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Original Article

Effects of Red Ginseng-Ejung-tang Water Extract on Cytokine Production in LPS-induced Mouse Macrophages

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Objectives: The purpose of this study was to investigate effects of Red Ginseng-Ejung-tang Water Extract (ER) on cytokine production in RAW 264.7 mouse macrophages stimulated by lipopolysaccharide (LPS).
Methods: Levels of various cytokines such as interleukin (IL)-6, IL-10, IL-2, IL-12p70, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, keratinocyte-derived chemokine (KC), tumor necrosis factor (TNF)-alpha, granulocyte macrophage colony-stimulating factor (GM-CSF) were measured by high-throughput multiplex bead array cytokine assay based on xMAP (multi-analyte profiling beads) technology.
Results: ER significantly decreased levels of IL-6, IL-10, IL-2, IL-12p70, VEGF, and MCP-1 for 24 hrs incubation at the concentrations of 25, 50, and 100 ㎍/mL in LPS-induced RAW 264.7 cells ($P < 0.05$). But ER did not exert significant effects on production of MIP-2, KC, TNF-α, and GM-CSF in LPS-induced RAW 264.7 cells.
Conclusions: These results suggest that ER has an anti-inflammatory property related with its inhibition of cytokine production in LPS-induced macrophages.

Key Words : Red ginseng, Ejung-tang, macrophage, inflammation, cytokine

Introduction

Ejung-tang (*Lizhong-tang* in Chinese) was first reported by 'Jang Gi (150-219)', a famous traditional Chinese doctor, in his classical medical book 'Sang-Han Theory[1)] to treat various gastrointestinal diseases such as chronic gastritis, gastroduodenal ulceration, gastrointestinal ulceration, chronic colitis, indigestion, gastric dilatation, stomach pain, gastroptosia, vomiting, watery diarrhea, fullness of the abdomen, anorexia, pale tongue with white coating, deep and thready pulse, which were together classified as 'Spleen *Yang* Deficiency' in traditional Korean medicine[1,2)]. *Ejung-tang* is composed of 4 different herbal drugs; Ginseng Radix, Atractylodis Rhizoma, Glycyrrhizae Radix, Zingiberis Rhizoma[1-5)] In Donguibogam, a classic traditional Korean medical guide, *Ejung-tang* was explained as an important drug for 'Spleen Yang Deficiency'[3)].

Traditionally in Korea, steamed Ginseng Radix has been termed red ginseng, while non-steamed Ginseng Radix has been termed white ginseng. Thus, *Ejung-tang* can be divided into red ginseng-*Ejung-tang* and white ginseng-*Ejung-tang* according to the type of Ginseng Radix[6,7)].

It is well known that lipopolysaccharide (LPS)

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stimulates macrophages to produce a large amount of cytokines such as interleukin(IL)-1, IL-6, IL-12, IL-17, IL-23, tumor necrosis factor-alpha(TNF-α), interferon-inducible protein(IP)-10, keratinocyte-derived chemokine(KC), macrophage inflammatory protein(MIP)-1alpha, MIP-1beta, MIP-2, monocyte chemoattractant protein(MCP)-1, MCP-3, other chemokines), vascular endothelial growth factor (VEGF), macrophage-colony stimulating factor(M-CSF), granulocyte macrophage-colony stimulating factor(GM-CSF), granulocyte colony-stimulating factor(G-CSF), platelet derived growth factor(PDGF)-BB, and basic-fibroblast growth factor(FGF)[8,9]. But the excessive production of cytokines by highly activated macrophages can lead to acute and chronic inflammatory disorders. Recently, many studies on anti-inflammatory effects of traditional medicines have been reported. Recently, both red ginseng-*Ejung-tang* water extract (ER) and white ginseng-*Ejung-tang* water extract (EG) were reported to increase the intracellular hydrogen peroxide production of RAW 264.7 mouse macrophages[5]. However, the inhibitory effect of ER on cytokine production of LPS-stimulated macrophages has not yet been fully reported.

In our study, we investigated that the inhibitory effect of red ginseng-*Ejung-tang* water extract (ER) on production of cytokines (IL-6, IL-10, IL-2, IL-12p70, VEGF, MCP-1, MIP-2, KC, TNF-α, and GM-CSF) in LPS-induced mouse macrophages RAW 264.7.

Materials and Methods

1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (PBS, pH 7.4), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, USA). LPS and all other chemicals were purchased from Sigma-Aldrich (St. Louis, USA). The multiplex bead-based cytokine assay kits used for the determination of cytokine concentration were purchased from Bio-Rad (Hercules, USA) and Panomics (Redwood City, USA)[5-7, 9-11].

2. ER Preparation

Red ginseng (No. 201006-9) was obtained from Korean Ginseng (Daejon, Korea). Atractylodis Rhizoma (No. 201006-10), Glycyrrhizae Radix (No. 201006-11), and Zingiberis Rhizoma (No. 201006-12) were obtained from Omniherb (Yeongcheon, Korea). Red ginseng (12.5 g), Atractylodis Rhizoma (12.5 g), Glycyrrhizae Radix (12.5 g), and Zingiberis Rhizoma (12.5 g) were mixed and extracted with 2 L of boiling distilled water at 100℃ for 2 hrs., then the extract was filtered and lyophilized. The powdered extract (ER) was dissolved in saline and then filtered through a 0.22-μm syringe filter before experiments[5-7, 9-11].

3. Cell culture

RAW 264.7 mouse macrophage cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM supplemented with 10% FBS containing 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C in a 5% CO_2 humidified incubator.[5-7, 9-11].

4. Multiplex cytokine assay

Cytokines released from treated RAW 264.7 macrophages for 24 hrs were measured in cell culture supernatants using a Luminex assay based on xMAP technology. This assay was performed with Bio-Plex cytokine assay kits (Bio-Rad) and Procarta cytokine assay kits (Panomics), Bio-Plex

200 suspension array system (Bio-Rad) as described previously[9,10]. Standard curves for each cytokine were generated using the kit-supplied reference cytokine samples. Production of the following cytokines was assessed: IL-6, IL-10, IL-2, IL-12p70, VEGF, MCP-1, MIP-2, KC, TNF-α, and GM-CSF.

5. Statistical analysis

The data from more than three independent experiments were represented as mean ± SD. Significant differences were examined using nonparametric Moses-test with SPSS 11.0 software (SPSS, Chicago, USA). The significant p value was 0.05.

Results

1. Effect of ER on IL-6 production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on IL-6 production in LPS-induced RAW 264.7 macrophages are represented in Fig. 1. ER significantly decreased the production of IL-6 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL (P < 0.05).

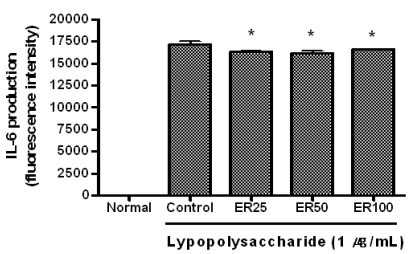

Fig. 1. Effect of ER on production of IL-6 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents P < 0.05 compared to the control.

2. Effect of ER on IL-10 production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on IL-10 production in LPS-induced RAW 264.7 macrophages are represented in Fig. 2. ER significantly decreased the production of IL-10 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL (P < 0.05).

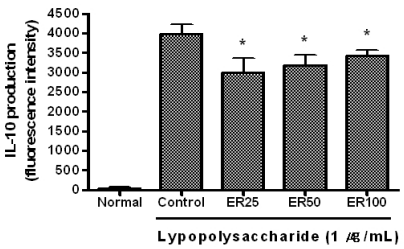

Fig. 2. Effect of ER on production of IL-10 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents P < 0.05 compared to the control.

3. Effect of ER on IL-2 production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on IL-2 production in LPS-induced RAW 264.7 macrophages are represented in Fig. 3. ER significantly decreased the production of IL-2 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL (P < 0.05).

4. Effect of ER on IL-12p70 production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on IL-12p70 production in LPS-induced RAW 264.7

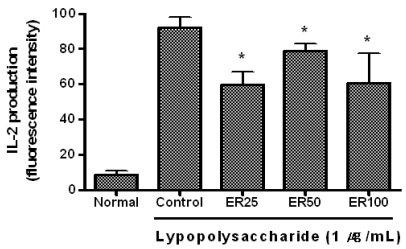

Fig. 3. Effect of ER on production of IL-2 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

macrophages are represented in Fig. 4. ER significantly decreased the production of IL-12p70 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL ($P < 0.05$).

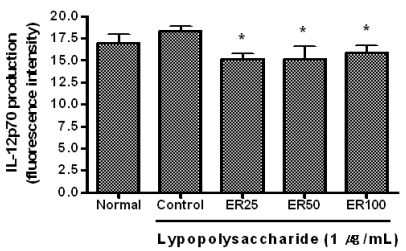

Fig. 4. Effect of ER on production of IL-12p70 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

5. Effect of ER on VEGF production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on VEGF production in LPS-induced RAW 264.7 macrophages are represented in Fig. 5. ER significantly decreased the production of VEGF in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL ($P < 0.05$).

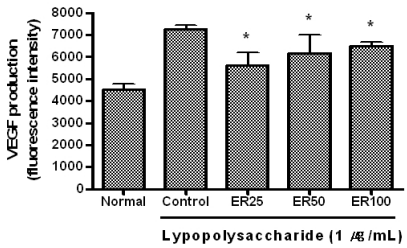

Fig. 5. Effect of ER on production of VEGF in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

6. Effect of ER on MCP-1 production of LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on MCP-1 production in LPS-induced RAW 264.7 macrophages are represented in Fig. 6. ER significantly decreased the production of MCP-1 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 ㎍/mL ($P < 0.05$).

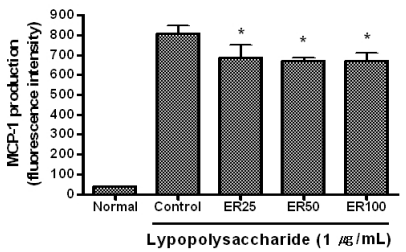

Fig. 6. Effect of ER on production of MCP-1 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

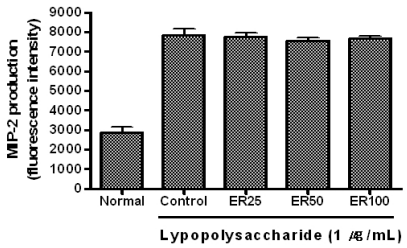

Fig. 7. Effect of ER on production of MIP-2 in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

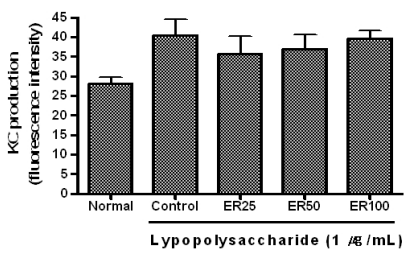

Fig. 8. Effect of ER on production of KC in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

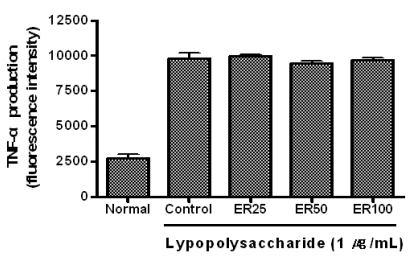

Fig. 9. Effect of ER on production of TNF-α in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

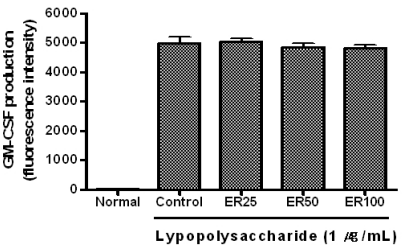

Fig. 10. Effect of ER on production of GM-CSF in LPS-induced RAW 264.7 cells.

Cells were incubated with LPS (1 ㎍/mL) and ER (0, 25, 50, 100 ㎍/mL) for 24 hrs. Results are represented as mean ± SD.
Normal: Normal group treated with culture medium only.
Control: Control group treated with LPS only.
* represents $P < 0.05$ compared to the control.

7. Effect of ER on production of MIP-2, KC, TNF-α, and GM-CSF in LPS-induced RAW 264.7 cells

The effects of 24 hrs treatments with ER on MIP-2, KC, TNF-α, and GM-CSF in LPS-induced RAW 264.7 cells are represented in Fig. 7, Fig. 8, Fig. 9, and Fig. 10. ER did not show any significant effect associated with production of MIP-2, KC, TNF-α, and GM-CSF in LPS-induced mouse macrophages.

Discussion

Macrophages, the most famous innate immune system players, produce various inflammatory mediators such as nitric oxide (NO), cytokines, and prostaglandins against various infectious pathogens such as bacteria, virus, fungus, and parasites[8,9].

LPS, a kind of endotoxin, was reported to stimulate macrophages to produce a large amounts of inflammatory mediators such as NO, prostaglandins, and cytokines[9].

It is well known that macrophages stimulated by LPS produce excessive cytokines such as IL-1alpha, IL-1, IL-6, IL-10, IL-12, VEGF, GM-CSF, TNF-α, MCP-1, MCP-3, IP-10, and

KC. These excessively produced and unregulated cytokines sometimes can provoke acute or chronic inflammatory disorders. It has been reported that IL-6 and IL-12 were increased in autoimmune diseases such as rheumatic arthritis and multiple sclerosis[15], and IL-10 was reported to be increased in systemic lupus erythematosus and Crohn's disease[16]. During lung inflammation, it has been also reported that MIP-2, MCP-1, M-CSF, IP-10, and GM-CSF were increased in the bronchoalveolar lavage fluid[17-19]. Appelmann et al. reported that VEGF and GM-CSF increase in endometriosis[20]. Delgado et al. reported that IP-10 and KC increase in neurodegenerative disease[21]. MCP-1, MCP-3, IL-1alpha, IL-2, IL-5, IL-9, IL-13, G-CSF, and KC were reported to be hypersecreted in airway hyperreactivity and chronic airway inflammatory diseases such as asthma[22-24].

Recently, it was reported that both ER and EG have immune-enhancing properties related with their increasing effects on the intracellular hydrogen peroxide production of RAW 264.7 mouse macrophages[5]. Both ER and EG have also been reported to have anti-inflammatory properties related with their inhibition of NO and hydrogen peroxide production in lipopolysaccharide (LPS)-induced mouse macrophages[6,7]. Seo et al.[25] have reported that the anti-allergic activities of the newly prepared Yijungtang (YJT; 人蔘 Ginseng Radix 4g, 附子 Aconiti Iateralis Preparata Radix 4g, 肉桂 Cinnamomi Cortex 4g, 黃芪 Astragali Radix 4g, 白茯苓 Hoelen 4g, 白朮 Atractylodis Rhizoma Alba 4g, 當歸 Angelicae Gigantis Radix 4g, 乾地黃 Rehmanniae Radix Preparat 4g, 木香 Aucklandiae Radix 4g, 甘草 Glycyrrhizae Radix 2g) may be mediated by down-regulation of Th2 cytokines, such as IL-4 and IL-13, through the regulation GATA-2, NF-AT2 and NF-kB transcription factors in RBL-2H3 mast cells, which means YJT may play an important role in recovering AD symptoms. However, the effects of ER on proinflammatory mediators secreted in LPS-stimulated macrophages have not been fully reported yet. In the present study, we investigated that the inhibitory effect of ER on production of cytokines such as IL-6, IL-10, IL-2, IL-12p70, VEGF, MCP-1, MIP-2, KC, TNF-α, and GM-CSF in LPS-induced mouse macrophages RAW 264.7.

Our experimental data showed that after 24 hrs ER treatments with LPS, ER significantly decreased the production of IL-6, IL-10, IL-2, IL-12p70, VEGF, and MCP-1 in LPS-induced mouse macrophages at the concentrations of 25, 50, and 100 μg/mL ($P < 0.05$ vs LPS alone). But ER did not show any significant effect on production of MIP-2, KC, TNF-α, and GM-CSF in LPS-induced mouse macrophages. This leads us to conclude ER might have anti-inflammatory properties related with its inhibition of cytokine production in LPS-induced macrophages. Because of this modulatory effect on some specific cytokines, ER could be a candidate for alleviation of inflammatory diseases, i.e. rheumatoid arthritis with increase of IL-6, autoimmune encephalomyelitis with increase of IL-12, asthma with increase of IL-2, lung inflammation with increase of MCP-1, Crohn's disease with increase of IL-10, and endometriosis with increase of VEGF. Further studies are needed to verify the precise mechanism regulating anti-inflammatory effect of ER on macrophages.

Conclusion

ER significantly decreased levels of IL-6, IL-10, IL-2, IL-12p70, VEGF, and MCP-1 for 24 hrs incubation at the concentrations of 25, 50, and 100 μg/mL in LPS-induced RAW 264.7 cells ($P < 0.05$). But ER did not exert significant effects on production of MIP-2, KC, TNF-α, and GM-CSF in

LPS-induced RAW 264.7 cells. These results suggest that ER has anti-inflammatory properties related with its inhibition of cytokine production in LPS-induced macrophages. The evaluation of mechanism for anti-inflammatory effect of ER needs more precise research.

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