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Effects of β-glucans from *Coriolus versicolor* on Macrophage Functions in Young and Aged mice

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Abstract - The immune system may play an important role in aging and the changes in the immune status are associated with treatment of various immunomodulators. This study examined the effects of β-glucans isolated from mushroom fungi, *Coriolus versicolor* on macrophages functions in young (8-weeks-old) and aged (82-weeks-old) male C57BL/6 mice. When peritoneal macrophages were treated with various concentrations of β-glucan (1-100 μg/ml) for 24 hrs, tumoricidal activity, NO production and phagocytic activity were significantly increased in the young mice, whereas there are no effects in the aged mice. These results suggest that β-glucans has differential effects on the macrophage functions in young and aged mice and age nutrition might need to be considered to select proper immunomodulator. In addition, β-glucan could be used clinically for the treatment of diseases such as cancer therapy in the young.

Key words - Beta-glucan, *Corious versicolor*, Aging, Macrophages

Introduction

Macrophages have been known to an important component of host defense mechanism against bacterial infections and cancer (Hahn and Kaufmann, 1981; Verstovsek *et al*., 1992). Large pools of macrophages, thought to be of mononuclear phagocyte origin, are located throughout the body and historically have been identified by different names including peritoneum (peritoneal macrophages), brain (microglia), bone (osteoclast) and liver (kupffer cells). For killing tumor cells, macrophages can be activated *in vitro* by a nonspecific, extracellular mechanism that might be important in host defense against neoplastic cells *in vivo* (Klimp *et al*., 2002). Peritoneal macrophages can be stimulated by a variety of agents, such as IFN-γ, lipopolysaccharides (LPS), and other microbial products (Gautam and Deodhar, 1989; Paulnock and Lambert, 1988). Some of these molecules have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and nitric oxide (NO), and to induce tumoricidal activity in macrophages. Activated macrophages also increase their phagocytic activity, release various molecules such as cytokines and reactive oxygen intermediates, and then carry out nonspecific immune responses (Choriki *et al*., 1989; Keller *et al*., 1990).

β-Glucans are naturally occurring (1→3)-β-D-linked polymer glucose, which are found in the cell wall of certain pathogenic bacteria and fungi (Muller *et al*., 1996; Williams *et al*., 1996). β-Glucans are widely used as dietary supplements and anticancer therapy, with well-established stimulating effects on the immune defense system. Many studies have demonstrated that β-glucans, either in the form of particulate or soluble, have stimulating effects on the innate immune cells (macrophages, neutrophils (PMN) and natural killer (NK) cells), on the antibacterial and anti-tumor activities, and on the production of cytokines (Di Luzio *et al*., 1979; Imura *et al*., 1985). β-Glucans can be isolated from almost every species of yeast. In addition, β-glucans can be isolated from bacteria, mushrooms, algae, or cereal grains. The structure of the β-glucan depends on both source and type of isolation. Different physicochemical parameters, such as solubility, primary structure, molecular weight, and branching play a role in the biological activities of β-glucans (Yadomae, 2000).

Aging is a natural phenomenon, accompanied by physical

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and memory dysfunction, and is also believed to be concomitant with immune dysfunction (Takeoka *et al*., 1996; Boehmer *et al*., 2004). Even though there have been several reports on the age-related phenotype changes in immune cell, the effects of immunomodulators on aged cells have not been studied well. Therefore, it would be interesting to examine the function of macrophages in young and aged mice. In this study, we determined whether the effect of β-glucans isolated from mushroom fungi, *Coriolus versicolor* on macrophage functions in aged animals was different from that of young ones.

Materials and Methods

Preparation of β-glucans from mushrooms

Mushroom β-glucans isolated from *Coriolus versicolor* were used. The process of isolating and purifying a water-soluble glucan from *C. versicolor* was achieved by hot water extraction, filtration, solvent precipitation, dialysis, and freeze-drying. Acidic fractions of the polysaccharide were separated from crude polysaccharides by DEAE-cellulose anion exchange chromatography at 0.7 M NaCl. The molecular weight of the proteo-heteroglycan after Sepharose CL-4B gel filtration chromatography was about 750 kDa. This product has been shown to contain a 85% purity level.

Aged mice housing and feeding

Male C57BL/6 mice (7 weeks) were obtained from the Charles River Breeding Laboratories (Japan). The animals were randomly distributed into five per group. The aged (82 weeks) and young (8 weeks) C57BL/6 male mice were used in this study. The mice were provided with water and food, ad libitum and quarantined under 12 h light : 12 h dark photoperiod in the animal care facility of the Sungkyunkwan University, Suwon, Korea. Animal care was performed following the Institute for Laboratory Animal Research (ILAR) guideline.

Chemicals

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). The XTT {2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt} cell viability assay kit was purchased from WelGENE (Daegu, South Korea). All the tissue culture reagents and β-glucans were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate, Sigma), and the endotoxin levels were found to be < 10 pg/ml.

Macrophage-mediated tumoricidal activity

The assay for macrophage-mediated tumoricidal activity was determined using A modification of the method reported by Mosmann (1983). Briefly, macrophages (1×10^5 cells/well) from mice first incubated in either medium alone or in medium supplemented with various doses of two reagents for 24 h in 96-well plates. Macrophages were washed with RPMI-FBS to remove two reagents and then co-incubated with B16 melanoma cells (1×10^4 cells/well; effector : target cell ratio of 10:1). After 24 h, plates were stained with crystal violet containing 10% formaldehyde for 15 min. Absorbance of each well at 540 nm was determined by using Molecular Devices microplate reader (Menlo Park, CA, USA). Cytotoxic activity is expressed as the percentage of tumor cytotoxicity by the following formula : [1-{OD of (target cells + macrophages) - OD of macrophages}/OD of target cells] × 100.

OD of target cells is the optical density of B16 melanoma cells and OD of macrophages is the optical density of macrophages.

Nitrite determination

The cells were treated with various doses of β-glucans for 24 h and the accumulation of nitrite in culture supernatants was measured using the assay system described by Ding *et al* (1988). 100 μl aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

NBT assay for phagocytosis

Phagocytosis was measured by nitro blue tetrazolium

(NBT) reduction assay (Okimura *et al*., 1986). Peritoneal macrophages were seeded in 96-well plates at a density 5×10^4 cells per well, treated with various concentration of β-glucans and cultured for 24 h. The cultured media was then removed and 50 μl of 5×10^6 particles/ml zymosan and 0.6 mg/ml NBT was added into each well. After an additional incubation for 1 h, wells were washed with cold D-PBS two times and the optical density of reduction product of NBT, a purple insoluble formazan, was determined at 540 nm using a microplate reader. It was not required to solubilize the formazan before taking the measurement of absorbance.

Statistical analysis

Each experiment was repeated at least two times, and the results of one representative experiment are shown. The results were expressed as means ± S.E.M. and analyzed via ANOVA. The significant values are represented by an asterisk (*$p < 0.05$ and **$p < 0.01$).

Results and Discussion

The immune system may play an important role in aging and the changes in the immune status are associated with treatment of various immunomodulators (Speziali *et al*., 2009). This study examined the immunomodulatory effects of β-glucans on the functions of peritoneal macrophages in young and aged mice.

To examine whether β-glucan treatments stimulate the tumoricidal activities in macrophages against target tumor cells, macrophages from the young and aged mice were co-cultured with B16 cells for 24 h. B16 tumor cells were used as target since they are known to be either TNF-α or NO sensitive. As shown in Fig. 1, β-glucans significantly increased tumoricidal activity of macrophages, peaking at 10 μg/ml in the young mice, whereas there is no effect on tumoricidal activity of macrophages in aged mice.

Activated macrophages have been known to produce various cytokines such as TNF-α, IL-1, IL-6 and NO that are involved in tumoricidal activity. Since NO is related to cytostatic or cytotoxic function of macrophages against a variety of tumors (Keller *et al*., 1990; Hibbs *et al*., 1987), we examined the effects of β-glucans on NO production by macrophages in the young and aged mice, respectively. As shown in Fig. 2, β-glucans increased NO production by the peritoneal macrophages in young mice, peaking at 10 μg/ml,

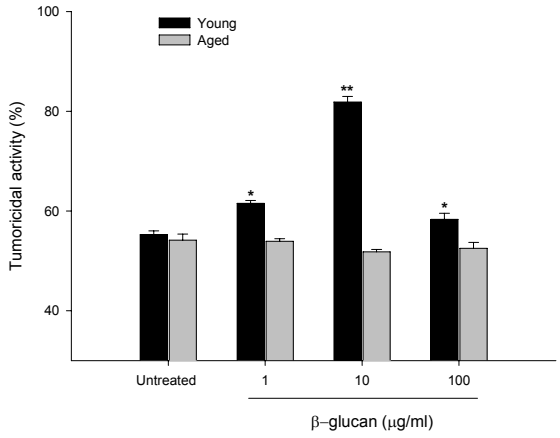

Fig. 1. Effects of β-glucans on macrophage-mediated tumoricidal activity in the young and aged. The peritoneal macrophages (1×10^5 cells/well) from the young and old mice were treated with β-glucans and co-cultured B16 target tumor cells (1×10^4 cells/well) for 24 h. Macrophage-mediated tumoricidal activity was determined by XTT assay. The data represents the means ± S.E.M. of quadruplicate experiments. *$p < 0.05$, **$p < 0.01$; significantly different from the control (no treatment).

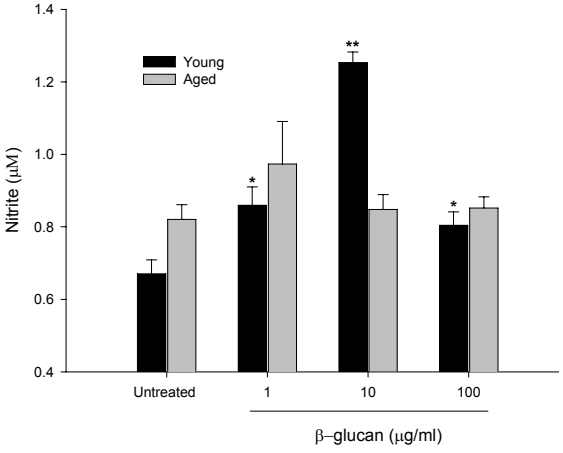

Fig. 2. The effects of β-glucans on production of nitrite in peritoneal macrophages from the young and aged mice. Macrophages (1×10^4 cells/well) were treated with various concentration of in β-glucans for 24 h. Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. The data represents the mean ± S.E.M. of quadruplicate experiments. *$p < 0.05$, **$p < 0.01$; significantly different from the control (no treatment).

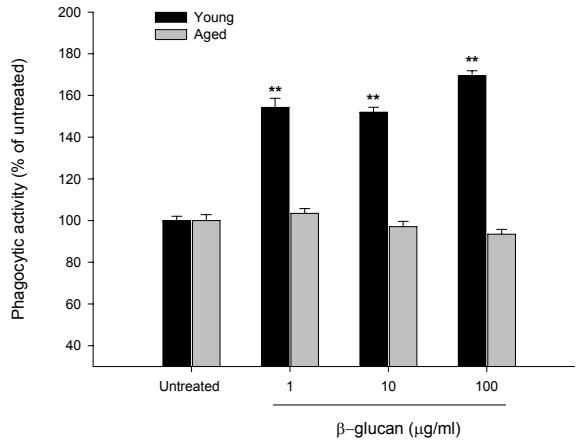

Fig. 3. Effects of β-glucans on phagocytosis in peritoneal macrophages from the young and aged mice. Macrophages (1×10^4 cells/well) were treated with various concentration of in β-glucans for 24 h, and then incubated with media containing zymosan (1×10^6 particles/ml) and NBT (0.6 mg/ml) for 1 h. Formazan formation was measured at 540 nm. The data represents the mean ± S.E.M. of quadruplicate experiments. **$p < 0.01$; significantly different from the control (no treatment).

same as the results of tumoricidal activity, while it had no effects in the aged mice macrophages. The differential effects of macrophages from young and old mice might be due to no expression of iNOS in old mice (Khare *et al*., 1997).

Interestingly, β-glucans showed better effect at 10 μg/ml than 100 μg/ml. It might imply that the proper concentration range of β-glucans needs to trigger related receptors. The tumoricidal activity induced by β-glucans in young mice appeared to be mediated by the production of NO because excessive formation of which from macrophages mediates the bacterial and tumoricidal actions has been well known.

Phagocytosis is the primary activity of macrophages, which is responsible for a diverse range of antimicrobial- and cytotoxic- activities, including respiratory burst, secretion of inflammatory mediators and antigen presentation. In this study, we examined the effects of β-glucans on the phagocytosis of macrophages in young and aged mice. As shown in Fig. 3, β-glucans significantly increased the phagocytic activity of peritoneal macrophage in the young mice, whereas there is no effects of phagocytic activity in the aged mice. Our data demonstrated that β-glucans from *C. versicolor* have immunomodulatory effects on macrophage functions, especially to tumoricidal and phagocytic activities in young mice.

Age-related involution brings about many alterations in immune system. Despite the fact that thymic atrophy and diminished output of T lymphocytes are the most recognized changes, it is increasingly evident that the effects of aging on altering immune system are widespread, extending from hematopoietic stem cells and earlier lymphoid progenitors to mature lymphocytes in secondary lymphoid organs and immune cells (Taub and Longo, 2005; Haynes *et al*., 2003; Miller and Allman, 2003; Benner *et al*., 1981; Miller, 1996). Our results also showed that β-glucans had several different effects on functions of peritoneal macrophages due to age-related alterations in immune system. This suggests that β-glucans might induce the production of different type of biological molecules from macrophages in young and aged mice.

Many researchers have believed that nutritional effects of natural products are similar in young and old age. It has been regarded as if one has good immunological effects in young, it might be good for the old as well. Recently, the incidence of cancer in young age had been increased. According to our data, β-glucans has differential immunomodulating effects on peritoneal macrophages in young and aged mice, β-glucans is more useful for young people to treat cancer therapy as a immunomodulators and furthermore, nutritional intake for elder people might need to be considered when to select proper immunomodulators.

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