The effect of extract from leaves and stalks of *Angelica gigas* on the innate immunity

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Abstract

The dried root of *Angelica gigas* (*A. gigas*) has been traditionally used as an oriental medicine, which is known to improve blood circulation and blood stasis. In the present study, leaves and stalks of *A. gigas* were used to investigate their effects on the innate immunity. The extracts were prepared from leaves and stalks of *A. gigas* and were fed to mice. The numbers of blood cells, total WBCs, neutrophils, lymphocytes, eosinophils and basophils were increased by 50% in mice fed with leaves extract of *A. gigas* compared to control mice. However, the numbers of blood cells were decreased when treated with stalks extract of *A. gigas*. The level of cholesterol and triglyceride in serum was markedly reduced in both mice group fed with leaves extract and stalks extract of *A. gigas* compared to control group (*P* < 0.01). There was no significant change in the level of albumin, total protein, phosphate and calcium in serum. Activity of cationic peptide was found to be diffused in the testicles of mice fed with leaves extract of *A. gigas* compared to control group, which might be due to increased lysozyme in testicle. The lysoplate assay and immunohistochemistry assay suggest that the extract of leaves and stalks of *A. gigas* are immunogenic, but the effects might be related with acquired immune response rather than innate immunity.

Key words: *Angelica gigas*, Lysozyme, Innate immunity, Blood assay, Cationic peptide

INTRODUCTION

The innate immunity is evolutionarily a part of protective system in most hosts and functions primarily to protect host from pathogenic microbes. The molecular module of innate immunity has been found both in plants and animals (Hoffmann et al, 1999; Charles and Rulsan, 2002). In 1921, Alexander Fleming discovered a substance from a drop of nasal mucus that can lays bacteria, named as Lysozyme (Fleming, 1922). Lysozyme is a molecule that has antimicrobial effects and its activities in innate immunity have been extensively studied. Lysozyme has more effects like antibacterial, antiviral, antitumor and immune modulatory activities (Vidal et al, 2005). The structure of lysozyme was elucidated by Canfield (1963), which consisting of 129 amino acid residues, including 10 carboxyl and 7 amino groups, 11 arginine residues, 6 tryptophan residues and 4 disulfide bonds. Lysozyme is produced by glandular serous cells, surface epithelial cells and macrophages in the human airway (Ganz, 2004; Konstan et al, 1982; Prager and Jollès, 1996) and is present at high concentration in tears, gastric juice and breast milk (Hankiewicz and Swierczek, 1974). Lysozyme catalyzes the hydrolysis of
the b (1-4) glycosidic bond of the bacterial peptidoglycan, a major component of the bacterial cell wall (Phillips, 1996).

There are three kinds of Angelica species such as A. gigas Nakai (Korean dang gui), A. sinensis (Chinese dang gui), and A. acutiloba (Japanase dang gui). A. gigas has dark purple-colored flowers, whereas A. sinensis and A. acutiloba have white-colored flowers (Ahn et al, 2008). A. gigas has been used as a traditional herbal medicine in oriental medicine, and is also one of the most popular herbal medicine in Asian countries, including Korea, Japan and China. The dried form of root of A. gigas is used in oriental medicine. It has been reported that A. gigas has pharmacological effects such as depression of cardiac contraction, decrease of the carotid arterial pressure and respiration (Chi and Kim, 1970). Recently, some scientists reported that A. gigas had anti-inflammatory effects (Shin et al, 2009). Although the root of A. gigas has been used in oriental medicine, study on the leaves and stalks of A. gigas have not been performed.

In the present study, the extraction was prepared from leaves and stalks of A. gigas, and their effects on the innate immunity were investigated.

**MATERIALS AND METHODS**

**Experimental animals**

Male ICR mice from Daehan biolink (Eumsung, Korea) were used for this experiment. Body weight of mice was 23–26 g. Each cage housed 5 mice in a room temperature with an alternating 12 hrs light-dark cycle. Food, extracted solution and water were available ad libitum. They were divided into 3 groups; control group, experiment 1 group and experiment 2 group. The extraction solution was diluted 2 times for group in this study. We have used an undiluted solution in group 2. A water was supplied to control group. Diluted solution and undiluted solutions were used instead of water for group 1 and group 2.

**Preparation of crude extraction from leaves and stalks of Angelica gigas**

The dried leaves and stalks was cut into thin slices. The extraction method was modified from the article of Choi et al (2003). Crude extract was prepared as follow. 1 kg of sliced leaves and stalks of A. gigas was mixed with 5 L of water. The mixture was boiled on 50°C for 3 hrs. The crude extracted solution was filtered through Whatman No 1. filter paper after cooling at room temperature.

**Body weight measurement of mice**

Body weight measure to watch the mice body weight fluctuation has carried out by group using a electric balance.

**Collection of blood and intestinal organs**

Mouse was administered intraperitoneally with 3.6% chloral hydrate 0.1 mL per 10 g of body weight for anesthesia. Blood was collected from abdominal vena cava and intestinal organs collection was carried out under the stereoscopic microscope. Blood analysis was performed using HEMAVET (Drew Scientific, USA) for measure of total WBC, lymphocytes, neutrophils, monocytes, eosinophils, basophils after homogenized the tube contained heparin solution for 30 mins. Serum analysis was carried out cholesterol, triglyceride, albumin, total protein, calcium, phosphate by serum analyst (Biochemical system SM 4000, Italy).

**Extraction of cationic peptide**

Cationic peptide extraction method was based on the method of Cole et al (1999). Extraction process is as follow, It takes 200 mg of organs and put into the 1.5 mL centrifuge tube. They were sonicated after adding 400 μL of 5% glacial acetic acid into the tube. To obtain the supernatnat after centrifugation at 13,000 rpm, add same volume of Macro-Prep CM cation exchange support (Bio-rad, USA) to the supernatent and mix gently for 3 hrs at room temperature. Removed the super-
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Table 1.

A variation of body weight after feeding a mouse on with *A. gigas* extracted solution (g: Mean±SE)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>The first body weight</td>
<td>25</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>Average</td>
<td>24.6±0.41</td>
<td>33.2±0.47</td>
<td>32.8±0.59**</td>
</tr>
</tbody>
</table>

*P < 0.05, **P > 0.05 (Compared to the control), determined by the student’s t-test. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

Table 2.

White blood cell (WBC) analysis of mice after feed on with *A. gigas* extracted solution (Mean±SE)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBCs</td>
<td>5.12±0.35</td>
<td>7.65±0.26*</td>
<td>2.31±0.19*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.56±0.12</td>
<td>1.38±0.05*</td>
<td>0.69±0.10***</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>4.30±0.38</td>
<td>7.02±0.16*</td>
<td>2.02±0.25*</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.18±0.09</td>
<td>0.23±0.05***</td>
<td>0.08±0.02***</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.06±0.03</td>
<td>0.52±0.08*</td>
<td>0.02±0.008***</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.03±0.02</td>
<td>0.08±0.007**</td>
<td>0.01±0.006**</td>
</tr>
</tbody>
</table>

*N: Normal. *P < 0.01, **P < 0.05, ***P > 0.05 (Compared to the control), determined by the student’s t-test. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

Cationic peptide quantity

BCA Protein assay kit (Pierce, USA) was used for Cationic peptide quantity that was modified the Lowry’s method (Lowry et al, 1951) to the measurement of total protein. The measurement was performed by following the manufacture's manual. The standard curve was produced first, and then the amount of cationic peptide was calculated using the standard curve after the OD read at 550 nm by the spectrophotometer (Biotek, USA).

Lysoplate assay

This experiment has carried out to measurement of the activity of cationic peptide called the substrate related to innate immunity. Assay method was used modified from method of the Osserman and Lawlor (1966). Briefly, 0.125 g of heat killed *Micrococcus lysodeikticus* (Sigma, USA) put into the 50 mL of 66 mM sodium phosphate buffer (pH 7.0) and homogenized. We called this solution A. We have made solution B that was 5% agarose gel using a low melting agarose (Sigma, USA) and 66 mM sodium phosphate buffer (pH 7.0). Solution B was maintained at 42°C. Assay plate was made in a square petri dish with 13 mm Grid (BD Falcon, USA) by final concentration of 1% was mixed sloution A and solution B. The same amount of cationic peptide put into the hole of solidificated gel and measured the diameter of cationic peptide diffusion after react for 18 hrs.

Immunohistochemistry study

The organs were fixed at 10% neutralized formalin solution. Immunostaining was performed using the histostain-sp kit (Invitrogen, USA). Five-micrometer sections were cut from the paraffin blocks and mounted on charged slides. The sections were deparaffinized, rehydrated, and treated with serum blocking reagent for 10 mins to block endogenous peroxidase activity. Sections then were rinsed and incubated with primary antibodies against lysozyme (Dako, Denmark).
Table 3. Serum analysis of mice after feed on with A. gigas extracted solution (Mean±SE)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Control</th>
<th>Exp 1</th>
<th>Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>76.2±4.54</td>
<td>36±2.33*</td>
<td>55.8±6.34***</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>172.4±4.87</td>
<td>125.8±6.18*</td>
<td>121.6±5.00*</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.66±0.25</td>
<td>2.59±0.19***</td>
<td>2.53±0.15***</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>2.83±0.2</td>
<td>2.76±0.12***</td>
<td>2.72±0.19***</td>
</tr>
<tr>
<td>Phosphate (mg/dL)</td>
<td>16.45±0.86</td>
<td>15.76±0.31***</td>
<td>16.78±1.11***</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>7.3±0.45</td>
<td>7.5±0.28***</td>
<td>8.52±0.26**</td>
</tr>
</tbody>
</table>

*P<0.01, **P<0.05, ***P>0.05 (Compared to the control), determined by the student’s t-test. Exp 1: A. gigas extracted solution was diluted 2 times with water. Exp 2: A. gigas extracted solution undiluted.

RESULTS

Body weight variation of mouse

The results are shown by Table 1, after feeding a mouse on with A. gigas extracted solution for 20 days. Body weight of experimental groups decreased slightly than the control group, but there were no significant variation of body weight in comparition with the control group.

Analysis of White blood cells and serum

The results of differential analysis on white blood cells (WBC) are shown in Table 2. The experiment 1 group increased about 50% more than the control group in the number of total WBCs but the experiment 2 group decreased by more than 50% compared to the control group. Significance of experiment 1 group and experiment 2 group was accepted by P value is under 0.01 each. There were significance between experiment 1 group and the control group by P value is under 0.01 but there is no significance between experiment 2 group and the control group because P value of experiment 2 group against with the control was more than 0.05 in the number of neutrophils. In the number of lymphocytes, the experiment 1 group increased more than the control group and the experiment 2 group decreased more than the control group. In significance value, they were accepted in the significance of lymphocyte number test because the P value is under 0.01 each. In the Monocyte, Eosinophil, the significance value was not accepted except for the experiment 1 group in the eosinophil. P value of experiment 1 group in the neutrophil was uner 0.01. In the basophil, the significance of the experiment 1 group was accepted, but the experiment was not.

Cholesterol level in serum was significantly decreased in the experiment 1 group compared to the experiment 2 group. The significance value of experiment 1 group was accepted but experiment 2 group was not accepted. Triglyceride level was significantly decreased in the experiment 1 group and the experiment 2 group. Significance value also was accepted in each of the experiment groups. In the level of albumin, total protein, phosphate, calcium, there were no difference in the level of the experimental groups with the control group. Calcium level of the experiment 2 group was increased more than the experiment 1 group and the control group (Table 3).

Lysoplate assay

The activity of cationic peptide are shown in Fig. 1. In the results, the cationic peptide activy shows no significant differences in the lung, liver, kidney and spleen

Fig. 1. Results of lysoplate assay to the cationic peptide activity.
Immunohistochemistry study

The results of the immunohistochemistry stain to see the lysozyme are shown in Fig. 2. The existence of lysozyme was no significance change by immunohistochemistry stain in the lung, liver, or the kidney for the control group, experiment 1 group and experiment 2 group. But the lysozyme was strongly detected in the testicle of experiment 1 group, while the lysozyme was not detected in the experiment 2 group.

**DISCUSSION**

*A. gigas* has been known to nourish the blood vessel and to improve blood circulation and stasis, and has been used as a herbal medicine in oriental medicine. It has been reported that *A. gigas* has anti-microbial and immune-modulating effects via B cell activation (Kumazawa et al, 1982). *A. gigas* increased IgM and IgG antibody titer against sheep erythrocytes (Kumazawa et al, 1985) and mediated the inflammation in mouse macrophage (Yoon et al, 2007).

In the present study, the effects of the leaves and stalks of *A. gigas* were investigated using animal. The total WBC, lymphocytes, monocytes, eosinophils, basophils were increased in mice fed with the leaves extract of *A. gigas* compared to control group. However, the numbers of these blood cells were decreased in mice fed with the stalks extract of *A. gigas*.

The serum levels of cholesterol and triglyceride were greatly reduced only in mice fed with the leaves extract of *A. gigas* compared to control group, but the change in the level of albumin, total protein, phosphate, and calcium was minimal in both animal groups. These results suggest that leaves extract of *A. gigas* induced more effects in innate immunity than stalks extract of *A. gigas*, which might be due to the disagreement of components between leaves and stalks of *A. gigas*.

The higher activity of cationic peptide was shown in the testicles of mice fed with leaves extract of *A. gigas*. The increase of cationic peptide and absence of lysozyme in testicle might be explained that the lysozyme...
was increased at early time in several cells like neutrophils, macrophage, and eosinophil, but be decreased later. These suggest that leaves extract of *A. gigas* could increase innate immunity and might be used as an adjuvant of vaccine.

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**REFERENCES**


