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Original Article

Anti-inflammatory Activity of *Carpinus tschonoskii* Leaves Extract in R848-stimulated Bone Marrow-derived Macrophages and Dendritic Cells

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The present study aims to evaluate the anti-inflammatory effect of methanol extract from leaves of *Carpinus tschonoskii* (CE) on R848-stimulated primary bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs). Primary BMDMs and BMDCs were used for pro-inflammatory cytokine production. Human embryonic kidney cell line 293T (HEK293T) was used to access NF-κB activity. In all cases, R848 was used to stimulate the cells. The CE (0~150 μg/ml) was treated to BMDMs, BMDCs, and HEK293T cells. CE pre-treatment in R848-stimulated BMDMs and BMDCs showed a dose-dependent inhibitory effect on pro-inflammatory cytokine (e.g., IL-12 p40, IL-6, and TNF-α) production as compared to non-treated controls. In NF-κB reporter gene assay, the CE pre-treatment inhibited NF-κB-dependent luciferase activity in a dose-dependent manner. Overall, our findings suggest that CE has significant inhibitory effect on pro-inflammatory cytokine production and deserve further studies concerning potentials of CE for medicinal uses.

Key Words: *Carpinus tschonoskii*, Pro-inflammatory cytokine, NF-κB, TLR7

INTRODUCTION

Innate immune response has a crucial role in protection of host against pathogen as well as autoimmune disorders (1, 2). Bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) are vital cellular components of innate immune system (3). In these cells, recognition of pathogen-associated molecular patterns (PAMPs) by Toll-like receptor (TLR) triggers activation of downstream signaling cascade including, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways, leading to production of pro-inflammatory cytokines and induction of adaptive immune response (1, 4~6).

Toll-like receptor 7 (TLR7) is responsible for recognition of single stranded RNA in endosomes, which is a common feature of viral genomes (4). R848 is a synthetic low molecular weight imidazoquinoline compound that can act as TLR7 ligand and activate immune cells via the TLR7-MyD88-dependent signaling pathway (7). In some auto-immune disorders, the TLR7-mediated immune cells activation is found to be triggered by mammalian self-RNAs incorporated into immune complexes (8, 9). In addition, overproduction of cytokines such as interleukin (IL)-6 have been reported to enhance colitis-associated cancer (CAC) tumorigenesis (10, 11).

The genus *Carpinus*, commonly known as Hornbeams, a woody flora, consists of over 40 species most of which are distributed in the northern hemisphere including parts of China and Korea (12). Phytochemical extract from the

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leaves of *C. tschonoskii* has been previously reported for its cytoprotective effects against H_2O_2-associated reactive oxygen species (ROS) damage by upregulation of catalase activity in V79-4 cells (13).

However, the effect of this plant extract on innate immune response has been barely defined in terms of its influence on primary murine BMDCs and BMDMs. Therefore, our present work investigates the anti-inflammatory effects of *C. tschonoskii* leaves extract (CE) on TLR7 agonist-stimulated BMDMs, BMDCs, and human embryonic kidney cell line 293T (HEK293T) cells.

MATERIALS AND METHODS

Plant extract preparation

The *C. tschonoskii* leaves extract was purchased from Korea Research Institute of Bioscience and Biotechnology (KRIBB). The plant extract from the leaves of *C. tschonoskii* (CE) was prepared as previously described (13). Briefly, the leaves were dried well in the shade, and grinded to obtain course powder. The dried powder (35 g) was extracted with HPLC grade MeOH (200 ml) under an automatic extractor for 20 min at 50℃, 1,500 psi using N_2 gas. A part of the filtrate which was separated from solid substances through defatted cotton-plugged funnel and completely concentrated under reduced pressure at 45℃. Finally, the resulting MeOH extract was dissolved in dimethyl sulfoxide (DMSO) and used for experiments.

Mice

C57BL/6 mice were from Orient Bio Inc. (Seongnam-si, Gyenggi-do, Korea). Mice were maintained under specific pathogen-free conditions. All mice were maintained and used in accordance with institutional and National Institutes of Health guidelines. All animal procedures were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of Jeju National University (#2010-0028).

Cell cultures and measurement of cytokine production

The BMDCs and BMDMs were derived from wild-type C57BL/6 mice. Briefly, bone marrow from tibia and femur was obtained by flushing with DMEM, and bone marrow cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 50 μM of 2-ME, and 2 mM of glutamine supplemented with 3% J558L hybridoma cell culture supernatant containing granulocyte-macrophage colony-stimulating factor for dendritic cells generation. In the meantime, the bone marrow cells were cultured for macrophages in DMEM medium containing 20% heat-inactivated FBS, 30% L929 cell culture supernatant containing macrophage colony-stimulating factor and 1% penicillin-streptomycin (Gibco, NY, USA). Cells were incubated in 48-well plates at a density of 1×10^5 cells/0.5 ml, and then treated with the CE for 1 h before stimulation with R848 (TLR7 ligand, 1 μM). Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA (BD PharMingen, CA, USA, R&D system, MN, USA) according to the manufacturer's instructions.

Cell viability assay

The cell viability was determined by standard procedure of 3-(4,5-dimethyl-2,5 thiazolyl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, the cells at a concentration of 5×10^4 cells were seeded on a 96-well culture plate. After incubation for 1 h at 37℃, cells were treated with CE at various concentrations for 18 h. Cells were added 0.2 mg MTT (Sigma, MO, USA) and then incubated for 4 h at 37℃. The plate was centrifuged and the supernatants were aspirated. The formazan crystals in each well were dissolved in 250 μl dimethyl sulfoxide (DMSO). Absorbance was measured at wavelength of 540 nm.

Luciferase assay

For NF-κB reporter assays, HEK293T cells were plated in 24-well plates and grown overnight. Cells were transfected using Fugene 6 (Roche, IN, USA) with a NF-κB reporter gene, pRLnull (Promega, WI, USA) and pcDNA3 (empty vector) or TLR7-encoding pcDNA3. Cells were incubated for 24 h, and then pre-treated with CE for 1 h before

Table 1. Effects of *Carpinus tschonoskii* leaves extract (CE) on cell viability

Extract (μg/ml)	Viability (%)[a]	
	BMDMs	BMDCs
0	100.0±1.2	100.0±2.1
25	111.3±5.2	99.3±3.4
50	109.6±7.1	103.1±2.2
100	112.1±6.3	97.5±2.5
150	113.7±3.2	109.3±3.1

[a] Bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) were treated with CE (25~150 μg/ml) for 18 hours and viability was measured using MTT assay. Results shown are the mean ± SD of an experiment done in triplicate and are representative of three separate experiments.

stimulation with R848 (1 μM). After further incubation for 18 h, cells were lysed in a passive lysis buffer (Promega), and firefly luciferase versus renilla activities were measured using a dual luciferase reporter assay system (Promega).

Data analysis

Results are expressed as means ± standard deviation (SD). Student's *t*-test was used to evaluate the data with the following significance levels: $*p < 0.05$, $**p < 0.01$. All assays were performed in at least three independent experiments.

RESULTS

Effects of *Carpinus tschonoskii* leaves extract on cell viability in BMDMs and BMDCs

In order to access the anti-inflammatory effects of CE, we first used a colorimetric MTT assay (Sigma) to confirm the effect of CE on cell viability. We observed little or no effect on cell viability of BMDMs and BMDCs (Table 1) at the concentration range of 25 to 150 μg/ml. Hence, CE (25~150 μg/ml) was used for further analysis.

Inhibitory effects of *Carpinus tschonoskii* leaves extract on IL-12 p40, IL-6, and TNF-α production in R848-stimulated BMDMs and BMDCs

Pro-inflammatory cytokines, including IL-12 p40, IL-6, and TNF-α, play critical roles in inflammatory and autoimmune diseases (14, 15). To evaluate the plant extract for anti-inflammatory activity, the CE was tested for the inhibitory effects on R848-stimulated IL-12 p40, IL-6, and TNF-α production in BMDMs using ELISA. R848 induced significantly increased IL-12 p40, IL-6, and TNF-α production in BMDMs (Fig. 1). The CE showed strong dose-dependent inhibition of IL-12 p40 and IL-6 production in R848-stimulated BMDMs. However, the CE exhibited moderate inhibitory effects on TNF-α production in R848-stimulated BMDMs.

We also evaluated the ability of CE to inhibit R848-stimulated IL-12 p40, IL-6, and TNF-α production in BMDCs using ELISA. R848 induced significantly increased IL-12 p40, IL-6, and TNF-α production in BMDCs (Fig. 2). The CE showed strong dose-dependent inhibition of IL-12 p40 and IL-6 production in R848-stimulated BMDCs. In addition, the CE exhibited modest inhibitory effects on TNF-α production in R848-stimulated BMDCs.

Taken together, these data suggest that CE has an inhibitory effect on pro-inflammatory cytokine production in R848-stimulated BMDMs and BMDCs.

***Carpinus tschonoskii* leaves extract treatment inhibited NF-κB reporter activity in HEK293T cells**

Activation of NF-κB pathway results in nuclear translocation and binding of this transcription factor to its target promoter sites (16). To investigate whether the CE had inhibitory effect on R848-stimulated NF-κB transcriptional activity, the NF-κB reporter gene assay was conducted (Fig. 3). The HEK293T cells transfected with empty pcDNA3 showed little or no NF-κB-dependent luciferase activity upon R848-stimulation. However, the HEK293T cells transfected with TLR7-expressing plasmid exhibited robust NF-κB-dependent luciferase activity upon R848 stimulation. In contrast, CE pre-treatment showed strong dose-dependent inhibition of NF-κB-dependent luciferase activity in HEK-293T cells transfected with TLR7-expressing plasmid (Fig. 3). Therefore, this data suggest that CE has an inhibitory effect on TLR7-dependent NF-κB activation upon R848 stimulation.

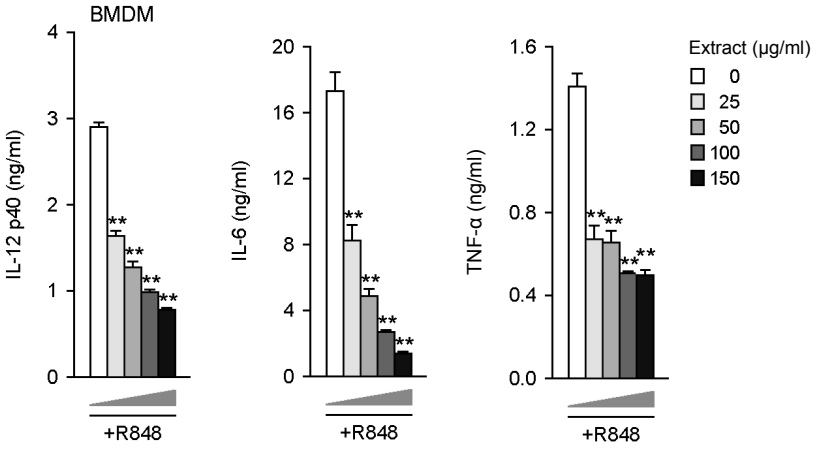

Figure 1. Inhibitory effects of *Carpinus tschonoskii* leaves extract (CE) on IL-12 p40, IL-6, and TNF-α production in R848-stimulated bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDM) were treated with CE (25~150 μg/ml) for 1 h before stimulation with R848 (1 μM). Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA. Results shown are the mean ± SD of an experiment done in triplicate and are representative of three separate experiments (*p < 0.05, **p < 0.01 versus CE-untreated cells stimulated with TLR7 ligand).

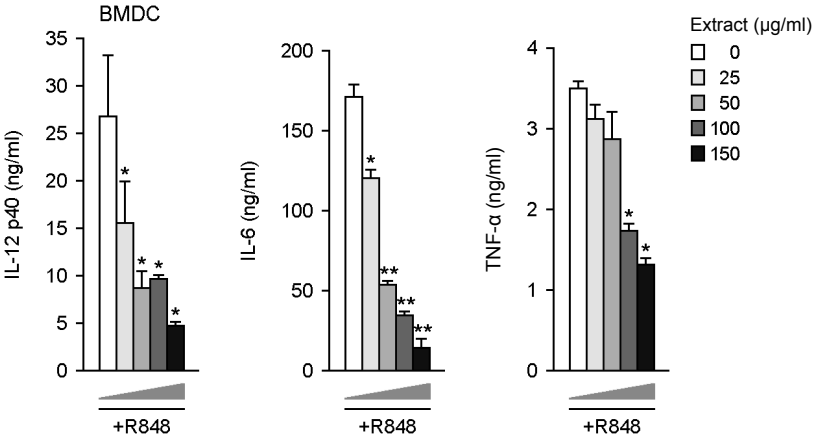

Figure 2. Inhibitory effects of *Carpinus tschonoskii* leaves extract (CE) on IL-12 p40, IL-6, and TNF-α production in R848-stimulated bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDC) were treated with CE (25~150 μg/ml) for 1 h before stimulation with R848 (1 μM). Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were determined by ELISA. Results shown are the mean ± SD of an experiment done in triplicate and are representative of three separate experiments (*p < 0.05, **p < 0.01 versus CE-untreated cells stimulated with TLR7 ligand).

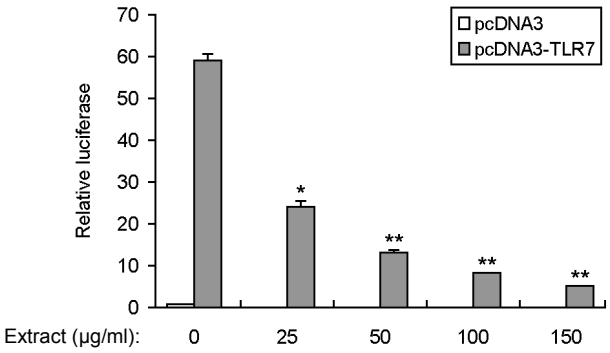

Figure 3. *Carpinus tschonoskii* leaves extract (CE) treatment inhibited NF-κB reporter activity in HEK293T cells. HEK293T cells were transfected with empty vector (pcDNA3) or TLR7-expressing plasmid (pcDNA3-TLR7), and then treated with CE for 1 h before stimulation with R848 (1 μM). Cell lysates were prepared and assayed for firefly luciferase versus renilla activities, and results were expressed as fold increases. All results shown are the mean ± SD of an experiment done in triplicate and are representative of three separate experiments. (*p < 0.05, **p < 0.01 versus CE-untreated cells stimulated with TLR7 ligand).

DISCUSSION

Activated BMDMs and BMDCs perform crucial func tions in immune and inflammatory responses via PAMPs-stimulated production of pro-inflammatory cytokines such as IL-12 p40, IL-6, and TNF-α (16). These pro-inflammatory cytokine play a crucial role in host defense and inflammatory response. IL-12 p40 has important immunoregulatory activities and is critical for the differentiation and generation of T helper 1 cells (17). It should be noted that since IL-12 is a key cytokine in Th1-mediated autoimmune responses, downregulation of IL-12 p40 production by the CE may ameliorate the autoimmune diseases (17, 18). IL-6 has broad cellular and physiological responses including hematopoiesis, inflammation, regulation of cell growth, proliferation, and differentiation (19, 20). However, IL-6 has been reported to

inhibit apoptosis and promote cell proliferation signal transducers and activators of transcription (STAT) 3 pathways during tumorigenesis (10, 11, 15). In the present study, pretreatment of CE showed a strong dose-dependent inhibition of IL-6 production in R848-stimulated cells. Hence, strong inhibitory property of this plant extracts against IL-6 production warrants further studies concerning potentials in future anti-inflammatory and anti-cancer application.

BMDMs and BMDCs both express TLR7 which is strongly activated by R848 in a MyD88-dependent pathway (7). This triggers activation of both MAPK and NF-κB pathways, leading to the production of inflammatory cytokines (21, 22). IL-12 p40, IL-6, and TNF-α are also NF-κB-responsive cytokines and transcriptional activity of NF-κB at their corresponding κB promoter sites is essential for their expression (4, 18). Our result from the NF-κB-dependent reporter gene assay in HEK293T cells also showed that CE inhibited NF-κB-dependent luciferase activity in HEK293T cells in a dose-dependent manner. Taken together, these findings suggest that inhibition of pro-inflammatory cytokines production by CE may correlate with blockage of NF-κB-dependent pathway. Hence, the CE-mediated anti-inflammatory activity represents a potential therapeutic use of the extract for inflammatory diseases. Our present results warrant further studies concerning potential uses of CE for anti-inflammatory diseases and inflammation-associated cancer.

In conclusion, our present study suggests that *Carpinus tschonoskii* leaves extract has an anti-inflammatory effect on production of pro-inflammatory cytokines, and TLR7-dependent NF-κB activation. Hence, identification and detail mode of action of the pure active components from the CE remains an interesting chapter for the future research.

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