[17:00 - 17:20]

Immunomodulating Activity of Fungal β-Glucan through Dectin-1 and Toll-like Receptor on Murine Macrophage

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ABSTRACT: β-Glucan is a glucose polymer that has linkage of β-(1,3), -(1,4) and -(1,6). As exclusively found in fungal and bacterial cell wall, not in animal, β-glucans are recognized by innate immune system. Dendritic cells (DC) or macrophages possesses pattern recognition molecule (PRM) for binding β-glucan as pathogen-associated molecular pattern (PAMP). Recently β-glucan receptor was cloned from DC and named as dectin-1 which belongs to type II C-type lectin family. Human dectin-1 is consisted of 7 exons and 6 introns. The polypeptide of dectin-1 has 247 amino acids and has cytoplasmic, transmembrane, stalk and carbohydrate recognition domains. Dectin-1 could recognize variety of beta-1,3 and/or beta-1,6 glucan linkages, but not alpha-glucans. In our macrophage cell line culture system, dectin-1 mRNA was detected in RAW264.7 cells by reverse transcription-polymerase chain reaction (RT-PCR). Dectin-1 was also detected in the murine organs of spleen, thymus, lung and intestines. Treatment of RAW264.7 cells with β-glucans of *Ganoderma lucidum* (GLG) resulted in increased expression of IL-6 and TNF-α in the presence of LPS. However, GLG alone did not increase IL-6 nor TNF-α. These results suggest that receptor dectin-1 cooperate with CD14 to activate signal transduction that is very critical in immunoresponse.

KEY WORDS: *Ganoderma lucidum*, β-glucan, macrophage, dectin-1, TNF- α, IL-6, LPS, RT-PCR.

INTRODUCTION

β-(1,3)-D-Glucans are glucose polymers found in the cell walls of fungi and bacteria and their conserved structures can be considered as classical pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). They are also known as biological response modifiers and have a variety of effects on the immune system, including anti-tumor (Ross *et al.*, 1999) and anti-infective activities such as against fungi (Williams *et al.*, 1978), against bacteria (Kokoshis *et al.*, 1978), against virus (Itoh *et al.*, 1997), and against protozoa (Cook *et al.*, 1982). Many β-glucans have immuno-pharmacological activity (Yadomae *et al.*, 1992), although the activity varies among different classes of glucans (Hashimoto *et al.*, 1997). The differences in activity are at least partially dependent on their structural parameters, such as solubility, molecular mass, degree of branching and conformation (Aketagawa *et al.*, 1993, Saito *et al.*, 1991, and Yoshioka *et al.*, 1992). β-Glucans possess anti-infective and anti-tumorigenic properties that stem from their ability to activate leukocytes, stimulate their phagocytic activity, and produce reactive oxygen intermediates, inflammatory mediators and cytokines, including TNF-α (Williams, 1997, and Ross *et al.*, 1999). Some data also suggested that β-glucans could promote T cell-specific responses, perhaps through triggering the secretion of IFN-γ, IL-6, IL-8, and IL-12 from macrophages (Di Luzio *et al.*, 1976), neutrophils (Morikawa *et al.*, 1985), and NK cells (Scaringi *et al.*, 1988) to kill sensitive tumor cells.

Dectin-1 is a β-glucan receptor, a Ca^{2+}-dependent lectin-like receptor, whose gene is located on mouse chromosome 6 and human chromosome 12. The dectin-1 mRNA is highly expressed in dendritic cells (Hernanz-Falcon *et al.*, 2001). The presence of dectin-1 on dendritic cell or macrophage has been controversial. Dectin-1 as a β-glucan receptor from a murine macrophage possessed a single C-type lectin-like carbohydrate recognition domains (CRD) connected to the transmembrane regions by a stalk and a cytoplasmic tail possessing immunoreceptor tyrosine-based activation motif (ITAM) (Ariizumi *et al.*, 2000). Murine and human dectin-1 protein has

244 and 247 amino acids (33-43 kDa glycoprotein), respectively. Dectin-1 was found to be widely expressed in mouse tissues (Brown *et al.,* 2001) and human tissues acted as a pattern recognition receptor, recognizing variety of carbohydrates containing β-1,3- and/or β-1,6-glucan linkages and intact *Saccharomyces cerevisiae* and *Candida albicans* (Brown *et al.,* 2001). In addition, the receptor could bind to T-lymphocytes (Ariizumi *et al.,* 2000) but at a site distinct from that which recognized β-glucans (Brown *et al.,* 2001).

MATERIALS AND METHODS

Reagents

β-Glucan of laminarin from *Laminaria digitata* and LPS were purchased from Sigma-Aldrich Co. (St. Louis, USA). β-Glucan of *Ganoderma lucidum* (GLG) was partially purified from the fruit bodies. Phycoerythrin hamster anti-mouse TNF receptor type II (p75) monoclonal antibody (mAb) was purchased from BD Biosciences Pharmingen (San Diego, USA). PCR primers were synthesized by Takara-Korea Biomedicals Inc. (Seoul, Korea). Two step RNA PCR kit was purchased from Takara Biomedicals (Japan). DMEM, RPMI, penicillin-streptomycin and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). AMV reverse transcriptase was purchased from Promega (USA).

Cell Culture

Mouse monocyte-macrophage RAW264.7 (ATCC, Rockville, MD) cells were maintained in DMEM medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat inactivated fetal bovine serum (JRH Biosciences Co., Lenexa, USA), penicillin(100 ㎍

/ml)-streptomycin (100 unit/ml) (Life Technologies, Inc.) and incubated at 37℃ in 5% CO_2, respectively.

RNA Isolation

Total RNA was extracted from the cultured RAW264.7 cells or mouse tissues with TRIzol reagent according to the manufacturer's instructions. The cells were lyzed by adding 1 ml of TRIzol reagent to a 6-well plate, and passing the cell lysate several times through a pipette. Mouse tissues were lyzed by adding 1 ml of TRIzol reagent and homogenized with homogenizer. The cell lysates were incubated for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. The 0.2 ml of chloroform was added and the lysates were shaken vigorously for 15 seconds and incubated at room temperature (RT) for 2 to 3 minutes. Centrifugation was performed at 12,000 × g for 15 minutes at 2 to 8 ℃. The aqueous phase was transferred to a fresh tube. The RNA was precipitated by mixing with isoprophyl alcohol. The RNAs were incubated at RT for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 2 to 8 ℃. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. They were mixed by vortexing and centrifuged at 7,500 × g for 5 minutes at 2 to 8 ℃. The RNA pellet was briefly dried, dissolved in RNase-free water, and stored at -70 ℃ until use.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Two step RNA PCR kit (Takara Biomedicals, Japan) was used for the reverse transcription from RNA to cDNA using AMV reverse transcriptase and subsequently amplified by utilizing AMV-optimized *Taq* DNA polymerase. The PCR primers were designed according to the sequence of mouse dectin-1 (MD-1, AF262985), TNF-α (NM-013693), and IL-6 (X54542).

The RT-PCR program consisted of 30 cycles of 30 seconds at 94 ℃ for denaturation, 30 seconds at 59, 65 and 55℃ for annealing MD-1, TNF-α and IL-6, respectively, and polymerized for 1 minute at 72 ℃. The PCR products were identified by 2 % agarose gel electrophoresis.

Flow Cytometry

Cells of 1×10^6 were washed twice with PBS, and then resuspended in PBS at a concentration of 1×10^6 cells/ml. One hundred $\mu\ell$ of cell suspension was transferred to 1.5 ml centrifuge tube. Cells were then incubated at room temperature in the dark for 15 minutes with 5 $\mu\ell$ of 1 μg/5 $\mu\ell$ phycoerythrin hamster anti-mouse TNF receptor type II (p75) mAb. After adding binding buffer or PBS (400 $\mu\ell$), cells were analyzed by flow cytometry with FACSCalibur™ (Becton Dickinson Co., USA). The percentage positive cells were determined cells that bound TNF receptor-PE. At least 5000 cells were analyzed for each sample.

RESULTS

Expression of Dectin-1 α and β mRNA in RAW264.7 Macrophage and Mouse Organs

To detect dectin-1 expression, we investigated RAW264.7 macrophage cell line by RT-PCR using MD-1 primers. Murine dectin-1 primers could detect both the full length, 757 bp (MD-1α) and a spliced variant isoform, 620 bp (MD-1β). The α type of dectin-1 was known to possess single C-type lectin-like domain, transmembrane region, stalk region, and cytoplasmic tail containing an ITAM but β type of dectin-1 has deleted stalk region. By the RT-PCR analysis, both of dectin-1 α and β mRNAs were expressed in RAW264.7 murine macrophage cell line (Fig. 1A). The upper and lower bands in Figure 1A were confirmed to be dectin-1α and dectin-

1β by the forward and reverse DNA sequence analyses after extraction of the bands from the agarose gel (Fig. 1B).

Then we further analyzed expression of dectin-1 α and β mRNAs on the various organs of mouse by RT-PCR analysis. Both of dectin-1 α and β mRNAs were also expressed in various organs such as thymus, lung, spleen, stomach and intestine isolated from ICR mice (Fig. 1C). Dectin-1 α and β were widely expressed in a variety of lymphoid organs with high expressions in thymus, spleen, and digestive organs such as stomach and intestine.

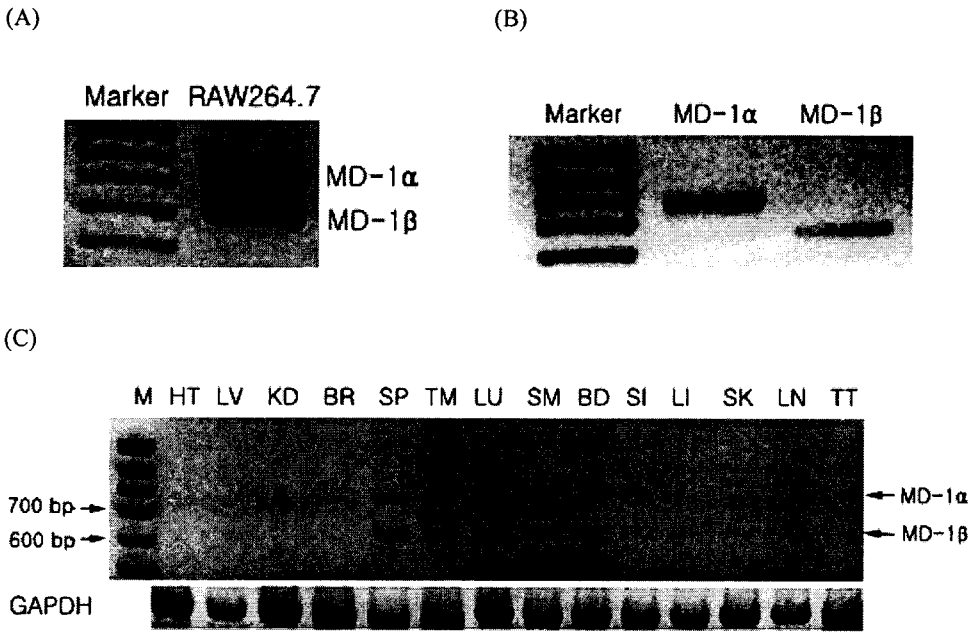

Fig. 1. RT-PCR expression analysis of the murine dectin-1 (MD-1) α and β with primers that could detect the full length (MD-1α) and splice variant (MD-1β). (A) Expression of MD-1 α and β mRNAs in RAW264.7 murine macrophage cell line. (B) Extracted and purified MD-1 α and β mRNAs for DNA sequence analysis. (C) Expression of MD-1 α and β mRNAs the murine organs of heart (HT), liver (LV), kidney (KD), brain (BR), spleen (SP), thymus (TM), lung (LU), stomach (SM), bladder (BD), small intestine (SI), large intestine (LI), skin (SK), lymph node (LN) and testis (TT) isolated from ICR mice. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control.

GLG Increased TNF-α and IL-6 Expressions in LPS Treated Macrophage

By stimulating macrophage together with LPS, β-glucan as a PAMP could activate macrophage and induce various cytokine production. We investigated the effect of GLG in RAW264.7 macrophage in the absence or presence of LPS for 12 hrs. Treatment of the cell with LPS alone did not effect on TNF-α expression. Stimulation of the cells with 100 and 300 μg/ml GLG were neither showed any increase in TNF-α expression when compared with that of the control. In the presence of LPS, however, 100 and 300 μg/ml GLG showed synergistic effect on TNF-α expression when compared with than that of the control. At 300 μg/ml GLG, TNF-α was induced much stronger than at 100 μg/ml GLG, indicating concentration dependent induction of TNF-α in macrophage by β-glucan treatment (Fig. 2A).

For the IL-6 expression analysis, single treatment of the RAW264.7 cell with LPS or GLG did not show any increase in IL-6 expression. However, 100 or 300 μg/ml GLG strongly induced IL-6 expression in the presence of LPS when compared with than that of the control.. 300 μg/ml GLG with LPS did not increase IL-6 expression when compared with than that of the 100 μg/ml GLG plus LPS treatment, indicating 100 μg/ml GLG is a maximum concentration for the IL-6 expression in the presence of LPS (Fig. 2B).

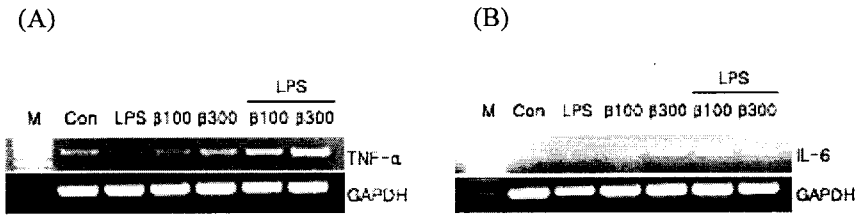

Fig. 2. β-Glucan induced (A) TNF-α and (B) IL-6 mRNAs expression in macrophage. RAW264.7 cells were treated with 100 or 300 μg/ml GLG in the absence or presence of LPS (100 ng/ml) for 12 hrs. Extracted RNAs were polymerized by RT-PCR analysis. Annealing temperatures of TNF-α and IL-6 were 55 ℃ and 65 ℃, polymerizing each with 28 cycles, respectively. GAPDH was used as control in RT-PCR.

FACS Analysis of TNF Receptor in β-Glucan and LPS Treated Macrophage

TNF-α is a cytokine produced by macrophages and T cells that has multiple functions in the immune response. The TNF-α functions as cell-associated or secreted proteins that interact with receptors of the tumor necrosis factor receptor (TNFR) family, which in turn communicates with the interior of the cell via components known as tumor necrosis factor receptor-associated factors (TRAFs). As shown in Figure 2, co-treatment with LPS and β-glucan induced TNF-α mRNA expression. Therefore, we investigated expression of TNF receptor by co-treatment of RAW264.7 cells with GLG and LPS. When detected by FACS analysis, stimulation of the cell with GLG for 6 hrs in the presence of LPS led TNF receptor type II expression to 72.0% when compared with those of control, LPS and GLG to 4.4%, 9.4% and 4.6%, respectively (Fig. 3).

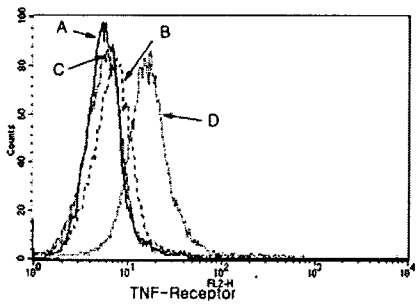

Fig. 3. FACS analysis of TNF receptor type II induced by LPS and β-glucan. RAW264.7 cell were treated with (B) LPS (100 ng/ml), (C) GLG (100 μg/ml), and (D) LPS (100 ng/ml) plus GLG (100 μg/ml) for 6 hrs. (A) is autofluorescence of the cell

Effect Ca^{2+} on Binding of β-Glucan to Dectin-1

β-Glucan binds to dectin-1 in the presence of Ca^{2+}. To investigate Ca^{2+}-dependent binding of β-glucan to dectin-1, EDTA as a Ca^{2+} -chelator, was treated in our system. RAW264.7 cells

were treated with GLG or laminarin for 12 hrs in the presence of 10 uM EDTA and/or LPS, Treatment of the cells with laminarin or GLG in the presence of LPS led to the increased expression of IL-6. However, addition of EDTA to this system, IL-6 mRNA expression was abolished, indicating requirement of Ca^{2+} for binding of β-glucan to dectin-1 (Fig. 4).

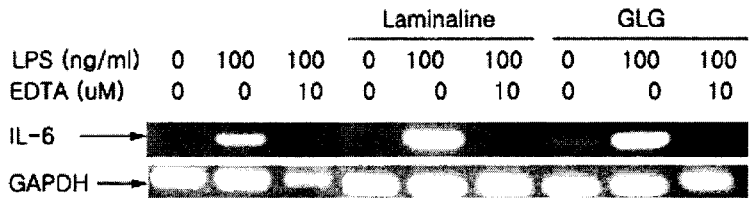

Fig. 4. Effect of EDTA in IL-6 mRNA induction by β-glucan. RAW264.7 cells were treated with LPS (100 ng/ml), EDTA (10 uM), laminarin (100 μg/ml) and GLG (100 μg/ml) for 12 hrs. GAPDH was carried out as control.

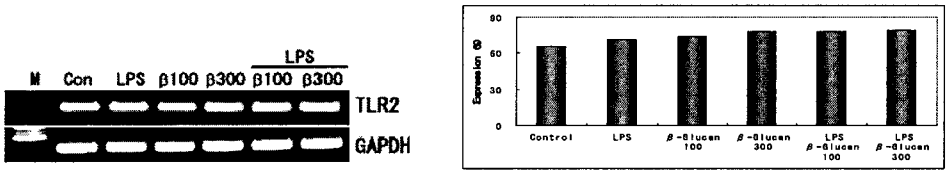

Fig. 5. Effect of LPS and β-glucan in Toll-like receptor 2(TLR2) expression in RAW264.7 cells.

DISCUSSION

Glucans are heterogeneous groups of glucose polymer found in the cell walls of plants, bacteria and fungi. β-Glucan has immuno-pharmacological activity (Yadomae *et al.*, 1992), and various biological activities including anti-tumor activity (Miura *et al.*, 1999). Czop found the

β-glucan receptor, and suggested that β-glucan could trigger lymphocytes activation by binding to β-glucan receptor (Czop and Kay, 1991). Therefore, we investigated that inflammatory cytokine gene expression by treatment with β-glucan of *G. lucidum* in macrophage in the presence of LPS. LPS or β-glucan alone showed almost no induction effects on TNF-α and IL-6 expression. However, LPS and β-glucan exerted synergistic increase in the expression of inflammatory cytokine genes of TNF-α and IL-6 in macrophage cell line.

Expression of dectin-1 was not yet clearly understood among immune cells. Ariizumi *et al.* identified a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning (Ariizumi *et al.*, 2000) and insisted that dectin-1 is expressed only on dendritic cells, not on macrophage cells. Taylor *et al.* found that dectin-1 was widely expressed on the surface of monocyte, macrophage and neutrophil lineages as well as dendritic cells (Taylor *et al.*, 2002). To identify dectin-1 expression on the macrophage among immune cells, we examined RAW264.7 murine macrophage. The RT-PCR analysis led to the detection of both dectin-1 α and dectin-1 β in murine macrophage RAW264.7 cell line. By the DNA sequence analysis of the gel extracted bands, upper band was confirmed to be dectin-1 α. On the other hand, the lower band in the gel electrophoresis was coincide with murine dectin-1 β sequence showing deletion of the stalk region of the dectin-1 ranging amino acid position from 68 to 112.

Among the various lymphoid organs of mouse, dectin-1 α and β were detected in the thymus, lung, spleen, stomach and intestine. The detection of dectin-1 seems to be derived from tissue macrophage/dendritic cell in thymus or spleen, alveolar macrophage in lung and macrophage of payer's patch in intestine.

Major functions of beta-glucan are antitumor and antifungal activities even in oral administration. The absorption of high-molecular-weights beta-glucan through gut barrier has been a subject of long rooted scientific debate. Recently, intestinal transportation mechanism of pathogenic particles or high-molecular-weights compounds began to be elucidated. Membraneous (M) cells of Payer's patch of the gut is responsible for the transportation of pathogens from the lumen into the gut barrier so as to stimulate DC or macrophages beneath the

GALT. M cells are interspersed on the Payer's patch and close contact with subepithelial lymphocytes and dendritic cells. M cells could take up antigens from the gut lumen by endocytosis. Then the antigens are released beneath M cells and taken up by antigen-presenting DCs. Captured by DC, antigens are subsequently presented to T lymphocytes at lymph nodes (Siebers and Finlay, 1996; Savidge, 1996). DC possesses receptor dectin-1 for binding to carbohydrates such as β-glucan. Not having β-glucanase, mammarian can not digest orally administered β-glucan which can reach to the intestine. Therefore, β-glucan might be able to activate immune system, leading to the secretion of inflammatory cytokines.

In conclusion, Our results demonstrate that β-glucan and LPS induce various changes typical for immune modulating genes such as TNF-α, TNF receptor and IL-6. Based on this result, β-glucan might be a promising agent because it is safe and potent anti-tumor efficacy. Future study may expand into new area of investigation such as structural motif responsible for interaction with dectin-1. Should the structural motif is elucidated, reinforced forms of β-glucan derivatives can be screened or even developed to stimulate human immune system.

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