Anti-inflammatory Effect of Oyaksungisan in Peripheral Blood Mononuclear Cells from Cerebral Infarction Patients

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Oyaksungisan, the herbal prescription composed of eleven herbs, has been widely used in treatment of cerebral infarct in Oriental Medicine. However, the mechanisms by which the herbal formula affects on the production of pro- and anti-inflammatory cytokines in cerebral infarction patients remain unknown yet. The secretory levels of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and IL-10 were significantly increased in both LPS and PHA-stimulated peripheral blood mononuclear cells (PBMCs) from cerebral infarction patients. However, pretreatment with Oyaksungisan significantly inhibited the secretion of pro- and anti-inflammatory in PBMCs. Also, Oyaksungisan induced a significant increase of transforming growth factor (TGF)-β1 in PBMCs. Thus, these data indicate that Oyaksungisan may be beneficial in the cessation of inflammatory processes of cerebral infarct through suppression of TNF-α, IL-1β, IL-6, and IL-10 and induction of TGF-β1.

Key words Oyaksungisan; cerebral infarct; pro-inflammatory cytokine; IL-10; TGF-β1

Oyaksungisan, a traditional prescription of Oriental Medicine, has been used in treatment of cerebral infarction patients to ameliorate and decrease morbidity and mortality after stroke. Oyaksungisan is consisted of eleven herbs, containing Ephedra sinica Staff, Citrus unshiu Markovich, Lindera strichnifolia Villars, Zingiber officinale Roscoe, Cnidium officinale Makino, Angelica dahurica Bentham et Hooker, Bombyx mori Linne, Citrus aurantium Linne, Plectodon grandiflorum A. De Candolle, Zizyphus jujuba Miller, and Glycyrrhiza glabra Linne. However, the mechanism of its therapeutic beneficial has not been well defined yet in cerebral infarction patients.

Cerebral infarct usually causes cerebral ischemic insults with irreversible deterioration of CNS behaviors. After the onset of cerebral ischemia, inflammatory process triggers the acceleration of the early onset and functions as a determinant factor in severity of cerebral damage, morbidity and mortality in neurodegenerative brain diseases.1,2 Usually, inflammation is occurred in tissues outside brain as the feature of redness, swelling, and heat. Under cerebral ischemia, acute phase of inflammation initiates to recruit the activated inflammatory cells, including as macrophages and lymphocytes, into the damaged brain lesions. Macrophages and lymphocytes, circulating immune cells, play an essential role to secrete pro-inflammatory cytokines and to activate inflammatory mediators in ischemic status. Other supporting cells, including astrocytes, microglia and endothelia, are also involved in inflammatory processes after cerebral ischemic stroke.

Pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, secreted in the ischemic region by activated immune cells, which drive inflammatory process and accelerates the additional inflammatory processes by inducing inflammatory molecules, such as intercellular adhesion molecules (ICAM), vascular cell adhesion molecules-1 (VCAM-1), and selectin. These inflammatory modulators recruit more circulating leucocytes which infiltrate into ischemic region and lead to further loss of neuronal cells and brain tissue and increase of cerebral infarct size.6—8 Interestingly, blockade of immune reaction and anti-inflammatory agents have been regarded as a potentially therapeutic beneficial in cerebral ischemia over the past decade. Therefore, specific inhibition on the role of pro-inflammatory molecules has been focused in treatment of cerebral infarction patients even though molecular mechanisms are not clearly demonstrated in prevention of subsequent neuronal damages during ischemia.9—11

Anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β1, have been identified to suppress the production of pro-inflammatory cytokine in protection of damaged brain tissues after ischemic stroke. Also, anti-inflammatory cytokines are associated with repairing damaged brain tissues.12—14

To identify the functional effect of Oyaksungisan, we herein investigated the regulatory roles of Oyaksungisan on immune response in peripheral blood mononuclear cells (PBMCs) from cerebral infarction patients.

MATERIALS AND METHODS

Reagents Ficoll-Hypaque, lipopolysaccharide (LPS), phytohemagglutinin (PHA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). RPMI 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) were bought from Gibco BRL (Grand Island, NY, U.S.A.). TNF-α, IL-1β, IL-6, IL-10, and TGF-β1 ELISA kits were obtained from R&D system (Minneapolis MN55413, U.S.A.).

Patients Patients with cerebral infarction were admitted and examined at the Department of Neurology, Wonkwang University Hospital from September 2004 to February 2005. The diagnosis of cerebral infarction was confirmed with computerized tomography (CT), magnetic resonance imaging (MRI), and specific clinical signs, including hemiparesis,
hemiplegia, slurred speech, and facial palsy etc. All patients gave an informed consent before participating in the research protocols, which was approved by the ethics committee of Wonkwang University hospital.

Preparation of Oyaksungisan The ingredients of Oyak-sungisan were *Epheidra sinica* Staff (24 g), *Citrus unshiu* Markovici (24 g), *Lindera strichnifolia* Villars (24 g), *Zingiber officinalis* Roscoe (24 g), *Cnidium officinale* Makino (16 g), *Angelica dahurica* Bentham et Hooker (16 g), *Bombbyx mori* Linne (16 g), *Citrus aurantium* Linne (16 g), *Plant-codon grandiforum* A. De Candolle (16 g), *Zizyphus jujuba* Miller (16 g), and *Glycyrrhiza glabra* Linne (4.8 g). An extract of Oyaksungisan was prepared by decocting the dried prescription of herbs with distilled water (100 g/l). The decoction was filtered, lyophilized, and kept at 4°C. The yield of extraction was about 10% (w/w). The Oyaksungisan powder of water extract was dissolved in sterile saline (100 mg/ml). The final concentration of 1.0, 0.5, 0.25 mg/ml Oyasungisan was used for the experiments. The plant materials were obtained and identified by Professor Byoug S. Moon from Oriental Medical Hospital, Wonkwang University, Korea.

Cell Culture PBMCs from heparinized venous blood of patients with cerebral infarction were isolated by Ficoll-gradient centrifugation, washed three times in PBS and resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% FBS inactivated for 30 min at 56°C. PBMCs were isolated by Ficoll-gradient centrifugation, washed three times in PBS and resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% FBS inactivated for 30 min at 56°C. PBMCs from cerebral infarction patients (n=10, mean age 56±12 years; 3 female and 7 males) were treated with Oyaksungisan for 24 h and cell viability was tested by MTT assay. To evaluate whether Oyaksungisan suppresses pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, PBMCs from cerebral infarction patients were pretreated with Oyaksungisan and further maintained with 0.1 µg/ml LPS and 10 µg/ml PHA for 24 h. The culture supernatant was collected to measure the secretion of pro-inflammatory cytokines by ELISA assay (Fig. 1). As shown in Fig. 2A, treatment with LPS and PHA resulted in a significant increase in secretion of TNF-α (control cells, 1.026±0.203 ng/ml; LPS and PHA-stimulated cells, 4.726±1.678 ng/ml). However, addition of PBMCs with Oyaksungisan significantly suppressed TNF-α production by LPS and PHA in a concentration-dependent manner (*p<0.05).

Also, treatment with LPS and PHA resulted in a significant increase in secretion of IL-1β in PBMCs (control cells, 0.204±0.056 ng/ml; LPS and PHA-stimulated cells, 2.734±

RESULTS

Oyaksungisan Significantly Inhibited the Production of Pro-inflammatory in Human PBMCs from Cerebral Infarction Patients We examined whether Oyaksungisan could regulate pro-inflammatory cytokines in PBMCs from cerebral infarction patients. At first, we confirmed that the concentration of Oyaksungisan used in this study showed no significant effect on viability of PBMCs at 1.0 mg/ml (Fig. 1). PBMCs from cerebral infarction patients (n=10, mean age 56±12 years; 3 female and 7 males) were treated with Oyaksungisan for 24 h and cell viability was tested by MTT assay. To evaluate whether Oyaksungisan suppresses pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, PBMCs from cerebral infarction patients were pretreated with Oyaksungisan and further maintained with 0.1 µg/ml LPS and 10 µg/ml PHA for 24 h. The culture supernatant was collected to measure the secretion of pro-inflammatory cytokines by ELISA assay (Fig. 2). As shown in Fig. 2A, treatment with LPS and PHA resulted in a significant increase in secretion of TNF-α (control cells, 1.026±0.203 ng/ml; LPS and PHA-stimulated cells, 4.726±1.678 ng/ml). However, addition of PBMCs with Oyaksungisan significantly suppressed TNF-α production by LPS and PHA in a concentration-dependent manner (*p<0.05).

Also, treatment with LPS and PHA resulted in a significant increase in secretion of IL-1β in PBMCs (control cells, 0.204±0.056 ng/ml; LPS and PHA-stimulated cells, 2.734±
0.809 ng/ml). As shown in Fig. 2B, pretreatment with Oyaksungisan dramatically down-regulated IL-1β secretion by LPS and PHA in PBMCs from cerebral infarction patients in a dose-dependent fashion (*p < 0.05). We also measured IL-6 production in PBMCs. Treatment with LPS and PHA caused a significantly increase of IL-6 production (control cells, 0.146 ± 0.056 ng/ml; LPS and PHA-stimulated cells, 0.844 ± 0.104 ng/ml), respectively (Fig. 2C). Oyaksungisan markedly suppressed IL-8 production in PBMCs treated with LPS and PHA.

**Oyaksungisan Significantly Inhibited the Production of IL-10 in Human PBMCs of Cerebral Infarction Patients**

To evaluate whether Oyaksungisan regulates the production of anti-inflammatory cytokines, PBMCs were pretreated with Oyaksungisan and maintained with 0.1 µg/ml LPS and 10 µg/ml PHA for 24 h. The culture supernatant was collected to measure the secretion of IL-10 by ELISA assay (Fig. 3). As shown in Fig. 3, treatment with LPS and PHA induced a significant increase in secretion of IL-10 (control cells, 0.08 ± 0.012 ng/ml; LPS and PHA-stimulated cells, 0.429 ± 0.087 ng/ml). Addition of PBMCs with Oyaksungisan significantly suppressed IL-10 production by LPS and PHA in a concentration-dependent manner (*p < 0.05).

**Oyaksungisan Significantly Increased the Production of TGF-β1 in Human PBMCs of Cerebral Infarction Patients**

Also, to assess whether Oyaksungisan produces TGF-β1, an anti-inflammatory cytokine, PBMCs were treated with Oyaksungisan for 24 h. The culture supernatant was collected to measure the secretion of TGF-β1 by ELISA assay (Fig. 4). As shown in Fig. 4, Oyaksungisan markedly up-regulated TGF-β1 secretion in PBMCs of cerebral infarction patients in a dose-dependent fashion (control cells, 30.75 ± 8.26 pg/ml, 1.0 mg/ml of Oyaksungisan-treated cells, 136.5 ± 18.63 pg/ml), (*p < 0.05).

**Characterization of the Principal Components of Oyaksungisan**

Oyaksungisan was analyzed by HPLC. Chromatogram of Oyaksungisan is shown in Fig. 5. Peaks of the principal components have not yet been identified in this study.
MAKINO protects neuronal cell death by Cnidium officinale grandiflorum A. De Candolle, reduced the production of TGF-β in PBMC of cerebral infarction patients. Oyaksungisan also increased the production of pro-inflammatory cytokines, including TNF-α and IL-1β, in PBMCs treated with LPS and PHA, which were significantly decreased by pretreatment with Oyaksungisan in PBMC of cerebral infarction patients. Oyaksungisan also induced the production of TGF-β1, an anti-inflammatory cytokine. Thus, we suggest that the one of the beneficial role of Oyaksungisan in treatment of cerebral infarction patients is associated with its regulatory effects on inflammatory process.

Oyaksungisan is consisted of eleven herbs, containing Ephedra sinica Staff, Citrus unshiu Markovich, Lindera stricnhifolia Villars, Zingiber officinale Roscoe, Cnidium officinale Makino, Angelica dahurica Bentham et Hooker, Bombus mori Linne, Citrus aurantium Linne, Plantcodon grandiflorum A. De Candolle, Zizyphus jujuba Miller, and Glycyrrhiza glabra Linne. These extracts of each herb have been used as oriental traditional medicine and reported their pharmacological effects as follow. Bupleurum falcatum Linne has the anti-inflammatory and anti-arthritis effects. Citrus unshiu Markovich has an anti-inflammatory effect and Zingiber officinale Roscoe is reported to modulate the cellular immune response in vitro and in vivo. Kim et al. reported Cnidium officinale Makino protects neuronal cell death by reducing nitric oxide production.

Under cerebral ischemia state, inflammatory cells infiltrate into the ischemic brain region and trigger to activate inherent brain cells, including astrocytes, microglia and endothelia. Inflammatory responses which affect these cells with substances, including pre-inflammatory cytokines, vasoactive substances and adhesion molecules, play an important role in the pathogenesis of cerebral lesions following cerebral ischemia.

The most important pro-inflammatory cytokines in post-ischemic inflammation include TNF-α, IL-1β, and IL-6. TNF-α and IL-1β have the major role to initiate the inflammatory response mediated by the induction of inflammatory metabolites and increased expression of adhesion molecules on the surface of endothelial cells. TNF-α and IL-1β secreted from brain cells share a high homology in structure and function. TNF-α and IL-1β are also responsible for the accumulation of inflammatory cells in the peripheral nucleus of cerebral infarct and induce a second inflammatory response mediated by IL-6. IL-6 is playing a central role in acute inflammatory processes exhibiting pro-inflammatory activities in many different brain pathologies including cerebral ischemia and excitotoxic brain damage. In addition, to ameliorate brain damages followed by stroke and ischemic attack, there have been reported various therapeutic trials, including anti-TNF-α antibody and TNF-α soluble receptor type 1, IL-1β receptor antagonist, and recombinant IL-1ra. Accumulated evidences have proved that the suppressive approaches of specific inflammatory mediators are believed in one of best solution to prevent leading to secondary ischemia and damage of brain cells under cerebral ischemia.

IL-10, a Th2-type response and an anti-inflammatory cytokine, modulates immune processes by inhibiting Th1-type response, which is associated with the function of antigen presenting cell. Especially, it reduces ischemic infarct size and the production of other pro-inflammatory mediators in brain after stroke. Through deactivation of macrophage-like cells and astrocytes, IL-10 plays a role of inhibiting secondary inflammatory processes by them within in brain. However, it has been reported cerebral infarction patients to have a significantly lower IL-10 serum level than people without.

TGF-β1, a Th3-type response and an anti-inflammatory cytokine, regulates immune processes through the suppression of Th1-type response. TGF-β1 is implicated in tissue repair, differentiation, and various immune functions. After ischemic stroke, TGF-β1 in brain functions on tissue repairing and angiogenesis. Wang et al. reported that it protects the cerebral hemispheres from damage induced by stroke.

In conclusion, our data demonstrate that Oyaksungisan functions to suppress the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in PBMCs from cerebral infarction patients. Simultaneously, Oyaksungisan induces a significant increase of anti-inflammatory cytokines. Taken together, Oyaksungisan has many functions on normalization of homeostatic balance between anti- and pro-inflammatory processes mediated by mediator, including cytokines.

Furthermore, works are undergoing to identify the crucial components of herbal constituents of Oyaksungisan and the precise signaling pathway of Oyaksungisan on inhibitory mechanism in vitro and in vivo animal model of brain ischemia.

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REFERENCES