Activation of inducible nitric oxide synthase by Kagamjuaguiem in peritoneal macrophages in mice

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Received June 16, 2006

Background & objectives: A Korean herbal formula Kagamjuaguiem (KJE) has been used for the purpose of the tumour therapy. However, its mechanism of action is not clear. Nitric oxide (NO) as a potent macrophage-derived effector molecule against a variety of tumours has received increasing attention. In this study, using mouse peritoneal macrophages, we have examined the mechanism by which KJE regulates NO production.

Methods: Peritoneal macrophages were cultured with recombinant interferon-γ (γIFN-γ) for 6 h. The cells were then stimulated with various concentrations of KJE. NO synthesis in cell cultures was measured by Griess method, and inducible NOS expression was measured by western blotting. The amount of tumour necrosis factor-α (TNF-α) secreted by the cell was measured by a modified enzyme-linked immunosorbent assay.

Results: When KJE was used in combination with γIFN-γ there was a marked co-operative induction of NO production. However, KJE had no effect on NO production by itself. The increased production of NO from rIFN-γ plus KJE-stimulated cells was almost completely inhibited by pre-treatment with pyrrolidine dithiocarbamate, an inhibitor of nuclear factor kappa B (NF-κB). Further, treatment of peritoneal macrophages with rIFN-γ plus KJE caused a significant increase in TNF-α production.

Interpretation & conclusion: Our findings demonstrate that KJE increases the production of NO and TNF-α by rIFN-γ-primed macrophages and suggest that NF-κB plays a critical role in mediating these effects of KJE.

Key words Kagamjuaguiem - macrophages - nitric oxide - tumour necrosis factor-α

Kagamjuaguiem (KJE), a traditional Korean medicine, has been developed for tumour therapy, and used clinically for various kinds of tumours. KJE is a modified prescription of Juaguiem, which has been used for the diseases of reproductive system, menstruation diseases, and menopausal disorders1,2. KJE was designed with the purpose of enhancing tumour treatment effect, and is currently used as a supplement during cancer therapy. However, its pharmacological mechanism has not been defined.

Nitric oxide (NO) is a highly reactive molecule produced from a guanidine nitrogen of NO synthase (NOS) enzymes3. In the recent past, NO has received
attention as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumours\(^6\). Evidence of tumour cell cytostasis and cytotoxicity was found in macrophage-tumour cell cocultures in which cytokine- and/or lipopolysaccharide (LPS)-stimulated macrophages inhibited metabolic functioning of co-cultured tumour cells\(^5\).

Three isoforms of NOS have been identified and are classified into two major categories, namely, constitutive and inducible NOS (iNOS). Neuronal and endothelial NOSs, which are constitutively expressed, are activated by calcium and calmodulin\(^6\). Of the three NO synthases, iNOS, the high-output isoform, is the most widely expressed in various cell types after its transcriptional activation\(^7\). Most importantly, iNOS is highly expressed in LPS-activated macrophages, and this contributes to the pathogenesis of septic shock\(^8\). NO may also be induced in target cells themselves yielding apoptotic cell death induced by autoexpression of iNOS\(^9\). The activity of NOS can be inhibited by \(\text{N}^\text{G}\)-monomethyl-L-arginine (\(\text{N}^\text{G}\text{MMA}\), substrate analogues) or \(\text{N}\text{a}-\text{Tosyl-Phe}\) chloromethyl ketone (TPCK, an inhibitor of iNOS)\(^10\).

The proinflammatory cytokine tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) regulates systemic responses to microbial infection or tissue injury\(^11\). These signals stimulate immune functions and induce expression of acute phase reactants in the liver, among other effects. Production of TNF-\(\alpha\) protein is enhanced by the presence of interferon-\(\gamma\) (IFN-\(\gamma\)). TNF-\(\alpha\) then acts as an autocrine signal to amplify IFN-\(\gamma\)-induced production of NO in macrophages\(^12\). Macrophages are a major source of cytokine such as TNF-\(\alpha\), and induction of cytokine gene expression by LPS occurs primarily at the level of transcription and involves the action of several transcription factors, including members of the nuclear factor-\(\kappa\)B (NF-\(\kappa\)B)/rel, C/EBP, Ets, and AP-1 protein families\(^13\). Especially, NF-\(\kappa\)B bound to specific consensus DNA elements present on the promoter of target genes initiates the transcription of TNF-\(\alpha\), iNOS, cyclo-oxygenase-2 and interleukin-6 (IL-6)\(^14\).

In the present study, we investigated the induction of NO and TNF-\(\alpha\) production by KJE in peritoneal macrophages treated with recombinant IFN-\(\gamma\) (rIFN-\(\gamma\)). To investigate the mechanism of KJE-induced NO and TNF-\(\alpha\) production, we examined the ability of NF-\(\kappa\)B inhibitors such as pyrrolidine dithiocarbamate (PDTC) to block the KJE-induced effect.

### Material & Methods

**Peritoneal macrophage cultures:** Thioglycollate (TG, Difco Laboratories, Detroit, MI, USA)-elicited peritoneal macrophages were harvested 3–4 days after i.p. injection of 2.5 ml TG to the mice (Orient Co., LTD., Sungnam, Gyeonggi-do, Republic of Korea) and isolated, as reported previously\(^15\). Using 8 ml of Hank’s balanced salt solution (HBSS, Gibco BRL, Grand Island, NY, USA) containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma, St. Louis, MO, USA), which was supplemented with 10 per cent heat-inactivated foetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA), in 24-well tissue culture plates (2.5 \(\times\) 10\(^5\) cells/well) incubated for 3 h at 37°C in an atmosphere of 5 per cent CO\(_2\), washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10 per cent FBS before treatment.

**Preparation of KJE:** KJE was prepared by decocting the dried prescription of herbs (114g) with 1 l distilled water. The decoction was made by 300 W decocting machine for 3 h. The decoction was filtered, lyophilized and kept at 4°C. Dilutions were made in distilled water then filtered through 0.45 μm syringe filter. The ingredients of KJE include 8 g of *Rehmanniae radix*, 6 g each of *Dioscoreae rhizoma*, *Mori radicis cortex*, *Lycii radicis cortex*, respectively, and 4 g each of *Poria*, *Lycii Fructus*, *Anemarrhenae Rhizoma*, *Fritillariae Bulbus*, *Ephedrae herbae*, *Armeniacae amarum semen*, *Schisandraceae fructus*, *Pinelliae rhizoma*, *Citri reticulatae pericarpium*, *Ponciri seu aurantii fructus*, *Platyctodi radix*, *Citri reticulatae virdie pericarpium*, *Perillae semen*, *Peucedani radix*, *Asteris radix*, *Farfarae flos*, *Ophiopogonis radix*, *Asparagus radix*, *Angelicae sinens radix*, *Scutellariae radix*, *Lilii bulbus*, *Paeoniae radix alba*, respectively. These plant materials were obtained from Oriental Medicine Hospital (Jeonju, Jeonbuk, Republic of Korea), Wonkwang University and authenticated by E.J. Park, College of Oriental Medicine, Wonkwang University. Their voucher specimens have been deposited at the Herbarium at the College of Oriental Medicine, Wonkwang University.

The amounts of KJE in dried plant for adult Korean (average body weight 60 kg) are about 5-7.5 g. The yield of powdered extraction is about 10 per cent (w/w). On this basis, the dose of KJE in powdered extraction for an adult person can be about 0.01 g/kg. The dose range of 0.01-1 mg/ml was chosen to see the dose-dependency.
Measurement of nitrite concentration: Peritoneal macrophages (2.5 × 10^6 cells/well) were cultured with rIFN-γ (10 U/ml) for 6 h. The cells were then stimulated with various concentrations of KJE. To examine the effect of inhibitors (N^6 MMA, PDTC), they were stimulated before rIFN-γ treatment for 30 min. NO synthesis in cell cultures was measured by a microplate assay method, as previously described. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulphanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader (Molecular Devices, Orleans, CA, USA). NO₂⁻ was determined using sodium nitrite as a standard. A control resident cells (2-4 × 10⁶ cells/well) were incubated for 6 h with rIFN-γ (R&D Systems, Minneapolis, MN, USA; 10 U/ml). The cells were then stimulated with KJE (0.1 and 1 mg/ml) or LPS (10 μg/ml) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer [62.5 mM Tris-HCl, pH 6.8, 2 per cent sodium dodecyl sulphate (SDS), 20 per cent glycerol, and 10% 2-mercaptoethanol]. Proteins in the cell lysates were then separated by 10 per cent SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5 per cent skim milk in phosphate-buffered saline (PBS)-tween-20 for 1 h at room temperature and then incubated with 1 μg/ml anti-iNOS antibodies (Santa Cruz Biotechnology, Inc., CA, USA) for 1 h. After washing in PBS-tween-20 three times, the blot was incubated with 1:2000 diluted anti-rabbit IgG, horseradish peroxidase linked whole antibody (Amersham Corp. Newark, NJ, USA) for 30 min, and the antibody-specific proteins were visualized using the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Western blot analysis: Peritoneal macrophages (5 × 10^6 cells/well) were incubated for 6 h with rIFN-γ (R&D Systems, Minneapolis, MN, USA; 10 U/ml). The cells were then stimulated with KJE (0.1 and 1 mg/ml) or LPS (10 μg/ml) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer [62.5 mM Tris-HCl, pH 6.8, 2 per cent sodium dodecyl sulphate (SDS), 20 per cent glycerol, and 10% 2-mercaptoethanol]. Proteins in the cell lysates were then separated by 10 per cent SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5 per cent skim milk in phosphate-buffered saline (PBS)-tween-20 for 1 h at room temperature and then incubated with 1 μg/ml anti-iNOS antibodies (Santa Cruz Biotechnology, Inc., CA, USA) for 1 h. After washing in PBS-tween-20 three times, the blot was incubated with 1:2000 diluted anti-rabbit IgG, horseradish peroxidase linked whole antibody (Amersham Corp. Newark, NJ, USA) for 30 min, and the antibody-specific proteins were visualized using the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Assay of TNF-α release: Peritoneal macrophages (2.5 × 10^6 cells/well) were incubated with rIFN-γ (10 U/ml), KJE, rIFN-γ plus LPS (10 μg/ml), and rIFN-γ plus various concentrations of KJE (0.01, 0.1, and 1 mg/ml) for 24 h. Then the amount of TNF-α secreted by the cells was measured using a modified enzyme-linked immunosorbent assay (ELISA), as described previously. ELISA for TNF-α was carried out in duplicate in 96- well ELISA plates (Nunc, Denmark). The plate was coated with each of 100 μl aliquots of anti-mouse TNF-α monoclonal antibodies at 1.0 μg/ml in PBS at pH 7.4, and was incubated overnight at 4°C. The plates were washed in PBS containing 0.05 per cent tween 20 and blocked with PBS containing 1 per cent BSA, 5 per cent sucrose and 0.05 per cent NaN₃ for 1 h. After additional washes, samples or TNF-α standards were added and incubated at 37°C for 2 h. The wells were then washed and each of biotinylated anti-mouse TNF-α was added and plates were incubated for 20 min at 37°C. Wells were again washed and 2,2'azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) tablets substrate was added. Colour development was measured at 405 nm using an automated microplate ELISA reader.

Statistical analysis: The experiments were repeated at least three times and data presented are the mean ± SE of three experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey, and Duncan post hoc test to express the difference among the groups. All statistical analyses were performed using SPSS v12.0 statistical analysis software. A value of P < 0.05 was considered significant.

Results

Effects of KJE on NO production in activated peritoneal macrophages: To determine the effects of KJE on viability of mouse peritoneal macrophages, we carried out 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl C-tetrazolium bromide (MTT) assay. When we treated the cells with KJE (1 mg/ml), the viability was 101.4 ± 4.5 per cent compared with non-treated group as 100 per cent. The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. KJE had no effect on NO production in resting mouse peritoneal macrophages. IFN-γ (10 U/ml) alone did not cause the induction of NO production as shown in previous reports. However, when IFN-γ was used in conjunction with LPS, there was marked augmentation in the production of NO. When mouse peritoneal macrophages were primed with murine rIFN-γ for 6 h and then treated with KJE, NO production was significantly increased compared with non-primed conditions (Fig. 1).

Effects of KJE on rIFN-γ-primed iNOS expression: Fig.2 shows the effects of rIFN-γ plus KJE treatments
on the expression of iNOS protein in mouse peritoneal macrophages. rIFN-γ plus KJE synergistically increased the expression of iNOS protein in mouse peritoneal macrophages.

**Inhibition of KJE-induced NO production by N^G^MMA**:
The production of nitrite by rIFN-γ plus KJE in mouse peritoneal macrophages was progressively inhibited with increasing amount of N^G^MMA. The KJE-induced accumulation of nitrite was significantly blocked by N^G^MMA (1-100 μM) (Fig. 3).

**Inhibition of KJE-induced NO production by PDTC**: As an approach to define the signaling mechanism of KJE on NO production, we examined the influence of NF-κB inhibitor, PDTC, in rIFN-γ plus KJE (1 mg/ml)-treated mouse peritoneal macrophages. Adding PDTC (50 μM) to the rIFN-γ plus KJE-treated mouse peritoneal macrophages decreased the synergistic effects of KJE on NO production significantly \((P<0.05)\) (Table I).

**Effects of KJE on rIFN-γ-induced TNF-α production**: We examined the synergistic co-operative effects of KJE on rIFN-γ-induced TNF-α production. KJE in

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**Fig. 1.** Dose-dependent effects of KJE on NO synthesis in rIFN-γ-treated peritoneal macrophages. Peritoneal macrophages (2.5 × 10^6 cells/well) were cultured with rIFN-γ (10 U/ml). The peritoneal macrophages were then stimulated with various concentrations of KJE for 6 h after incubation. After 48 h of culture, NO release was measured as a form of nitrite. NO (nitrite) released into the medium is presented as the mean ± S.E. of duplicate determinations from three separate experiments. *P < 0.05, **P < 0.001 compared with rIFN-γ alone.

**Fig. 2.** Effects of KJE on the expression of iNOS by rIFN-γ plus KJE-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The peritoneal macrophages were then stimulated with KJE (0.1 mg/ml and 1 mg/ml) or LPS (10 μg/ml) for 12 h. The protein extracts were prepared, and then samples were analyzed for iNOS expression by Western blotting. The experiment was repeated three times, and the data shown are from one such experiment. 1, blank; 2, rIFN-γ; 3, LPS; 4, rIFN-γ + LPS; 5, KJE (1 mg/ml); 6, rIFN-γ + KJE (0.1 mg/ml); 7, rIFN-γ + KJE (1 mg/ml).

**Fig. 3.** Effects of N^G^MMA on KJE-induced nitrite accumulation in the cultured medium of peritoneal macrophages. Peritoneal macrophages (2.5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml) plus various concentrations of N^G^MMA. The peritoneal macrophages were then treated with KJE (1 mg/ml) and cultured for 48 h. NO release was measured as a form of nitrite. NO (nitrite) released into the medium is presented as the mean ± SE of duplicate determinations from three separate experiments. *P < 0.05, **P < 0.001 compared with control (absence of N^G^MMA).
pathways can be envisioned as a means to influence various immune response conditions. Addition of NF-κB inhibitor, PDTC (50 μM), to the rIFN-γ plus KJE (1 mg/ml)-treated mouse peritoneal macrophages decreased the synergistic effects of KJE on TNF-α production significantly (Table II).

Discussion

In this study, we demonstrated that KJE in combination with rIFN-γ could stimulate NO production in mouse peritoneal macrophages. KJE had a significant dose-dependent effect on NO production at a concentration of 0.1, and 1 mg/ml in rIFN-γ-treated mouse peritoneal macrophages. Our results suggest that KJE may provide a second signal for synergistic induction of NO production in mouse peritoneal macrophages.

NO from macrophages was shown to inhibit cellular respiration in target cells. Moreover, NO derived from Kupffer, NK cells, and endothelial cells also participate in tumoricidal activity against many types of tumors. Thus NO production via iNOS activation by KJE indicates its various activities such as antimicrobial, antitumoural, and antiviral under specific conditions in vivo.
The mechanism by which TNF is able to kill target cells involves the binding of TNF to TNF receptors. Two distinct TNF receptors, which are present on the majority of cell types and tissues have been described, and are known as TNF receptors type 1 and 2 (TNF-R1, TNF-R2). Binding to TNF-R1 can induce cytotoxicity, antiviral activity, fibroblast proliferation, and induction of NF-κB. Binding to TNF-R2 induces thymocyte and cytotoxic T-lymphocyte proliferation. Taken together; TNF and NO are considered as the most important mediators directly involved in tumour cell killing.

Signal transduction pathway of NO production has been previously reported and it is shown that LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation. NF-κB is now known to be ubiquitously expressed and to play a major role in controlling the expression of protein involved in immune, inflammatory and acute phase responses. Expression of iNOS and TNF-α genes is dependent on the activation of NF-κB. We found that the addition of NF-κB modulator, PDTC, inhibited the synergistic effect of KJE with rIFN-γ on NO and TNF-α production. These results suggest that KJE increases NO and TNF-α production through NF-κB activation.

The combination of IFN-γ and KJE acted synergistically to upregulate NO production and iNOS activity. The promoter of the iNOS gene contains many regulatory regions that act together to bind transcription factors; there are two NF-κB sites that are mainly targets of LPS-induced signaling, and another two for IFN-related transcription factors such as IRF-1 and Stat1α. This suggests that the synergistic upregulation of iNOS activity by KJE plus IFN-γ may be due to activation of NF-κB by the KJE, and of IRF-1 and Stat1α by the IFN-γ. Therefore, the effect of KJE alone was little on the production of NO directly, and KJE had IFN-γ triggering effect on NO production in mouse macrophages.

We suggest that IFN-γ priming KJE administration may be effective on cancer therapy. Windbichler et al. reported that the inclusion of IFN-γ in the first-line chemotherapy of ovarian cancer yielded a benefit in prolonging progression-free survival, and IFN-γ played an important role in amplifying the effects of other drugs. In the clinical trials, patients received 0.1 mg of IFN-γ subcutaneously. Our results showed the dose-dependent effects of KJE, and KJE had a maximal effect on NO production at a concentration of 1 mg/ml in rIFN-γ-treated mouse peritoneal macrophages. This suggests that the combined application of IFN-γ and KJE would be a helpful strategy for cancer therapy, but the clinical dose has to be determined after close investigation.

In conclusion, our results demonstrate that KJE acts as an accelerator of peritoneal macrophage activation by rIFN-γ via a process involving L-arginine-dependent NO production, and it increased the production of TNF-α significantly via NF-κB activation. Although the precise mechanism by which KJE promotes NO and TNF-α production induced by rIFN-γ remains to be elucidated, NO and TNF-α production by KJE might explain its beneficial effect in the treatment of tumours.

Acknowledgment

This work was supported by the Korea Research Foundation grant of the Korean Government Ministry of Education and Human Resource Development (MOEHRO), KRF-2004-042-E00160.

References


