Note

Enhancement of the Immunostimulatory Activity of 1,25-Dihydroxyvitamin D3-Differentiated HL60 Cells with an Arabinoxylan from Wheat Bran

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We found that a wheat bran arabinoxylan (WBA) activates the phagocytosis of 1,25-dihydroxyvitamin D3-differentiated HL60 cells; however, its mechanism was unknown. Hence, we investigated the molecular mechanism involved in the WBA-induced increase of immunostimulating activity using inhibitors. The results indicated that the WBA-induced increases in phagocytic activity and IL-1β production were inhibited by cathepsin B and caspase-1 inhibitor. Moreover, IL-1β production was inhibited by a phagocytosis inhibitor.

Keywords: wheat bran arabinoxylan, phagocytosis, IL-1β, cathepsin B, caspase-1

Introduction

LPS and β-glucan are well known as polysaccharides that have immunomodulating activities, and induce innate immunity via activation of CD14/TLR4 and Dectin-1/TLR2, respectively, on antigen-presenting cells (APCs) such as dendritic cells and macrophages (Miyake, 2003; Gantner et al., 2003). Arabinoxylans are also well known as polysaccharides with immunomodulating activities (Ghoneum and Matsuura, 2004; Ghoneum, Matsuura and Gollapudi, 2008). However, its mechanism remains unknown. Recently, we found that an arabinoxylan from wheat bran (WBA) activates the phagocytosis of macrophage-like cells (Monobe et al., 2008), and the degree of the activity was dependent on its molecular mass (those under 5 kD had no activity). Hence, we inferred that WBA uptake by phagocytosis recognizing a large molecule might induce phagocytic activation.

Foreign matters taken up by phagocytosis are digested by proteases such as cathepsin B, and the activation of cathepsin B induces the innate immune system via activation of caspase-1 (Sharp et al., 2009). However, compared to larger particles, nanoparticles smaller than 100 – 200 nm have a higher capability of evading phagocytosis by alveolar macrophages (Peters et al., 2006). Recently, it was reported that caspase signaling regulates phagocytic activity in macrophages, such as dendritic cells (Ho et al., 2009). Hence, in this study, we investigated the roles of cathepsin B and caspase activation in the induction of WBA-induced phagocytic activity.

Materials and Methods

WBA preparation The WBA was prepared as previously described (Monobe et al., 2008). Briefly, wheat bran was stirred vigorously in hot water (50°C) for 3 min, and then water-soluble substances were removed by gauze filtration. The washed wheat bran was then autoclaved (120°C, 1.8 bar, 15 min). The autoclave-treated washed bran was digested with hemicellulase (Cellulase RS; Yakult Pharmaceutical, Tokyo, Japan) at 50°C for 20 min; the enzyme reaction was stopped by heating at 90°C for 15 min, and the supernatant after centrifugation was lyophilized. The sugar content of the obtained pale yellow powder (WBA) was determined to be 80% by the phenol-sulfuric acid method using xylose as a standard. Acid hydrolysis of WBA in 2 M trifluoroacetic acid at 105°C for 1.5 h gave xylose, arabinose and glucose as neutral sugars at a molar ratio of 100 : 27 : 40, respectively. Hence, the arabinoxylan content of WBA was estimated to be 60%. The molecular mass distribution of WBA was determined to be in the range of 0.2 – 500 kDa by HPLC (column; SHODEX SUGAR KS-803, mobile phase; H₂O, detection; refractive index, standard; pullulan). The physico-chemical

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and cathepsin B released from phagolysosomes activates caspase-1 in an NALP3-dependent manner (Hornung et al., 2008). NALP3 is a cytoplasmic receptor that controls the activity of caspase-1 through the formation of multi-molecular complexes (NALP3 inflammasome).

Next, we investigated the possibility that production of IL-1β is induced by WBA. As shown in Figure 2A, WBA induced intracellular IL-1β production in VD3-differentiated HL60 cells. Then, we investigated the involvement of cathepsin B and caspase signaling in the production of intracellular IL-1β using inhibitors. As shown in Figure 2B and C, the induction was inhibited by a caspase signaling inhibitor (Z-Asp-CH2-DCB) and a cathepsin B inhibitor (CA-074 Me).

It was found that WBA promotes not only phagocytosis, but also intracellular IL-1β production through cathepsin B and caspase activation in VD3-differentiated HL60 cells. The activation of caspase-1 by macromolecules such as silica or properties of WBA are summarized in Table 1.

**Phagocytosis assay** The phagocytosis assay was performed as previously described (Monobe et al., 2007). Briefly, to differentiate cells along the monocytic pathway, HL60 cells, a human acute promyelocytic cell line, were cultured in RPMI 1640 medium supplemented with 120 nM 1,25-dihydroxyvitamin D3 (VD3). VD3-differentiated HL60 cells were seeded in plates. Next WBA (100 µg/mL) and a 1% suspension of YG-labeled microspheres (458 nm excitation, 540 nm emission; Polysciences, Inc., Warrington, PA) was added, and the solution was incubated at 37°C for 16 h. The cells were fixed with 2% formaldehyde and resuspended in PBS. The rate of phagocytosis was measured with a flow cytometer.

**Cytokine analysis** Cytokine analysis was performed as follows: VD3-differentiated HL60 cells (10^6 cells/250 µL/well) were seeded into 48-well plates. The cells were treated with 25 µL of WBA for 6 h, and brefeldin A (Wako) was added 2 h before harvest. Inhibitors (CA-074 Me and Z-YVAD-FMK; Calbiochem, Z-Asp-CH2-DCB; Enzo Life Sciences) were added 5 min before WBA treatment (100 µg/mL). The cells were fixed in paraformaldehyde, permeabiled and labeled with phycoerythrin (PE)-conjugated IL-1β antibody (eBioscience Inc., CA). The expression of intracellular IL-1β was detected by flow cytometry (Cell Lab Quanta; Beckman Coulter, Brea, CA), and analyzed using EPICSXL software (Beckman Coulter).

### Results and Discussion

First, we investigated the involvement of cathepsin B in WBA-induced phagocytosis. As shown in Fig. 1A, the increase in phagocytic activity was significantly inhibited by a cathepsin B inhibitor (CA-074 Me). Next, we investigated the involvement of caspase signaling in WBA-induced phagocytosis. As shown in Fig. 1B, the increase in phagocytic activity was significantly inhibited by a caspase family protease inhibitor (Z-Asp-CH2-DCB), and even more inhibited by a caspase-1 inhibitor (Z-YVAD-FMK) (Fig. 1C). These results suggest that WBA induces the phagocytosis of macrophage-like cells through cathepsin B and caspase-1. Cathepsin B, a lysosomal cysteine protease, plays an important role in the digestion of foreign materials in phagosomes, and their metabolism.

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**Table 1.** The physico-chemical properties of WBA.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Pale yellow powder</td>
</tr>
<tr>
<td>Sugar content</td>
<td>80%</td>
</tr>
<tr>
<td>Neutral sugar</td>
<td>Xyl : Ara : Glc = 100 : 27 : 40</td>
</tr>
<tr>
<td>Arabinofuranosan content</td>
<td>60%</td>
</tr>
<tr>
<td>Molecular mass range</td>
<td>0.2 – 500 kDa</td>
</tr>
</tbody>
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**Fig. 1.** Inhibition of phagocytic activity by cathepsin B or caspase inhibitors.

VD3-differentiated HL60 cells were incubated with beads in the presence of WBA (100 µg/mL) and inhibitors. (A) CA-074 Me (100 µM; Cathepsin B inhibitor). (B) Z-Asp-CH2-DCB (200 µM; a caspase family protease inhibitor). (C) Z-YVAD-FMK (40 µM; a caspase-1 inhibitor). Phagocytosis activity in the absence of WBA (control) was normalized to 100%. Values are the means ± SD, n = 3. Different letters indicate a significant difference (p < 0.001, as assessed by the Bonferroni/Dunn test).
Enhancement of Immunostimulatory Activity by Arabinoxylan

Figure 2. Inhibition of intracellular IL-1β production by cathepsin B inhibitor, caspase inhibitors or cytochalasin B.

VD3-differentiated HL60 cells were incubated in the presence (solid line histogram) or absence (dashed line histogram) of WBA (100 µg/mL) and inhibitors. IL-1β expression in cells was determined by flow cytometry with PE-conjugated IL-1β antibody (clone CRM56). (A) Absence of inhibitor, (B) Z-Asp-CH2-DCB (200 µM; a caspase family protease inhibitor), (C) CA-074 Me (100 µM; Cathepsin B inhibitor), (D) cytochalasin B (5 µM; a phagocytosis inhibitor).

aluminum first requires that the macromolecules be uptaken by macrophages through spontaneous phagocytosis (Hornung et al., 2008); therefore, we next investigated the relationship between phagocytosis and the intracellular IL-1β production induced by WBA. As shown in Figure 2D, the WBA-induced intracellular IL-1β production was inhibited by cytochalasin B, a well-characterized inhibitor of phagocytosis. Cytochalasin does not inhibit IL-1β production induced by non-macromolecule NALP3 activators, ATP or R838 (Hornung et al. 2008). Our results were similar to the results of Hornung et al. (2008), who reported that the silica crystals- or aluminum salts-induced IL-1β production is inhibited by cytochalasin, CA-074Me and caspase-1 inhibitor (z-YVAD); therefore, it is suggested that WBA uptake via spontaneous phagocytosis induces an innate immune reaction through cathepsin B/caspase signaling. The degree of the activity induced by WBA was dependent on its molecular mass (those under 5 kD had no activity) (Monobe et al., 2008). Thus, a high molecular mass of WBA may be an important factor for WBA-induced activation; however, high molecular mass alone cannot account for the macrophage activation since uptake of fluorescence latex beads (ϕ 2 µm) does not activate macrophage-like cells (Monobe et al., 2007). We infer that the activation mechanism by WBA is similar to that of silica crystals. Phagocytosis of silica crystals leads to lysosomal destabilization, probably due to physical stimulation, and activates the NALP3 inflammasome (Hornung et al., 2008). The WBA-induced enhancement of immunostimulatory activity may be related to NALP3 inflammasome activation via phagosomal destabilization due to the surface structure of the WBA molecule.

We infer that spontaneous WBA uptake by macrophages may represent the initiation of immune system activation, which induces cathepsin-caspase signaling and enhances IL-1β production and phagocytosis; however, further studies are needed to clarify the surface structure of the WBA molecule, WBA uptake by cells, and the gene expression involved in WBA-induced activation of the immune system.

References
weight dependence of an arabinoxylan derived from wheat bran. 
