International Journal of Biological Macromolecules 60 (2013) 399–404

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Immune enhancing effect of a Maillard-type lysozyme-galactomannan conjugate via signaling pathways

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ARTICLE INFO

Article history:
Received 7 January 2013
Received in revised form 6 June 2013
Accepted 10 June 2013
Available online 17 June 2013

Keywords:
Galactomannan
Lysozyme
Maillard reaction
Immune-modulating effect

ABSTRACT

We studied the immune-modulating effect of Maillard-type lysozyme-galactomannan conjugate (LGC). LGC significantly induced nitric oxide, and expressions of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 on the murine macrophage Raw 264.7 cell line. In the mechanism of LGC, while extracellular signal-regulated kinase (ERK) was important for the induction of TNF-α, IL-1β and IL-8, the phosphorylation of C-Jun NH2-termianl kinase (JNK) contributed to the induction of TNF-α and IL-1β to a greater degree. These cytokines were less sensitive to the inhibition of p38. Nuclear factor (NF)-κB was involved in the induction of TNF-α and IL-1β. These data indicate that LGC has immune-modulating effects via JNK, ERK and NF-κB pathways, and that LGC may contribute to host immune defense.

1. Introduction

The immune system protects organisms from infection through layered defenses of increasing specificity. As a general overview of how the system is composed, physical barriers prevent pathogens such as bacteria and viruses from entering the organism [1], and the immune system is made up of the two major categories of innate and adaptive immunity [2]. The most primitive arm of the system is referred to as innate immunity, which mainly involves the action of cells such as macrophages, and controls infection in a nonspecific fashion. Macrophages and their precursors, monocytes and neutrophils, make up the major cellular components of the innate immune system. The other component of the immune system, adaptive immunity system exhibits specificity against the foreign agents, and utilizes B and T lymphocytes which respond to challenge by foreign particles in a much more specific manner [3].

Macrophages are the primary defense cells of our body, and protect our body by enhancing the immune system [3]. It is suggested that this stimulatory effect, such as increasing production of certain cytokines from macrophages, may assist the body in defending against infection [4]. Activated macrophages enhance proliferation, phagocytosis, nitric oxide (NO) and cytokine production [5]. Increased NO production by macrophages against a variety of pathogens can induce immunostimulatory activity in macrophages [6]. Moreover, NO is involved in various physiological processes, including cytotoxicity against tumor cells, antioxidant protective activity through scavenging the superoxide radical, immune response, inflammatory reaction, potentiation of matrix degradation, bone metabolism and immune regulation [7]. In addition, the immune activities of macrophages indicate the release of an impressive panel of cytokines, including interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β [8]. Among those cytokines, evidence suggests the involvement of TNF-α, IL-1β, IL-8 and IL-1 Ra in immune induction in a rabbit model [9]. Of note, it has been reported that IL-8 is a cytokine which is primarily involved in chemotoxis, and recruitment of monocyte and T-lymphocytes to the site of infection [10]. On the other hand, the signaling pathways are known for the macrophage-related cytokines by various stimuli in different cell types [5]. NF-κB activates immune cells by up-regulating the expression of many cytokines essential for

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECL, electrochemiluminescent; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; G, galactomannan; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, C-Jun NH2-termianl kinase; L, lysozyme; LGC, lysozyme-galactomannan conjugate; MAPKs, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; PVDF, polyvinylidene difluoride; RH, relative humidity; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.06.007

immune response [11]. Also, the activated pathways of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38 and C-Jun NH2-terminal kinase (JNK) are involved in macrophage-related cytokines by various stimuli in different cell types [5].

Natural sources enhancing innate immunity may play beneficial roles in human health. Galactomannan is a polysaccharide consisting of a mannose backbone with galactose side groups [12]. It has been reported that it has immune-modulating effects [13]. On the other hand, lysozyme (L), which is a glycoside hydrolase enzyme [14], has antimicrobial activity toward Gram positive bacteria because it attacks peptidoglycans [15]. In mammalian and fish cells, L plays an important defense role in the earlier stages of bacterial infection by enhancing phagocytosis [16–18]. It is widely distributed in food such as cow milk, fish and egg [17]. On the other hand, in order to improve or produce new functional and biological properties, conjugation of proteins via Maillard reaction between an amino group and a reducing sugar under controlled conditions has been successfully applied to generate useful glycoproteins for industrial applications [19]. It has been reported that lysozyme-galactomannan conjugate (LGC) has protective effects against *Edwardsiella tarda* infection in carp, *Cypyinus carpio* L. [20,21], antibacterial effects against *Escherichia coli*, and has excellent emulsifying properties [22].

To our best knowledge, no detailed immune enhancing activities of the conjugation of proteins with polysaccharides have been carried out to date. In this study, we hypothesized that Maillard-type LGC possesses immune modulating activity compared to L alone. We used the murine monocyte Raw 264.7 cell line as a model. This study demonstrates that LGC increases NO production, as well as the expression of TNF-α, IL-1β and IL-8 in monocytes, and that its mode of action is related to the signal transduction pathways.

2. Materials and methods

2.1. Materials

High glucose Dulbecco's modified Eagle's medium (DMEM) cell culture media, penicillin streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Galactomannan, L, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Bay 11-7082, SB 239063 and SP 600125 were purchased from Sigma–Aldrich (MO, USA). PD 98059, phospho-SAPK/JNK antibody, SAPK/JNK antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Also, β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BCA protein assay kit was purchased from Thermo scientific (Rockford, IL, USA).

2.2. Experimental methods

2.2.1. Glycation of lysozyme (L) with galactomannan (G)

Maillard-type LGC was produced by mixing L and G in water at a weight ratio of 1:5, after which it was lyophilized. The dried sample was kept at 60 °C for 7 days at 79% relative humidity (RH) using saturated KBr solution in a desiccator according to a previously published method [23]. The freeze-dried LGC and L samples were dissolved in deionized water at a protein concentration of 500 mg/mL. Because LGC is difficult to dissolve, the solution was gently mixed, and was then centrifuged at 2500 × *g* for 10 min to remove any undissolved materials. The protein content in the supernatant was determined by the BCA method [24].

2.2.2. Determination of the degree of LGC

The fluorescence of the LGC was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm using VICTOR3^{TM} (Perkin Elmer, USA) spectrofluorometer. SDS-PAGE was then performed according to the method of Laemmli (Laemmli, 1970) with 5.0% stacking gel and 15.0% separating gels. Samples were mixed with an equal volume of SDS sample buffer [20% (v/v) glycerol, 2% (v/v) β-mercaptoehnanol, 0.04% (v/v) bromophenol blue, 4% (w/v) SDS and 125 mM Tris–HCl (pH 6.8)], and were boiled for 5 min. 20 μL of the mixed solutions were the put on the gel, and the procedure was run for 3 h at 100 V. After electrophoresis, gel was fixed with 50% methanol, followed by Coomassie brilliant blue R-250 staining. Dual color size marker (Bio-Rad, Hercules, CA, USA) was used as the molecular weight standard.

2.2.3. Cell culture and sample preparation

A murine monocyte/macrophage leukemia cell line, Raw 264.7 was purchased from the Korean cell line bank (Seoul, Korea). Cells were grown in high glucose DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 U/mL of streptomycin (Gibco, BCL, Burlington, Ontario, CA) at 37 °C under 5% CO2 in air. Samples were gently mixed in media and were centrifuged at 2500 × *g* for 10 min to remove any undissolved materials, after which the filtered supernatants were analyzed for protein concentration.

2.2.4. Cell viability assay

Cell viability was determined using the micro tetrazolium technique [25]. Cells were plated in 96 well plates (2×10^5 cells/well) and starvation medium without FBS overnight (8–12 h), and then with treated sample for 24 h. After treatment, 40 μL of DMEM without FBS and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to each well, followed by incubation for 3 h. The MTT solution was carefully removed from the wells to avoid loss of formazan crystals, before they were dissolved with 200 μL of DMSO. Absorbance was measured at 540 nm. Values of untreated controls were set to 100% of viability. For each experiment, MTT assays were performed in triplicate.

2.2.5. Measurement of nitric oxide (NO)

To measure the concentration of NO produced during the 24 h incubation period, the stable conversion product NO, nitrite (NO_2^-) was measured [26]. 50 μL of supernatant from each well was transferred to another 96 well plate, and 100 μL of the Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% (w/v) naphthylethlenediamine dihydrochloride in 5% (v/v) H_3PO_4] was added. The mixture was then incubated at room temperature for 5 min, after which the absorbance of the mixture (at 540 nm) was determined with a microplate reader (EL-808, Bio-Tek instrument, Inc.).

2.2.6. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

Cytokines secreted into culture media were measured using each specific enzyme immunoassay according to the manufacturer's instructions. We measured TNF-α (KOMA Biotech, Seoul, Korea), IL-1β (eBioscience, San Diego, CA, USA), IL-8 (IBL, Gunma, Japan) levels by ELISA. Proteins (50 μg) from cell lysates were separated by 10% SDS-PAGE, and were electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated with specific primary antibodies, and secondary antibodies were then conjugated with horseradish peroxidase. The bands were visualized using the electrochemiluminescent (ECL) detection system, and the band density was determined by Image J software (National Institute of Health, Bethesda, MD, USA).