2010) showed that β(1-3)-D-glucans successfully helped to keep the stress hormone corticosterone at almost normal levels. Based on the pleiotropics effects of β(1-3)-D-glucan, we further evaluated some less-studied effects of β(1-3)-D-glucan. To be certain that the observed effects are not caused by a single batch of β(1-3)-D-glucan, we compared four different types of β(1-3)-D-glucan.

Material and methods

Animals

Female, 8 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

Materials

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES buffer, glutamine, antibiotics, anthralin, human transferrin, insulin, dexamethasone, 3-isobutyl-1-methylxantine, Limulus lysate test E-TOXATE, Nile red and Oil red O were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

β(1-3)-D-glucans

Yeast-derived insoluble β(1-3)-D-glucan #300 were purchased from Transfer Point (Columbia, SC), yeast-derived Now BETA β(1-3)-D-glucan from Now Foods (Bloomingdale, IL), grain-derived Glucagel T from GraceLinc (Christchurch, New Zealand), mushroom-derived Krestin from (Kureha Chemical Industries, Tokyo, Japan), and Epicor from Embria Health Sciences, Ankeny, IA. Using the *Limulus* lysate test, we determined the LPS contamination to be below 0.005 U/ml.

Cell line

Human immortalized nontumorigenic keratinocyte cell line HaCaT was maintained in RPMI-1640 medium containing HEPES buffer supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, in plastic disposable tissue culture flasks at 37°C in a 5% CO_2/ 95% air incubator. 3T3-L1 preadipocytes were obtained from the ATCC (Manassas, VA) and cultivated the same way as HaCaT cells.

Mechanical wounding of confluent HaCat cells-Scratch wound assay

HaCaT cells were grown to confluent monolayer on 5 cm Petri dish. The *in vitro* wound assay was performed after washing the cells with phosphate buffered saline (PBS). Three scratches per dish were performed in a rectangular pattern with the tip of 5 ml pipette. Cells were then washed three times with PBS to remove cellular debris before incubation with serum-free medium for 24 h at 37°C (Buth

et al. 2004). β(1-3)-D-Glucan was used at concentration range 0.1–10 μg/ml. As a control, non-scratched cells were treated identically as described for the scratched cells. Multiple photographs of the wound were obtained using the TE-FM Epi-Fluorescence system attached to a Nikon Inverted microscope eclipse TE300 and the percentage of cellular recover areas were analysed using the MetaMorp 6.2 software (Universal Imaging).

Ear swelling assay

The ear swelling assay was performed according to Lange et al. (1998). Briefly, freshly prepared concentrations of anthralin were suspended in 70% ethanol/olive oil (4:1) and irritative dermatitis was elicited by applying 10 μl of anthralin suspension to the dorsal surface of both ear pinna. The ears of separate animals treated with ethanol/olive oil vehicle served as controls. A 20 μl of β(1-3)-D-glucan solution (1 μg/ml) has been applied to dorsal surface of both ears of experimental animals at various intervals. In some groups, experimental group consisted of animals fed with β(1-3)-D-glucan. At the end of experiment, ear thickness measurements were made using an Oditest precision caliper, model D1000 (Dyer Co., Lancaster, PA) at various time intervals.

Cell culture and induction of adipocyte differentiation

3T3-Li preadipocytes were maintained and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. These cells were differentiated into adipocytes as described (Poulain-Godefroy et al. 2010). Briefly, 2-day postconfluent 3T3-Li preadipocytes (designated day 0) were fed RPMI medium containing 10% FCS, 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxantine for 2 days. Cells were then fed medium supplemented with FCS and 5 μg/ml insulin until day 10. Mature adipocytes were determined by the accumulation of cytoplasmic lipid droplets using light microscopy using a scoring method for evaluating leukocyte alkaline prosphatase activity in histology with 6 droplets threshold (Hirota et al. 2010).

In vitro cell proliferation assay

For growth experiments, cells were harvested by centrifugation and washed three times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics and 10 μg/ml of human transferrin. Cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/ml (150 μl/well) in the absence or presence of different concentrations of β(1-3)-D-glucans in triplicate wells. After 3 or 5 days in culture, the proliferation was evaluated using Biotrak cell proliferation ELISA system measuring incorporation of BrdU in actively dividing cells (Amersham Pharmacia Biotech, Piscataway, NJ). Cells were incubated in BrdU-containing medium at 37°C for 2 h, then fixed and incubated with blocking solution for 30 min. Peroxidase-labelled anti-BrdU was added and incubated for 90 min. Wells were washed 3X and chromogenic peroxidase substrate was added to each well and incubated for 30 min. The optical density was measured at 450 nm using an SLT ELISA reader (Tecan, Research Triangle Park, NC).

Results

Four different β(1-3)-D-glucans were used in this study: Glucagel T barley β(1-3)-D-glucan is a mixed link (1→3, 1→4)-ß-D-glucose polymer, in which cellotriosyl and cellotetraosyl residues occur in a ratio of ~3:1. The natural purification process yields a reduced molecular weight β(1-3)-D-glucan (typically ~130 kDa) that is more readily hydrated than other conventionally purified β(1-3)-D-glucans. The typical carbohydrate content is 85–90%. β(1-3)-D-Glucan #300 is a proprietary (1→3, 1→6)-ß-D-glucan purified from *Saccharomyces cerevisiae* by Biothera for Transfer Point and even when corresponding to the β(1-3)-D-glucan sold under WGP name(s), the Glucan #300 has much higher purity (app. over 96%). Epicor is also isolated from yeasts, however the information about its purity is not available. Krestin is mushroom-derived β(1-3)-D-glucan, also known under the name PSK. NOW β(1-3)-D-glucan is a mixture of both insoluble β(1-3)-D-glucans from yeast and soluble β(1-3)-D-glucans from mushrooms. All these β(1-3)-D-glucans have been extensively studied in past (Vetvicka and Vetvickova 2008, 2009; Vetvicka and Vancikova 2010).

β(1-3)-D-Glucan has been implicated in accelerated wound healing, so we decided to test our β(1-3)-D-glucans on keratinocyte scratch test. Preliminary data showed no changes in proliferation of HaCaT cells by any of the tested β(1-3)-D-glucan (data not shown). The confluently grown HaCaT monolayers were scratch-wounded and further incubated in the presence of individual β(1-3)-D-glucans in serum-free medium to test the ability of the cells to regenerate a monolayer. Twenty-four hours post wounding, the treatment of β(1-3)-D-glucan increased the percentage of cellular recoverage area compared to control, with the highest activity achieved with β(1-3)-D-glucan #300 followed by NOW β(1-3)-D-glucan and PSK (Fig. 1).

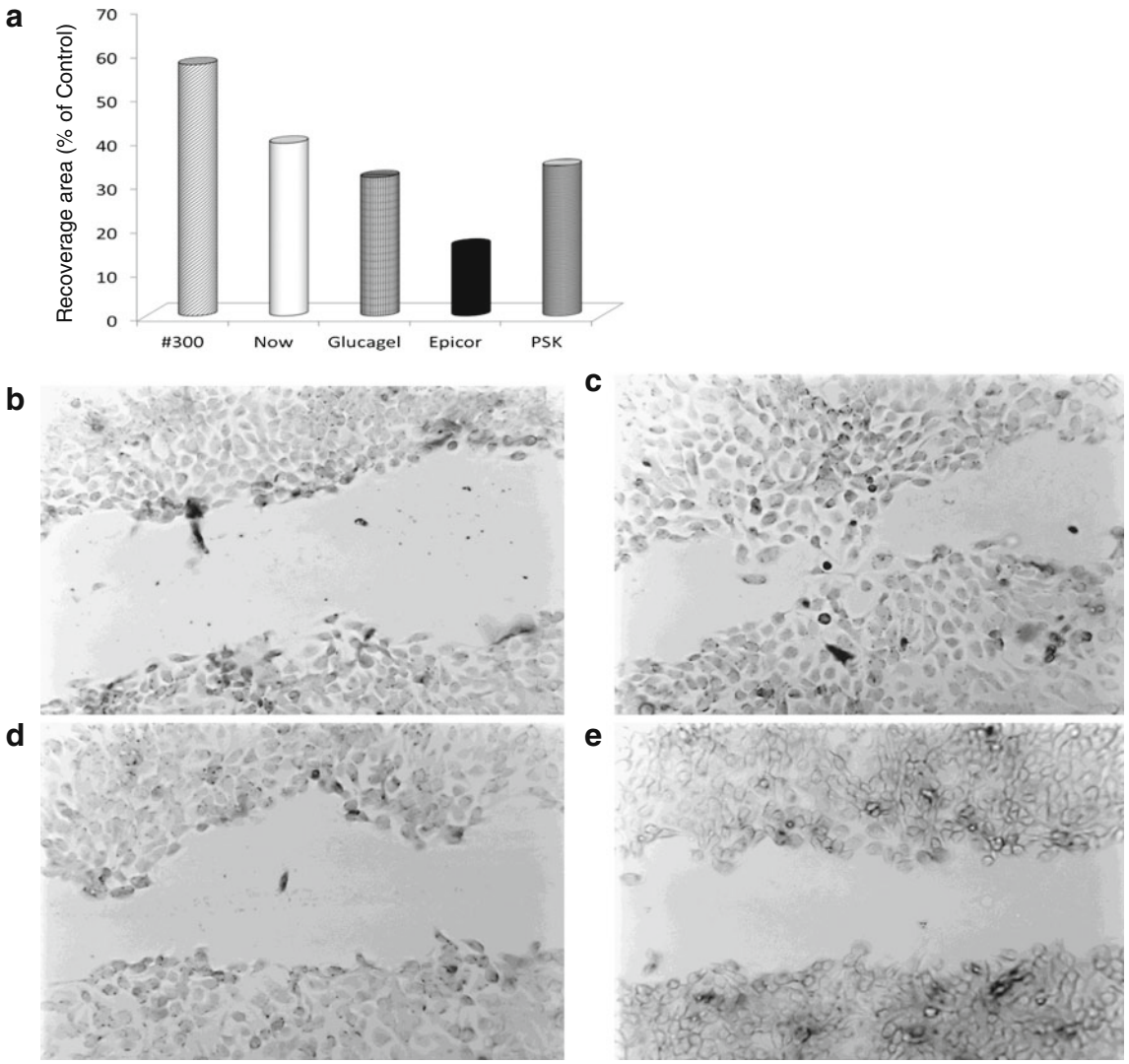

Fig. 1 Effect of β(1-3)-D-glucan on regeneration from scratch wounding of HaCaT cells. Confluent HaCaT cells were scratch-wounded, washed and allowed to regenerate in serum-free medium only (control) or in presence of 1 μg/ml of β(1-3)-D-glucan for 24 h and photographed (**a**). *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level. Multiple photographs of the wound were obtained and the percentage of cellular recovery areas was determined using image analysis software. The percentage of cellular recovery area to that of control was measured. Combined result of three independent experiments is shown: B–control, C–Glucan #300, D–Epicor, E–Glucagel T

To investigate the influence of β(1-3)-D-glucan on preadipocyte proliferation, we grew human 3T3-L1 preadipocytes in serum containing basal medium supplemented with increasing doses of β(1-3)-D-glucan. When tested either on day 3 or day 5, no differences in proliferation of preadipocytes were found (data not shown). However, a different situation was found when we measured the effects of β(1-3)-D-glucans on adipogenic differentiation (Fig. 2) assessed by morphologic counting of lipid-laden cells. Cells were treated with increasing doses of β(1-3)-D-glucan for the first 4 days of adipogenic differentiation. In cultures treated with medium only (similarly to cultures treated with 0.1 μg/ml of β(1-3)-D-glucan), app. 90–95% of cells underwent adipogenic differentiation as assessed by staining intracellular lipids with oil red O on day 14 of adipogenic differentiation. Whereas all β(1-3)-D-glucans showed dose-dependent inhibition of adipogenic differentiation, the strongest (up to 60% inhibition) was observed with Glucan #300, followed by Glucagel T (30 to 45% inhibition). These findings were further conformed by measuring the incorporation of the lipophilic dye Nile red into cellular lipids (Fig. 3).

To evaluate the potential effects of β(1-3)-D-glucan we used an established anthralin-induced skin inflammation test. First, we injected tested β(1-3)-D-glucan ip. 30 min before irritation. Results summarized in Fig. 4 show that only Glucan #300 consistently inhibited the skin irritation for up to 24 h. From the rest of tested samples, Glucagel's

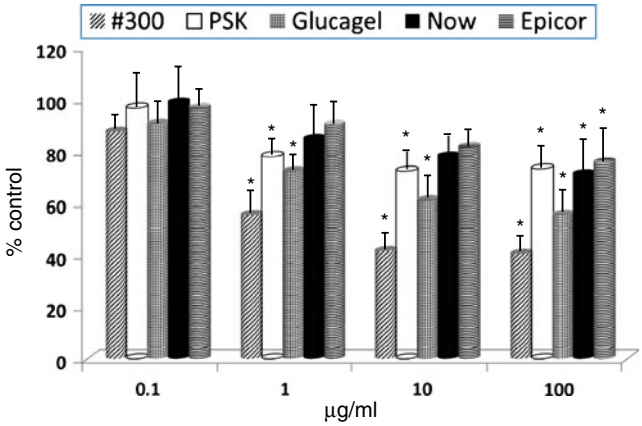

Fig. 2 Effects of β(1-3)-D-glucan on preadipocyte differentiation. Rate of differentiation was determined as described in Material and Methods. Data are expressed as mean values ± SD of three independent experiments performed in triplicates. *Represents significant differences between control (PBS) and glucan samples at $P \leq 0.05$ level

activity lasted 12 h, whereas NOW β(1-3)-D-glucan showed inhibition only at the 12 h interval. Epicor and PSK has only limited activity. Similar situation was achieved when β(1-3)-D-glucans were administered orally for 2 weeks. Again, Glucan #300 demonstrated long-lasting inhibition, with NOW β(1-3)-D-glucan and Glucagel showing limited activities (Fig. 5).

Discussion

β(1-3)-D-Glucan's role as an immunomodulator has been well documented, for over 50 years. Numerous studies (currently more than 6,000 publications) have subsequently shown that β(1-3)-D-glucans, either particulate or soluble,

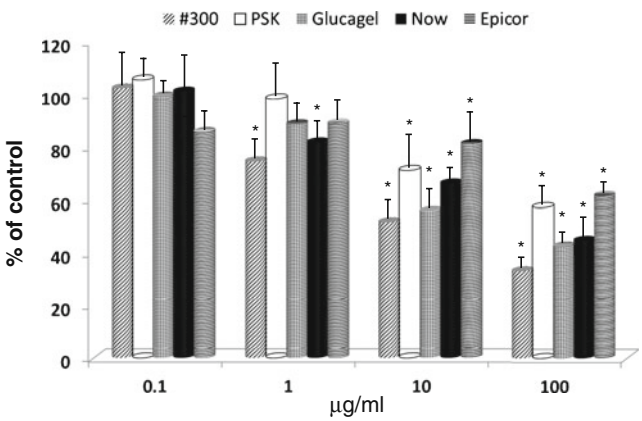

Fig. 3 Effects of β(1-3)-D-glucan on accumulation of triglycerides. Lipid content was determined on day 10 with Nile Red. Data are expressed as mean values ± SD of three independent experiments performed in triplicates. *Represents significant differences between control (PBS) and glucan samples at $P \leq 0.05$ level

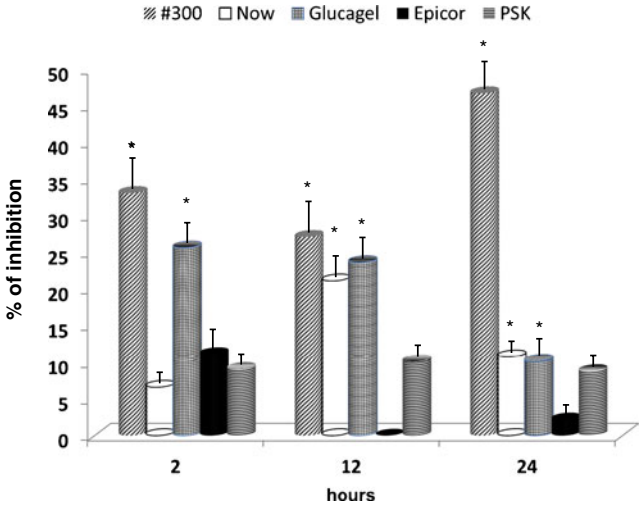

Fig. 4 Effects of β(1-3)-D-glucan on anthralin-induced ear swelling in BALB/c mice. β-Glucans were administered 30 min before irritation and the thickness was measured at 2, 12, and 24 h. Results are expressed as % of control (anthralin) level. Mean values ± SD of three independent experiments performed in triplicates. *Represents significant differences between control (PBS) and glucan samples at $P \leq 0.05$ level

exhibit immunostimulating properties, including antibacterial and anti-tumor activities (for review see Novak and Vetvicka 2009). There are various natural sources of β(1-3)-D-glucans; however, they are most often prepared from fungal cell walls. Additional sources of β(1-3)-D-glucan involve mushrooms, grain and seaweed. Despite the fact that β(1-3)-D-glucan is the most studied natural immunomodulator, due to the lack of comprehensive reviews

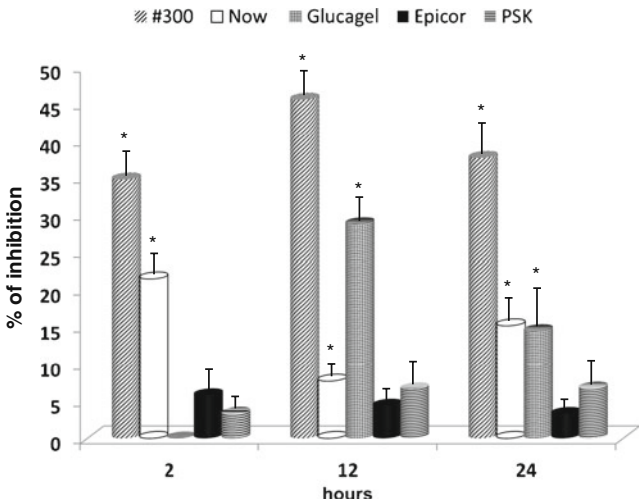

Fig. 5 Effects of β(1-3)-D-glucan on anthralin-induced ear swelling in BALB/c mice. β-Glucans were administered by daily oral dose for 14 days before irritation and the thickness was measured at 2, 12, and 24 h. Results are expressed as % of control (anthralin) level. Mean values ± SD of three independent experiments performed in triplicates. *Represents significant differences between control (PBS) and glucan samples at $P \leq 0.05$ level

comparing the biological effects of β(1-3)-D-glucan isolated from various sources, no final conclusions about the optimal source or biochemical characteristics have been reached.

Despite the extensive amount of scientific reports about β(1-3)-D-glucans and their biological activities, most of the studies are focused either on the description of newly isolated β(1-3)-D-glucans or on the description of their classical biological activities such as effects on phagocytosis, cytokine production or infection immunity. The present study is a follow-up of the previous comparison of the most common effects of a large group of β(1-3)-D-glucans (Vetvicka and Vetvickova 2010). We used the most effective β(1-3)-D-glucans and focused on their less studied biological activities.

To allow investigation of the effect of β(1-3)-D-glucan on the wound healing process, simple scratch-wound model was used. The effect of exogenously added β(1-3)-D-glucans has been tested on the regeneration of monolayer. We have seen the remarkable influence of the presence of three types of β(1-3)-D-glucan on the regeneration of monolayer. Since the β(1-3)-D-glucans do not influence the proliferation of keratinocytes, we hypothesize that β(1-3)-D-glucan enhances the regeneration process of wound healing both by paracrine stimulation of the cytokines. Whereas wound healing effects of β(1-3)-D-glucan are usually considered to be result of enhanced macrophage functions (Browder et al. 1988), our experimental design using a single cell monolayer suggests either the stimulation of collagen synthesis (Wei et al. 2002) or increased production of cytokines (Vashishta et al. 2007).

Recently, β(1-3)-D-glucan has been found to be involved in obesity, most probably via the increases in anorexigenic hormone Y-Y level (Beck et al. 2009). In addition, numerous papers suggested the obesity links of various modulators in adipocyte differentiation (Rayalam et al. 2009, Kong et al. 2010). We therefore hypothesized that β(1-3)-D-glucan might affect calorie restriction by a reduction of body fat and investigated the biological effects on fat cell biology. Adipose tissue size is determined by both the volume and the number of adipocytes (Fischer-Posovszky et al. 2007). The number of adipocytes can increase due to the proliferation of preadipocyte and subsequent adipogenic differentiation. Our data showed that whereas β(1-3)-D-glucan has no effect on viability or proliferation of mature adipocytes, it can significantly inhibit adipogenic differentiation.

Chemically-induced acute inflammatory mouse ear model has generally been used as one of the classic methods for detecting the efficacy of anti-inflammatory agents. Although β(1-3)-D-glucan is a well-established stimulator of immune reactions (Novak and Vetvicka 2009), which might be considered to worsen or even induce inflammation, β(1-3)-D-glucan also activate macrophages that can remove cellular debris resulting from oxidative damage, thus speeding the recovery of damaged tissue (Di Renzo et al. 1991). Immunomodulators are also known to reduce pre-existing inflammation (Ramprasath et al. 2006). In addition, β(1-3)-D-glucan has been used in therapy of rheumatoid arthritis (Bauerova et al. 2009). Our data showed that β(1-3)-D-glucan can significantly reduce the acute inflammation both when used locally or prophylactically by long-term oral administration. Similar data were independently shown after one oral dose of β(1-3)-D-glucan (Kim et al. 2007).

Data presented in this study clearly demonstrated significant differences in activities among individual types of β(1-3)-D-glucans. In addition, it is clear that individual β(1-3)-D-glucans can be highly active in one particular part of immune reaction and mediocre in other parts of immune reaction. The only β(1-3)-D-glucan consistently showing the highest activity in all tested reactions was Glucan #300, which corresponded with the previous studies (Vetvicka and Vetvickova 2008, 2009, 2010). In addition, we showed that besides the well-established effects of β(1-3)-D-glucans on immune reactions, this natural immunomodulator has multiple pleiotropic functions such as inhibition of adipogenic differentiation and inhibition of acute inflammation. Studies seeking detailed information on the mechanisms of these actions are currently in progress.

Conflict of interest There is no conflict of interest.

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