Effects of *Armillaria mellea* Extract on Macrophage and NK Cell Activity

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**Objective**: The purpose of this study was to investigate the effects of *Armillaria mellea* extract (AME) on immune modulation focused on anti-cancer activity.

**Methods**: To prove the effects of AME, we performed NO assay, NK cytotoxicity assay and RT-PCR of cytokine related with macrophage and NK cell activity.

**Results**: AME increased NO production produced by macrophages in part. AME also enhanced the NK cell activities in destroying target cells (YAC-1 cells). AME up-regulated gene expression of IL-1, iNOS, TNF-α in RAW 264.7 cells and IL-1, IL-2, IFN-γ, TNF-α in splenocytes, respectively.

**Conclusion**: From the above results, we assumed that AME is a potential drug for anti-cancer by activation of the macrophages and NK cells.

**Key Words**: *Armillaria mellea* extract (AME), NK cell, macrophage

**Introduction**

The main function of the immune system is the defense of the host organism against infectious agents and malignant cells. Among these immune systems, macrophages and NK cells do prior roles relatively for cancer-specific activities\(^1\)\(^{2}\).

It has been well known that innate and cellular immunity participate in tumor defence mechanisms, such as tumor incidence, growth, metastasis or final clinical outcome. Macrophages kill the enemies mainly by releasing the NO\(^3\) and NK cells destroy the MHC I deficient-tumors. These mechanisms have been focused for cancer treatment in immunotherapy.

In addition, macrophage and NK cells cooperate through the production of cell mediates, for example, interleukin-2 (IL-2), interferon gamma (IFN-γ)\(^4\)\(^5\). These instances of cooperation and modulation are some of major factors to prevent from evading immune surveillance of cancer. Hence, if any candidate is able to enhance the activities of macrophage and NK cell, it is considered as a potentially useful agents for cancer\(^6\)\(^9\)\(^\)\(^12\).

Recently, the immune modulating properties of
various herbal plants explaining the anti-tumor effects have been experimented on extensively and reported over the world. Administration of these oriental herbs is known to inhibit the tumor growth or and incidence, and prolong the tumor bearing rodent survival in transplanted experimental models and also restore the lowered host defense immunity. In our study, to investigate the effects of *Armillaria mellea* extract (AME) on tumor-related immunity, we analyzed the macrophage and NK cell activity through the NO release and NK cytotoxicity including gene expressions of cytokines.

**Materials and Methods**

1) **Materials**

*Armillaria mellea* was received from Daejeon Oriental Medical Hospital. Fifty grams of *Armillaria mellea* was mixed with 2 L of distilled water and left for 1 h at room temperature, and the whole mixture was then boiled for 2 h. The mixture was filtered and then lyophilized. The yield of Armillaria mellea extract (AME) was 10.5% (w/w) in terms of the dried medicinal herbs. M-MLV RT, Taq polymerase, dNTP and 5X TBE buffer were obtained from Bioneer (Cheongwon, Korea). Other chemicals were purchased from Sigma (St. Louis, USA).

2) **Experimental animals**

Specific pathogen-free BALB/c and Sprague-Dawley rats weighting 200-220g were obtained from a commercial animal breeder (Daehan BioLink, Korea). The animals were housed under normal laboratory conditions (22 ± 2°C and 40-60% relative humidity) with 12 h light/dark cycle with free access to standard rodent food and water.

3) **Cell culture**

RAW 264.7 cell was obtained from Korea Research Institute of Bioscience and Biotechnology (KIRBB) and cultured in RPMI 1640 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml streptomycin and 100 units/ml penicillin.

4) **NO assay**

Macrophages were isolated from rat injected intraperitonealy with 50 ml cold D-PBS, then followed centrifugation for 5 min at 400 × g and RBC lysis. The collected cells were washed with PBS and resuspended with RPMI 1640 medium (Sigma, U.S.A) containing 10% bovine serum albumin. Macrophages (1 × 10⁶ cells) were plated in 24-well plates (Nunc, Rosekilde, Denmark) and treated with various concentration of AME (1, 10, 100 µg/ml) and LPS (0.5 µg/ml) and incubated at 37°C with 5% CO2. NO formation was measured as the stable end product nitrite (NO₂⁻) in the culture supernatant with Griess reagent. Briefly, an aliquot of culture supernatant (100 µl) was added to each well of 96-well plates and mixed with the same volume of Griess reagent (1:1[v/v]; 0.1% N-[1-naphthyl]ethylenediamine dihydrochloride in H₂O, 1% sulfanilamide in 5% H₂PO₄), and then the A₅₄₀ was read with microplate reader (Molecular device, USA). Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards. By adding AME to Griess reagent, it was confirmed that AME did not interfere with the nitrite assay.

5) **NK cytotoxicity assay**

⁵¹Cr release assay was performed as described previously with modifications. Spleen cell suspensions were prepared in ice-cold RPMI 1640 medium from BALB/c mice. Hundred microliter of the splenocyte suspension containing 4 × 10⁶, 2 × 10⁶ or 1 × 10⁶ cells/well was plated onto the round bottom 96-well plate (4 well per group) with various concentration of
AME (0.1, 1, 10 μg/ml) and IL-2 (300 U/ml). These cells were incubated for 16 h at 37°C with 5% CO₂ and prepared as effector cells.

Pure NK cell were isolated using MACS system. Hundred microliter of NK cell suspension containing (1 × 10⁵, 5 × 10⁵ and 2.5 × 10⁴ cell/well) was plated onto the round bottom 96 well plate (4 well per group) with various concentration of AME (0.1, 1, 10 μg/ml) and IL-2 (300 U/ml). These cells were incubated for 12 h at 37°C with 5% CO₂ and prepared as effector cells.

On the other hand, YAC-1 cells (5 × 10⁶) were cultured for using as target cells of NK cell. After labeling the target cells by incubating for 2 h (37°C, 5% CO₂) with ⁵¹Cr (200 uCi), and washing and lysis of unhealthy cell, labelled target cells were centrifuged for 5 min at 400 × g, and adjusted to 2 × 10⁵ cell/ml. Fifty microliters of target cell suspension was added to effector cells and incubated for 4 h. Maximum leased groups were added with 50 μl of 2% NP-40, and spontaneous leased group with 50 μl of complete medium. At 4 h, the cells were concentrated by centrifugation for 10 min at 500 × g and cell-free supernatant were extracted from each well for assessment of radioactivity. Then gamma irradiation was assessed in a scintillation counter (Packard Instruments, USA). The percent of specific lysis was calculated by the following equation:

\[
\text{Specific killing activity (\%)} = \frac{\text{AME release-spont. release}}{\text{max. release-spont. release}} \times 100
\]

6) RT-PCR for IL-1, IL-2, IFN-α, TNF-α in splenocytes

BALB/c mice were sacrificed and spleens were removed to PBS. After RBC lysis and washing, spleen cells (2 × 10⁶) were treated with various AME (1, 10, 100 μg/ml) with or without LPS (0.5 μg/ml) in 6 well plate and incubated for 6 h at 37°C with 5% CO₂. Total RNA was isolated by the TRIzol® reagent and all process of first strand cDNA and polymerase chain reaction were done according to the manufacturer’s instructions.

Briefly, PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95°C for 10 min; then 35 cycles at 95°C for 1 min, 60°C for 40 seconds and 72°C for 40 seconds. The PCR products were run on a 1% agarose gel in 0.5×TBE buffer. The used primers were described in Table 1.

7) RT-PCR for IL-1, IFN-γ, TNF-α in RAW 264.7 cells

RAW 264.7 cells (5 × 10⁶) were plated into 6 well and the cells treated with various concentration of AME (1, 10, 100 μg/ml) with or without LPS (0.5 μg/ml) and incubated for 6 h at 37°C with 5% CO₂. Total RNA was

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**Table 1. Oligonucleotide Sequences of Primers**

<table>
<thead>
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<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product(bp)</th>
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<tr>
<td>β-actin</td>
<td>Sense</td>
<td>5’-ACC GTG AAA AGA TGA CCC AG-3’</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5’-TCT CAG CTG TGG TGA AG-3’</td>
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</tr>
<tr>
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<td>Sense</td>
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<td></td>
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<tr>
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isolated by the TRIzol® reagent and all process of first strand cDNA and polymerase chain reaction were done according to the manufacturer's instructions. The used primers were described in Table 2.

8) Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis of the data was carried out by Student’s t-test. A difference from the respective control data at the levels of $p<0.05$, $p<0.01$ and $p<0.001$ was regarded as statistically significant.

Results

1) NO production

NO production was assayed after 12, 24 and 36 hr at various concentrations of AME. NO production was increased just at 100 μg/ml in time-dependent manner but, did not affect in low concentration below 10 μg/ml (Fig. 1).

The effect of AME on rat peritoneal macrophages activated by LPS was investigated. NO production was

Table 2. Oligonucleotide Sequences of Primers

<table>
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<th>Product(bp)</th>
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<tr>
<td>β-actin</td>
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<td>285</td>
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<tr>
<td></td>
<td>Antisense</td>
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<td>IL-1</td>
<td>Sense</td>
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<td>5'-TGG AGG ACT CCT CCC AGG TA-3'</td>
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<td></td>
<td>Antisense</td>
<td>5'-TCG AAG ACT CCT CCC AGG TA-3'</td>
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Fig. 1. Effect of AME on NO production

Rat peritoneal macrophages were treated with AME (1, 10, 100 μg/ml) and PBS (control). NO production was measured on 12, 24 and 36 h after treatment. Each data represents the mean ± SD. Statistically significant value compared with control by t-test. (**: $p<0.01$, ***: $p<0.001$).
Effects of Armillaria mellea Extract on Macrophage and NK Cell Activity

1) NO production

Rat peritoneal macrophages activated by LPS (0.5 μg/ml) were treated with AME (1, 10, 100 μg/ml) and PBS (control). NO production was measured on 12, 24 and 36 h after treatment. Each data represents the mean ± SD. Statistically significant value compared with control by t-test. (*: p<0.05).

2) NK cell activity

AME showed a significant effect on NK cytotoxic activity compared with control at 1 and 10 μg/ml of concentration in all ratios of effector cell : target cell. As

Fig. 2. Effect of AME on NO production

Fig. 3. Effect of AME on NK cell activity
shown in Fig. 3, in ratio of 400 (effector cell) : 1 (target cell), AME (0.1, 1, 10 \( \mu g/ml \)) increased the NK cytotoxic activity significantly. Cytotoxicity at 10 \( \mu g/ml \) of AME increased by 28% which is three fold higher value than that of control.

We also investigated the effect of AME on pure NK cell cytotoxicity. As shown in Fig. 4, AME did not increase cytotoxicity of NK cell at all concentration.

3) Gene expression of IL-1, IL-2, IFN-\( \gamma \), TNF-\( \alpha \) in splenocytes

To investigate gene expression of cytokines in mouse splenocytes, splenocytes were treated with various concentration of AME (1, 10, 100 \( \mu g/ml \)) for 6 h. Another plate was treated with AME and LPS (0.5 \( \mu g/ml \)) together.

As shown in Fig. 5, IL-1 gene expression was increased by 11, 22 and 23% respectively compared with control in dose-dependent manner. When AME treated with LPS together, treated group was shown little up-regulation of IL-1 gene expression compared with control group.

AME up-regulated IL-2 gene expression by 36, 86 and 96% respectively in dose-dependent manner (Fig. 5).

IFN-\( \gamma \) gene expression was not increased in groups treated with 1 and 10 \( \mu g/ml \) of AME. Only in 100 \( \mu g/ml \) treated group, gene expression was increased by 42% compared with control group. But in AME group treated with LPS together, IFN-\( \gamma \) gene expression was increased by 31, 51 and 46% respectively compared with control group (Fig. 5).

TNF-\( \alpha \) gene expression was increased by 2.4, 2.6 and 2.6 fold respectively compared with control. But when AME treated with LPS together, differences from other groups were not shown (Fig. 5).

4) Gene expression of IL-1, iNOS, TNF-\( \alpha \) in RAW 264.7 cells

To investigate cytokine gene expression in RAW 264.7 cells, RAW 264.7 cells were treated with various concentration of AME (1, 10, 100 \( \mu g/ml \)) for 6h. Another plate was treated with AME and LPS (0.5 \( \mu g/ml \)) together.
IL-1 gene expression was increased a little. Maximum gene expression was shown in 10 μg/ml AME treated group. When AME treated with LPS together, Maximum gene expression was shown also in 10 μg/ml of AME treated group (Fig. 6).

Inducible nitric oxide synthase (iNOS) gene expression was increased by 1.8, 2.5 and 1.9 fold respectively compared with control. But when AME treated with LPS together, differences from other groups were not shown (Fig. 6).

TNF-α gene expression was increased by 11, 18 and 23% respectively compared with control. But when AME treated with LPS together, differences from other groups were not shown (Fig. 6).

**Discussion**

It is well known that most cancer cells are less immunogenic, then not susceptible to any component of
the immune system. Because cancer is weak immunogenically, usually, nonspecific immune responses are of greater significance than others. And the cancer cell is more sensitive to a cell-mediated response extensively by macrophage and NK cell, so it also can be killed nonspecifically by activated macrophages and NK cells.1

Macrophages and NK cells should be widely distributed throughout the body and able to extravasate and migrate to various tissue sites. They should be exceedingly responsive to recruiting signals, activation signals, and costimulatory triggers. They should possess lytic machinery and be prepared to employ, at any given time without previous notice, more than one strategy for elimination of cancer cells.21-23

Macrophages are responsible for the nonspecific cellular response. Among the many roles of macrophage in immune system, production of cytokines is one of the important functions. They can be activated by lymphokines and other cell mediator to kill tumor cells by producing TNF and NO.9 NK cells possess the ability to kill certain cancer cells, particular of hematopoietic origin, and normal cells infected with virus by lysing target cells and providing and early source of immunoregulatory cytokines.9-13

As above described, it is unquestionably believed that host immunity is one of the most important properties to control the development, growth, metastasis, recurrence and treatment of cancer. Recently, there are evidences that many human cancer cells express antigens that can induce cellular and humoral responses in the host. Though they are ineffective and undetectable, immunotherapy have been tried to eradicate the cancer cell clinically and in laboratory, instead of chemotherapy or radiation these days.9

Meanwhile, there are many therapeutics and a thousand of plant candidates of cancer agents in the oriental medicine field. The effects of immune modulating and anti-cancer activity using various herbal plants have been experimented extensively and reported over the world. Theoretically, major prescriptions and treatment of oriental medicine are likely to belong to immunotherapy in western medicine. AME has been used as a medicinal stuff for a long time in oriental medicine and it has been shown to have clinical efficacy. In this present study, we aimed to elucidate properties of AME by enhancement of macrophage and NK cell activities and some related cytokine gene expressions.

We evaluated the effects of AME on activation of macrophage by detection of NO release from rat peritoneal macrophage treated with AME. NO production significantly increased in time-dependent manner at AME 100 ug/ml. However, the other AME treated groups did not affect NO production in rat peritoneal macrophages activated. In gene expression of cytokines, IL-1 gene expression was increased a little. But, iNOS and TNF-α was significantly increased respectively compared with control. Activated macrophage express iNOS, its product NO have major role in bactericidal and tumoricidal function. iNOS has been shown to play a central role in the animal immune system, its transcriptional level and activity are induced during the activation of immune responses by endotoxins and cytokines, such as IL-1, IFN-γ or TNF-α.24-25 These results imply that AME activate cell-mediated response by macrophage.

To investigate the effects of AME on activation of NK cell, we measured cytotoxicity on Yac-1 cell which are loss of class I MHC molecules and gene expression of cytokines related with NK cell activity (IL-1, IL-2, IFN-γ). AME showed a significant effect on NK cytotoxic activity compared with control in all ratios of effector cell : target cell. Although AME had no influence on IL-1 gene expression in RAW 264.7 cells, it showed a significant increase IL-1 in
spleenocytes. Also, AME up-regulated IL-2 gene expression in dose-dependent manner. However, IFN-γ gene expression was only increased in 100 μg/ml treated group compared with control group. NK cell is prior to defense and kill the cancer cell because NK cell have properties to selectively lyse transformed cell with diminished class | MHC molecule which is commonly observed. Augmentation of NK cell activity in visceral organs enhances resistance to the growth of metastasis, then NK cells may represent a first line of host defense against the primary and secondary cancer26). In NK cell activity related with cytokines, IFN-γ is one of the most important immune mediators in tumor immunity. IFN-γ can be secreted by NK cell or activated Th1 cell, and strongly activate macrophage and NK cell itself, which mainly roles in tumor cell destroying18,26. IL-1 is produced by many cell types in response to damage, infection or antigens. It also influences NK cell cytoidal activity increases19. These results indicate that AME may have antitumorigenic property by promotion of the NK cell activity to lysis the YAC-1 cells and activation of IL-1 and IFN-γ.

As about results, it could be concluded that AME has significant properties to activate macrophages and NK cells by promoting related cytokines like IL-1, IL-2, IFN-γ, iNOS and TNF-α gene expression. In conclusion, AME may present the anticancer effects by modulating immune response specific to cancer. It can be a potential anticancer agent, even should be investigated for their mechanisms of action and clinical evaluation.

References

15. Zee-Cheng RK. Shi-quan-da-bu-tang (ten significant tonic decoction), SQT. A potent Chinese biological


