Food and Chemical Toxicology 51 (2013) 411–418

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Promoting effect of polysaccharide isolated from *Mori fructus* on dendritic cell maturation

Bo Ram Shin [a,1], Hyung Sook Kim [a,1], Mi Jung Yun [a], Hong Kyung Lee [a], Yeon Jin Kim [a], Sung Yeon Kim [b], Mi Kyeong Lee [a], Jin Tae Hong [a], Youngsoo Kim [a], Sang-Bae Han [a,*]

[a] College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea
[b] College of Pharmacy, Wonkwang University, Chonbuk 570-749, Republic of Korea

ARTICLE INFO

Article history:
Received 8 August 2012
Accepted 18 October 2012
Available online 27 October 2012

Keywords:
Mori fructus
Dendritic cells
TLR4
NF-κB
MAPK

ABSTRACT

Maturation of dendritic cells (DCs) is usually attenuated in the tumor microenvironment, which is an important immunological problem in DC-based immunotherapy of cancer. In this study, we report the effect of a *Mori fructus* polysaccharide (MFP) on DC maturation. MFP was treated to DCs generated from mouse BM cells. MFP induced phenotypic maturation of DCs, as proven by the increased expression of CD40, CD80/86, and MHC-I/II molecules. MFP induced functional maturation of DCs, in that MFP increased the expression of IL-12, IL-1β, TNF-α, and IFN-β, decreased antigen capture capacity, and enhanced allogenic T cell stimulation. MFP efficiently induced maturation of DCs from C3H/HeN mice having normal toll-like receptor4 (TLR4), but not DCs from C3H/HeJ mice having mutated TLR4, suggesting that TLR4 might be one of the membrane receptors of MFP. As a mechanism of action, MFP increased phosphorylation of mitogen-activated protein kinase (MAPKs), and nuclear translocation of NF-κB p65 subunit, which were important signal molecules downstream from TLR4. These data suggest that MFP induces DC maturation through TLR4 and MFP can be used as an adjuvant in DC-based cancer immunotherapy.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells that initiate the majority of immune responses, including tumor-specific T cell responses (Bennaceur et al., 2009). DCs are generated from either lymphoid or myeloid bone marrow progenitors through intermediate DC precursors (Ardavin et al., 2004). DCs are positioned at different portals of the human body, such as the mucosal surface and blood, where they can encounter the invading pathogens (Wu and Liu, 2007). After the uptake of antigen and exposure to inflammatory regents, immature DCs mature and migrate to the T cell areas of secondary lymphoid organs. Mature DCs down-regulate antigen uptake and processing capacity, but can sensitize naïve T cells through major histocompatibility (MHC) and co-stimulatory molecules (Kim et al., 2007).

The body's natural defenses are attenuated during neoplastic disease development and DC maturation is inhibited by tumor-derived factors, such as vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), ilterleukin-10 (IL-10), prostaglandin E2 (PGE2), and gangliosides (Gabrilovich et al., 1996; Katsenelson et al., 2001; Smyth et al., 2001; Terabe et al., 2003). Immature DCs usually accumulate in tumor-bearing mice (Ishida et al., 1998) and in cancer patients (Nestle et al., 1997). Also, several key deficiencies in maturation, migration to lymph nodes, and T cell activation by DCs have been observed in the tumor microenvironment (Lapteva et al., 2007). Defective DCs in tumor microenvironments are an important immunological problem that limits the success of cancer immunotherapy (Kim et al., 2010b).

This scenario suggests that overcoming mal-maturation of DCs in cancer patients is an important issue in cancer therapy. Experimentally, many studies attempted to find inducers of DC maturation using monocyte-conditioned medium, PGE2, toll-like receptor (TLR) ligands, and inflammatory cytokines, such as TNF-α, IL-6, and IL-1β (Ardeshna et al., 2000; Jonuleit et al., 1997; Mittal and Prasadarao, 2008; Reddy et al., 1997). In addition, plant-derived polysaccharides have been used as inducers of DC maturation (Kim et al., 2006; Kanazawa et al., 2004; Kim et al., 2010b; Ferreira et al., 2010; Harada et al., 2003). The proteoglycan from

Abbreviations: CR3, complement receptor 3; DCs, dendritic cells; ERK, extracellular signal-regulated kinase; IκB, inhibits NF-κB; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MFP, *Mori fructus* polysaccharides; TLR, toll-like receptor.

* Corresponding author. Address: College of Pharmacy, Chungbuk National University, 52 Naesoodong, Heungduk, Cheongju, Chungbuk 361-763, Republic of Korea. Tel.: +82 43 261 2815; fax: +82 43 268 2732.
E-mail address: shan@chungbuk.ac.kr (S.-B. Han).

[1] These authors contributed equally to this work.

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2012.10.018

Phellinus linteus (Kim et al., 2004), a polysaccharide from *Cordyceps militaris* (Kim et al., 2006), polysaccharide K (PSK) from *Coriolus versicolor* (Kanazawa et al., 2004), and β-glucan from *Sparassis crispa* (Kim et al., 2010b) induce DC maturation. Among them, polysaccharides isolated from *Grifola frondosa* have been assessed in clinical trials of cancer immunotherapy (Ferreira et al., 2010; Harada et al., 2003).

Here, we provide another candidate to increase DC maturation. *Mori fructus* is the fruit of *Morus alba* L. (Moraceae), which is widely cultivated in China, Japan, and Korea. The root bark, leaf, and fruit had been used popularly as an herbal medicine, and have been evaluated for their antioxidant and antimicrobial properties (Wang et al., 2012). With regard to the immunopharmacological aspect, the water extract of *M. fructus* activates macrophages through TLR4 (Yang et al., 2009). However, the effect of *M. fructus* polysaccharide (MFP) isolated *M. fructus* on dendritic cells is still unknown, which prompted us to investigate its effect on DC functions including maturation, cytokine production, and allo-T cell activation. In addition, we examined the underlying mechanisms related with DC activation by MFP.

2. Materials and methods

2.1. Materials

Female C57BL/6, BALB/c, C3H/HeN, and C3H/HeJ mice (6–8 weeks old) were obtained from Korea Research Institute of Bioscience and Biotechnology (Chungbuk, Korea). Mice were housed in specific pathogen-free conditions at 21–24 °C and 40–60% relative humidity under a 12 h light/dark cycle. All animals were acclimatized for at least 1 week prior to the experiments. All experimental procedures were approved by the Animal Experimentation Ethics Committee of Chungbuk National University. Anti-mouse antibodies against CD11c, CD40, CD80, CD86, and MHC-I/II were purchased from BD Pharmingen (San Diego, CA, USA) and those against extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs) were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipopolysaccharide (LPS), polymyxin B (PMB), and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Crude polysaccharide (MFP) from *M. fructus* was prepared as described previously (Kim et al., 2007). Briefly, the dried fruit was pulverized into powder. The sample was then successively extracted twice with hot water (65–70 °C) each time for 10 h. The extract was combined and concentrated under reduced pressure to small volumes. The crude polysaccharide was precipitated by adding 4 volumes of ethanol. The precipitate was collected by centrifugation and washed twice with ethanol. The precipitate was then suspended in water and lyophilized to yield crude polysaccharide, named as MFP. No endotoxin was detected at 100 μg/ml of MFP as determined by the LAL test (Wako Pure Chemicals, Osaka, Japan).

2.2. Generation of bone marrow-derived DCs

DCs were generated from bone marrow (BM) cells obtained from 6–7-week-old female mice (Kim et al., 2007). Briefly, BM cells were flushed out from femurs and tibias. After lysing red blood cells, whole BM cells (2×10^5 cells/ml) were cultured in 100-mm^2 culture dishes in 10 ml/dish of complete medium containing 2 ng/ml GM-CSF. On culture day 3, another 10 ml of fresh complete medium containing 2 ng/ml GM-CSF was added, and on day 6 half of the medium was changed. On day 8 non-adherent and loosely adherent DCs were harvested by vigorous pipetting and used as immature DCs (iDCs). iDCs recovered from these cultures were generally >85% CD11c$^+$, but not CD3$^+$ and B220$^+$.

2.3. Phenotype analysis

Phenotypic maturation of DCs was analyzed by flow cytometry. Cell staining was performed using a combination of FITC-conjugated anti-CD40, anti-CD80, anti-CD86, or anti-MHC plus PE-conjugated CD11c antibodies. Cells were analyzed using a FACSCalibur flow cytometer and data were analyzed using CellQuest Pro (BD Biosciences, San Jose, CA, USA). Forward and side scatter parameters were used to gate live cells.

2.4. Endocytosis assay

To analyze the endocytosis of DCs, 4×10^5 DCs were incubated at 37 °C for 1 h with 0.4 mg/ml FITC-dextran (42,000 Da, Sigma–Aldrich, St. Louis, MO, USA). After incubation, cells were washed twice with cold washing buffer (PBS containing 0.5% BSA) and stained using PE-conjugated anti-CD11c antibody. Double stained DCs were analyzed by flow cytometry. In addition, parallel experiments were performed at 4 °C to determine the nonspecific biding of FITC-dextran to DCs (Kim et al., 2011a). MFP or LPS were pretreated with 2,000 U/ml of polymyxin B (PMB) for 1 h and then used to treat DCs.

2.5. Cytokine assay

Total RNA was isolated using TRIZOL™ Reagent (Molecular Research Center, Cincinnati, OH, USA). For RT-PCR, single-strand cDNA was synthesized from 2 μg total RNA. The primer sequences used were as follows: interleukin (IL)-12, sense, 5′-AGA GGT GGA CTG GAC TCC CGA-3′, antisense, 5′-TTT GGT GCT TCA CAC TTC AG-3′; IL-1β, sense, 5′-ATG GCA ATG TTC CTG AAC TCA ACT-3′, antisense, 5′-CAG GAC AGG TAT AGA TTC TTT CCT TT-3′; tumor necrosis factor TNF-α, sense, 5′-AGG TTC TGT CCC TTT CAC TCA CTG-3′, antisense, 5′-AGA GAA CCT GGG AGT CAA GGT A-3′; interferon (IFN)-β, sense, 5′-CCA CAG CCC TCT CCA TCA ACT ATA AGC-3′, antisense, 5′-AGC TCT TCA ACT GGA GAG CAG TTG AGG-3′; β-actin, sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, and antisense 5′-TAA AAC GCA GCT CAG TAACAG TCC G-3′. PCR products were fractionated on 1% agarose gels and stained with 5 μg/ml ethidium bromide. After analyzing band areas using a Chemi Doc™ XRS$^+$ (BIO RAD, USA) and Image Lab Software. Cytokine levels of IL-1β, IL-12, and TNF-α in culture supernatants were measured using commercial immunoassay kits (R&D Systems, Minneapolis, MN, USA) (Kim et al., 2010b).

2.6. Western blots

Lysates were prepared from total cells or nuclear as previously describe (Kim et al., 2010b). Detergent-insoluble materials were removed, and equal amounts of protein were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to pure nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tween 20 plus Tris-buffered saline for 1 h and then incubated with an appropriate dilution of primary antibody in 5% bovine serum albumin (in Tris-buffered saline containing Tween 20) for 2 h. Blots were incubated with biotinylated antibody for 1 h and further incubated with horseradish peroxidase–conjugated streptavidin for 1 h. Signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7. Mixed leukocyte reaction (MLR)

Responder T cells were purified from the spleen of BALB/c mice by negative depletion using biotinylated antibodies for B220, GR-1, and CD11c (BD Pharmingen, San Diego, CA) and Dynabeads M-280 streptoavidin (Invitrogen, Dynal, Inc., Oslo, Norway), as previously described (Kim et al., 2010b). Purity was typically more than 90%. DCs were generated from the BM cells of C57BL/6 mice and were treated with 40 μg/ml mitomycin C (MMC) for 1 h. MMC-treated DCs were added to 1×10^5 T cells in U-bottom 96-well plates. Allogenic T cells were pulsed with ^{3}H-thymidine (113 Ci/nmol, NEN, Boston, MA) at a concentration of 1 μCi/well for the last 18 h and harvested on day 3 using an automated cell harvester (Innotech, Dottikon, Switzerland). The amount of ^{3}H-thymidine incorporated into cells was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland). Cytokine levels of IL-2 and IFN-γ in culture supernatants were measured using commercial immunoassay kits (R&D Systems, Minneapolis, MN, USA) (Kim et al., 2010b).

2.8. Statistics

Data represent the mean ± STD of more than three samples and all experiments were performed more than three times. Standard deviations (STD) were calculated using the Student's *t*-test and *p* values were calculated using ANOVA software (GraphPad Software, San Diego, CA).

3. Results

3.1. MFP induces the phenotypic maturation of DCs

DCs were generated from BM precursors by using 2 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF). On day 8 of culture, non-adherent and loosely adherent cells (i.e., immature DCs) were harvested from cultures and further activated with lipopolysaccharide (LPS) or MFP for 24 h. An analysis of cell surface makers showed that more than 85% of cells were CD11c$^+$, but not CD3$^+$ or B220$^+$ (data not shown). MFP dose-dependently increased the expression of CD40, CD80, CD86, and MHC-I/II (Fig. 1), which are known maturation markers of DCs. MFP-treated DCs showed a mature morphology with long dendritis, but untreated DCs showed short dendritis (Fig. 1E). MFP or LPS did not affect cell viability during the incubation (data not shown). These results are