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Original Article

# Effects of *Inonotus obliquus* Extracts on Immunomodulating Activity

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**Aim :** This study aimed at elucidating the effects of *Inonotus obliquus* on anti-tumor effects *in vivo* and immune-based characterization of the mushroom as a potential candidate for cancer remedy.

**Methods :** To investigate the immunomodulatory effects of *Inonotus obliquus*, we investigated macrophage functions and NK cell activities through the measurement of NO production of macrophage, NK cell cytotoxicity and expressions of cytokines and genes regulating immune responses, in addition to pulmonary metastasis model *in vivo*.

**Results :** *Inonotus obliquus* showed general cytotoxicity at high concentrations over the 100 ㎍/㎖ on the both of normal and cancer cell lines. *Inonotus obliquus* showed both inhibitory and promotive effects on pulmonary colonization of CT-26 cell depending on period or route of administration *in vivo*.

**Conclusion :** From these results, it cannot be concluded that *Inonotus obliquus* has cancer-specific activity. Furthermore, *Inonotus obliquus* has the provability to show adverse effects differently according to the concentration and the method of administration.

***Key Words*** : *Inonotus obliquus*, immunomodulating activity, cytotoxicity

## Introduction

A number of patients have used complementary and alternative medicine (CAM) to improve their health condition and CAM has been considered as one of new cancer therapies, demonstrating positive results in recent years[1-4]. Among numerous remedies in CAM, medicinal mushrooms have been used as supplements for conventional treatments showing lower efficacy and higher rate of side effects, such as chemotherapy and radiotherapy[5]. So far, various medicinal mushrooms have been intensively investigated for medicinal effects *in vivo* and *in vitro*, and new potentials for anti-cancer and immunoregulating activity have been identified [6-7].

Recently, *Inonotus obliquus*(Chaga mushroom) has been taken by many patients suffering from cancer, and recognized as a new anti-cancer herbal-medicine. There have been a number of studies exploring its components, and about it santi-tumor activities against several types of tumor cells[8-17]. However, little is yet known about the biological functions of *Inonotus obliquus* and its action mechanisms of anti-

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tumorigenic effects.

This study aimed at elucidating the effects of *Inonotus obliquus* on anti-tumor effects *in vivo* and immune-based characterization of the mushroom asa potential candidate forcancer remedy. To investigate the immunomodulatory effects of *Inonotus obliquus*, we investigated macrophage functions and NK cell activities through the measurement of NO production of macrophage, NK cell cytotoxicity and expressions of cytokines and genes regulating immune responses, in addition to pulmonary metastasis model *in vivo*.

## Materials and Methods

### 1. Materials

*Inonotus obliquus* was obtained from Daejeon Oriental Medical Hospital. Dried *Inonotus obliquus* was powdered with a grinder. Ten grams of the powder were mixed with 0.5 L of distilled water and left for 1 hr at room temperature, and the whole mixture was agitated for 2 hr at 80℃. The mixture was centrifuged for 30 min at 2000×g and supernatant was concentrated with vacuum evaporator (BÜCHI, Switzerland) and then lyophilized. The yield IOE was 6.5 % (w/w) in terms of the dried medicinal herbs. M-MLV RT, taq. polymerase, dNTP and 5X TBE buffer were obtained from Promega (Madison, USA). Other chemicals were purchased from Sigma (St Louis, USA).

### 2. Experimental animals

Specific pathogen-free BALB/c mice were obtained from a commercial animal breeder (Daehan BioLink, Korea). The animals were housed under normal laboratory conditions (23±2℃ and 40-60 % relative humidity) with 12 hr light/dark cycle with free access to standard rodent food and water.

### 3. Cell culture

RAW 264.7 and HT1080 cells were obtained from Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Hep G2 and CT-26 cells were obtained from Korean Cell Line Bank (Seoul, Korea), and human fibroblast, 7250 cells were obtained from National Cancer Institute/NIH (USA). The cells were cultured in DMEM (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS), 100 units/㎖ of streptomycin and 100 units/㎖ of penicillin.

### 4. Measurement of cytotoxicity

HT1080, Hep G2, 7250, and CT-26 cells (1×103) were seeded into a 96-well plate and cultured overnight. The cells were treated with IOE (0, 25, 50, 100, 200 ㎍/㎖). Cell counting kit-8 (CCK-8, 20 ㎕) was added into each well. Three hours later, 150 ㎕ of each culture medium was collected to determine optical density. Cell proliferation was determined at days 0, 2, 4 and 6.

### 5. Nitric oxide (NO) assay

RAW 264.7 cells (5×105) were plated in 24-well plates (BD, NJ, USA) and treated with IOE (0, 0.2, 2, 20, or 200 ㎍/㎖) and LPS (1 ㎍/㎖) and incubated at 37℃ with 5 % CO2. NO secretion was measured by analyzing its stable end product, nitrite (NO2-) in the culture supernatant with Griess reagent. Briefly, an aliquot of culture supernatant (100 ㎕) was added to each well of a 96-well microliter plate 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 a microplate reader (Molecular Device, USA).

Nitrite concentration was determined by using dilutionsof sodium nitrite in culture medium as standards. By adding IOE to standard solutions of sodium nitrite, it was confirmed that IOE did not interfere with the nitrite assay.

### 6. mRNA expressions of IL-1$\beta$, IL-10, TNF-$\alpha$ and iNOS in RAW 264.7 cells

RAW 264.7 cells (4×105) were plated into 6-well plates and treated with various concentrations of IOE (0, 0.2, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖) and incubated for 12 and 24 hr at 37℃ with 5 % CO2. Total RNA was isolated by the easy-BLUE reagent (Intron, Korea) and reverse transcription and polymerase chain reactions were done according to the manufacturer's instructions. The primers used are described in Table 1.

### 7. 51Cr release assay in NK cells

51Cr release assay was performed as described previously with modifications. Spleen cell suspensions were prepared in ice-cold DMEM from BALB/c mice. One hundred microliters of the splenocytes containing 4×106, 2×106 or 1×106 cells/well were plated onto the round bottom 96-well plate (4 wells per group) with various concentrations of IOE (0, 0.2, 2, 20, or 200 ㎍/㎖) and IL-2 (300 U/㎖). These cells were incubated for 14 hr at 37℃ with 5 % CO2 and prepared as effector cells.

Meanwhile, YAC-1 cells (5×106) were cultured as target cells of NK cells. After labeling the target cells by incubating for 2 hr (37℃, 5 % CO2) with 51Cr (200 μCi), the labeled target cells were centrifuged for 5 min at 400×g, and adjusted to 2×105 cells/㎖. Fifty microliters of cell suspension (1×104 cells) were added to the effector cells and incubated for additional 4 hr. Maximum leased groups were added with 150 ㎕ of 2 % NP-40, and spontaneous leased group with 150 ㎕ of complete medium. After 4 hr, the cells were concentrated by centrifugation at 500×g for 10 min, and cell-free supernatant was

**Table 1.** Oligonucleotide Sequences of Primers

| Gene | Primer | Sequence | Product (bp) |
|---|---|---|---|
| IL-1β | Sense Antisense | 5‘-AAG CTC TCA CCT CAA TGG A-3’<br>5‘-TGC TTG AGA GGT GCT GAT GT-3’ | 302 |
| IL-2 | Sense Antisense | 5‘-TGC TCC TTG TCA ACA GCG-3’<br>5‘-TCA TCA TCG AAT TGG CAC TC-3’ | 391 |
| IL-4 | Sense Antisense | 5‘-TCA ACC CCC AGC TAG TTG TC-3’<br>5‘-TGT TCT TCA AGC ACG GAG GT-3’ | 254 |
| IL-10 | Sense Antisense | 5‘-TCC TTG GAA AAC CTC GTT TG-3’<br>5‘-TCT CTT CCC AAG ACC CAT GA-3’ | 389 |
| TNF-α | Sense Antisense | 5‘-CTC CCA GGT TCT CTT CAA GG-3’<br>5‘-TGG AAG ACT CCT CCC AGG TA-3’ | 195 |
| IFN-γ | Sense Antisense | 5‘-GGA TAT CTG GAG GAA CTG GC-3’<br>5‘-GAG CTC ATT GAA TGC TTG GC-3’ | 250 |
| TGF-β | Sense Antisense | 5‘-TGA GTG GCT GTC TTT TGA CG-3’<br>5‘-TTC TCT GTG GAG CTG AAG CA-3’ | 310 |
| iNOS | Sense Antisense | 5'-TGG TGG TGA CAA GCA CAT TT-3'<br>5'-CTG AGT TCG TCC CCT TCT CTC C-3' | 229 |
| β-actin | Sense Antisense | 5‘-ACC GTG AAA AGA TGA CCC AG-3’<br>5‘-TCT CAG CTG TGG TGG TGA AG-3’ | 285 |

harvested from each well for assessment of radioactivity. Then gamma irradiation from each well was assessed in a scintillation counter (Packard Instruments). The percentage of specific lysis was calculated by the following equation:

$$\text{Specific killing activity (\%)} = \frac{\text{IOE release - spont. release}}{\text{max. release - spont. release}} \times 100$$

### 8. mRNA expressions of IL-1$\beta$ , IL-2, IL-4, IL-10, IL-12, TNF-$\alpha$, IFN-$\gamma$ and TGF-$\beta$ in splenocytes

BALB/c mice were sacrificed and spleens were subjected to cold phosphate buffered saline (PBS). After the spleen cells were washed twice with PBS, the prepared cells were plated on the 6-well plates. The splenocytes (2×107 cells) were treated with various concentrations of IOE (0, 0.2, 2, 20 ㎍/㎖) or LPS (1 ㎍/㎖) and incubated for 6 and 12 hr at 37℃with 5 % CO2. Total RNA was isolated by the easy-BLUE reagent (Intron, Korea) and reverse transcription and polymerase chain reactions were done according to the manufacturer's instructions.

Briefly, PCR amplification was carried out in a thermal cycler using a protocol of initial denaturing step at 95℃ for 10 min; then 27 cycles for β-actin and 35 cycles for other genes at 95℃ for 1 min, 60℃ for 40 seconds and 72℃ for 40 seconds. The PCR products were run on a 1 % agarose gel in 0.5×TBE buffer. The primers used are described in Table 1.

### 9. Cytokine expressions of IL-2, IL-10, TNF-$\alpha$ and IFN-$\gamma$ in splenocytes

BALB/c mice were sacrificed and spleens were subjected to cold PBS. After the spleen cells were washed twice with PBS, the prepared cells were plated on the 6-well plates. The splenocytes (5×106 cells) were treated with IOE (0, 0,2, 2, 20 ㎍/㎖), Con A (0.5 ㎍/㎖), or LPS (0.5 ㎍/㎖) in 24-well platesand incubated for 24 and 48 hr at 37℃with 5 % CO2. Supernatant was harvested and cytokines were determined by ELISA kit (BD, USA).

### 10. Splenocyte proliferation

BALB/c mice were sacrificed and spleens were removed aseptically. After lysing RBC, the cells were washed twice with PBS. The splenocytes (1×106 cells) were seeded into a 96-well plate and treated with IOE (0, 0.2, 2, or 20 ㎍/㎖) with 3 ㎍/㎖ Con A (T cell mitogen), or 3 ㎍/㎖ LPS (B cell mitogen). The plate was incubated for 72 hr at 37℃ with 5 % CO2. Cellular proliferation was determined by using CCK-8.

### 11. Pulmonary colony assay

Mice were divided into 2 groups (10 mice per each group). The mice were orally administrated with 20 and 200 ㎎/㎏ of IOE for 4 days before CT-26 injection (2×104 cells) and the mice in the control group were orally administrated with distilled water. In additional experiment, the mice were orally administrated with 50 and 200 ㎎/㎏ of IOE for a total 14 days before and after CT-26 injection. CT-26 cells (2×104) were inoculated via tail vein and pulmonary colonization was examined at day 14 after CT-26 inoculation. The mice were sacrificed 14 days after tumor inoculation, lungs were removed, and the tumor colonies in the lung were counted to check the capability of tumor metastasis.

### 12. Statistical analysis

Statistical analysis of the data was carried out by Student's t-test, and results were expressed as the mean ± SD. A difference from the respective

control data at the levels of $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$ and $p<0.00001$ was regarded as statistically significant.

## Results

### 1. Cytotoxicity

7250, HT1080, Hep G2, and CT-26 cells (1×103) were seeded into a 96-well plate, and were treated with various concentrations of IOE (0, 25, 50, 100, 200 ㎍/㎖) to determine the cytotoxic effects of IOE on the various cell lines including normal and cancer cell lines. As shown in Fig. 1, the proliferation of 7250 cells was significantly inhibited at 200 ㎍/㎖ of IOE on all of the cell lines used in this experiment. On day 6, it was also inhibited by IOE treatment at the low concentration of 100 ㎍/㎖, which indicated that IOE has toxic effect on normal cells as well as cancer cells at high concentrations. In HT1080 cells, cytotoxic effect was shown at the concentrations of 25 and 200 ㎍/㎖ of IOE on days 4 and 6. At 50 ㎍/㎖ IOE on day 6, the proliferation of HT1080 cells was also inhibited. As IOE showed an increase of cell proliferation at low concentration, but a decrease at high concentration, it is suggested that IOE has cytotoxic effect on the proliferation of HT1080 cells concentration-dependently. The proliferation of Hep G2 cells was also inhibited significantly at the concentration of 100 ㎍/㎖

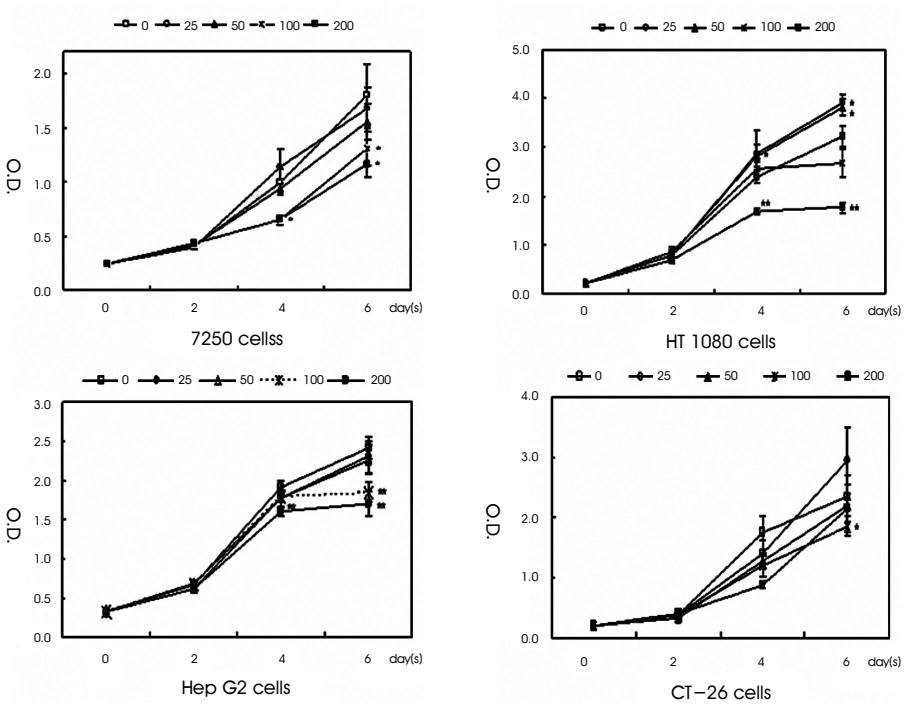


**Fig. 1.** Cytotoxic Effect of IOE on various cell lines. Human fibroblast cell line, 7250 cells ($1\times10^3$), HT1080 cells ($1\times10^3$), Hep G2 cells ($1\times10^3$) and CT-26 cells ($1\times10^3$) were seeded into a 96-well plate and cultured overnight. The cells were treated with IOE (0, 25, 50, 100, 200 ㎍/㎖). OD450-560 was determined at 3 hr after CCK-8 addition. Each data represents the mean ± SD. Statistically significant value compared with control by T-test. (*: $p<0.05$)

IOE on day 6, and at 200 ㎍/㎖ IOE on days 4 and 6. IOE inhibited the proliferation of CT-26 cells at 200 ㎍/㎖ IOE on day 4, and at 100 ㎍/㎖ IOE on day 6.

### 2. NO production

To investigate the ability of macrophage activation of IOE, NO production was measured after treatment of various concentrations (0, 0.2, 2, 20, 200 ㎍/㎖) in mouse macrophage cell lines, RAW 264.7 cells. The accumulated nitrite, estimated by the Griess method, in the culture medium was used as an index for NO synthesis from these cells. After treatment with LPS for 24 hr, nitrite concentration markedly increased about 3-fold (7-8 nmole). When cells were treated with various concentrations of IOE, nitrite production was significantly increased at concentrations of 20 and 200 ㎍/㎖ in a concentration-dependent manner (Fig. 2).

### 3. Changes in gene expression in RAW 264.7 cells

To investigate the effects of IOE on the regulation of cytokine gene transcription in RAW 264.7 cells, the cells were treated with various concentrations of IOE (0, 0.2, 2, 20, 200 ㎍/㎖) for 12 or 24 hr, and measured the expression of mRNA expression of various pro-inflammatory cytokines and iNOS by using RT-PCR assay. After treatment with LPS (1 ㎍/㎖) for 12 or 24 hr as a positive control, the mRNA expression of IL-1β, IL-10, and iNOS dramatically increased (Fig. 3).

When cells were treated with various concentrations of IOE, IL-1β mRNA expression was up-regulated in a dose-dependent manner, increasing by over 10-fold compared with control value. In addition, IL-10 mRNA expression was also up-regulated with high concentrations of 20 and 200 ㎍/㎖ IOE at 24 hr, but not with a low concentration of 2 ㎍/㎖ IOE. However, TNF-αgene expression was not affected by IOE treatment. With the assumption that the increase of NO production by IOE in RAW 264.7 cells would be caused by an up-regulation especially in the iNOS gene transcription, the effect of IOE on the iNOS mRNA expression was examined in cells treated with various concentration of IOE for 12 or 24

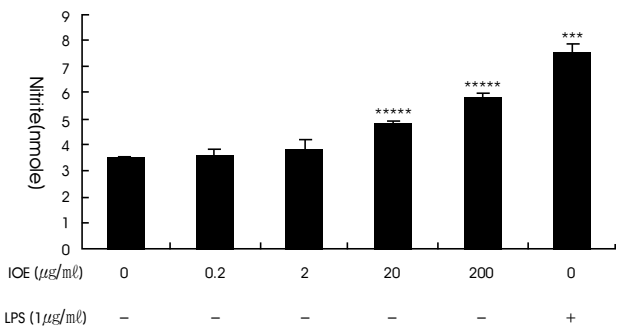


**Fig. 2.** Effect of IOE on NO production. RAW 264.7 cells were treated with IOE (0, 0.2, 2, 20, 200 ㎍/㎖) or LPS (1 ㎍/㎖). Accumulated nitrite in culture medium was determined by the Griess reaction. Each data represents the mean ±SD. Statistically significant value compared with control by T-test. (***: $p<0.001$, *****: $p<0.00001$)

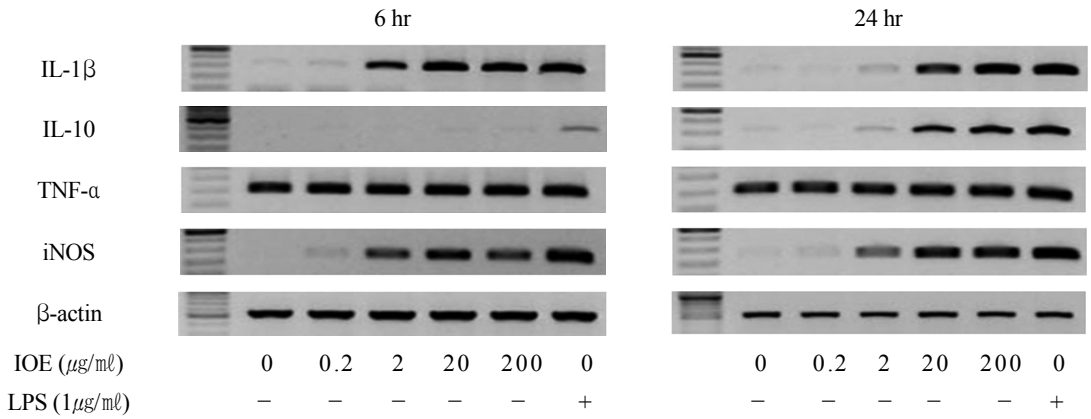


**Fig. 3.** Changes in gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with IOE (0, 0.2, 2, 20, 200 μg/mℓ) or LPS (1 μg/mℓ) for 12 and 24 hr. Total RNA was isolated and RT-PCR was performed as described in Materials and Methods.

hr. As expected, treatment with IOE increased iNOS mRNA expression in a concentration-dependent manner without changes in the amount of β-actin mRNA, an internal control, indicating the specific up-regulation of iNOS gene transcription by IOE.

### 4. NK cell activity

To investigate whether IOE might be involve the modulation of NK cell activity, the effect of IOE of the NK cell cytotoxicity on the prepared splenocytes by measuring 51Cr release assay was measured. An increased ratio of effector and target cells (up to 200:1) showed dramatic increase with the ability of cell lysis by effector cells (Fig. 4). At all concentrations of IOE (0.2, 2, 20 μg/mℓ), NK cells had significant cytotoxic activity compared with control concentrations of

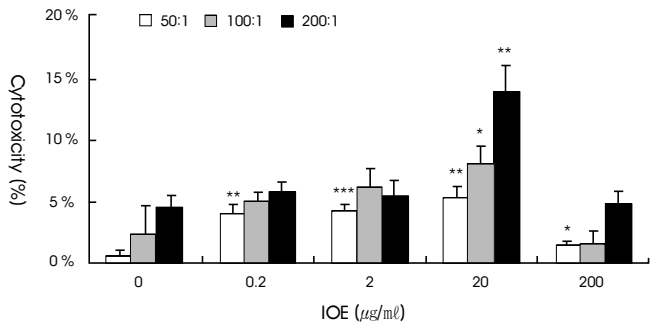


**Fig. 4.** Effect of IOE on NK cell activity. Spleen cells (effector cell) were treated with IOE (0, 0.2, 2, 20, 200 μg/mℓ) and IL-2 (300 U/mℓ) for 14 hr. YAC-1 cells (target cell) labeled with $^{51}Cr$ were mixed to effector cells for 4 hr. Cell-free supernatant containing released $^{51}Cr$ was counted by using gamma scintillating counter. Each data represent the mean±SD. Statistically significant value compared with control by T-test. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

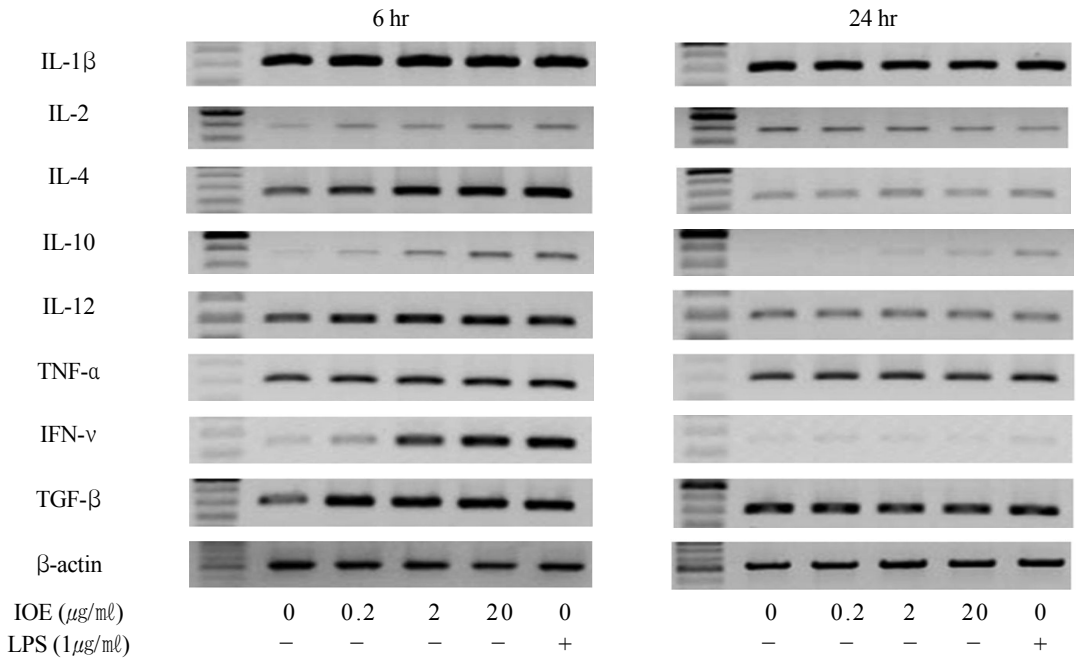


**Fig. 5.** Changes in gene expression in splenocytes. Splenocytes were treated with IOE (0, 0.2, 2, 20 μg/mℓ) or LPS (1 μg/mℓ) for 6 and 12 hr. Total RNA was isolated and RT-PCR was performed as described in Materials and Methods.

IOE, which indicating that IOE had a stimulatory effect on NK cell activity. Especially, at a ratio of 200:1, the NK cytotoxic activity increased significantly by 13.8 % at 20 μg/mℓ IOE compared with control (4.6 %). However, NK cytotoxic activity at 200 μg/mℓ of IOE was less than that at the concentrations below 20 μg/mℓ.

### 5. Changes in gene expression in splenocytes

To investigate the role of IOE on the regulation of cytokine gene transcription in murine immunologic system, splenocytes were isolated from BALB/c mice and treated with various concentrations of IOE (0, 0.2, 2, 20 μg/mℓ) or LPS (1 μg/mℓ) for 6 and 12 hr, and the mRNA expression of various inflammatory cytokines were assessed by RT-PCR (Fig. 5). When cells were treated with IOE at the concentration of 0.2, 2 and 20 μg/mℓ, there were not affected in mRNA expression of IL-1β and TNF-α in splenocytes. However, the mRNA expression of IL-2 as well as IL-4, IL-10, IL-12 genes were dramatically up-regulated after 6 hr treatment in a dose-dependant manner, which suggested that IOE might contribute the immunomodulative effects on immune function.

In addition, to further examine the IOE's effect on the immunomodulative functions, its effect on IFN-γ mRNA expression was also assessed. As shown in Fig. 5, treatment of IOE on the splenocytes with various concentrations resulted in the up-regulation of IFN-γ mRNA expression remarkably and dose-dependently; especially at 2 μg/mℓ IOE, the amount of mRNA expression increased by almost 5 times of control. Furthermore, the mRNA expression of TGF-β was also

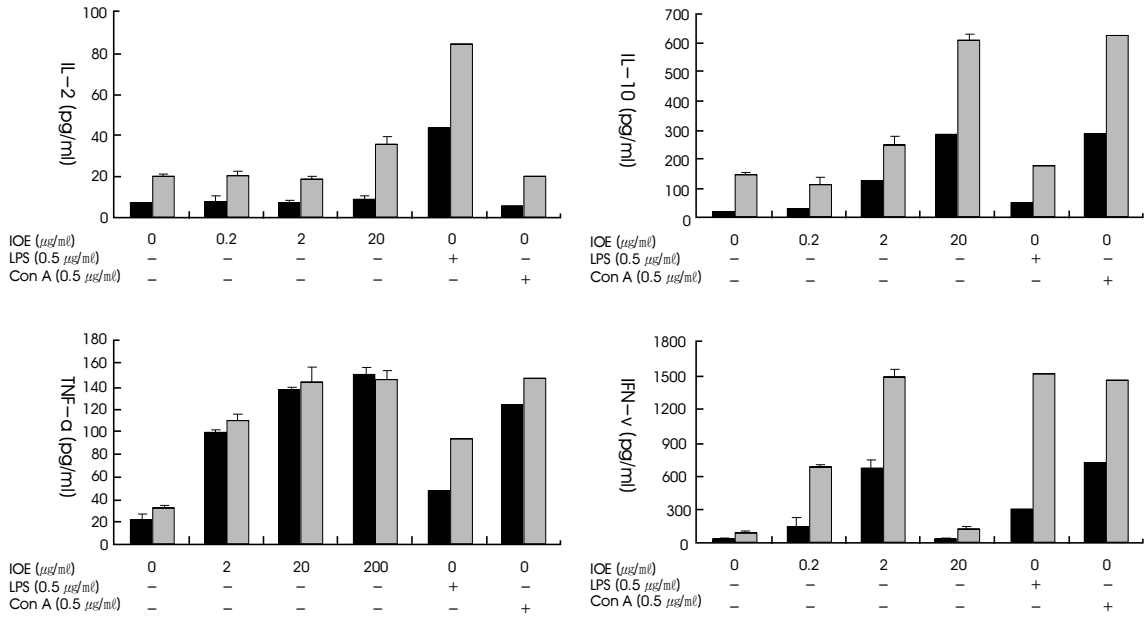


**Fig. 6.** Protein expression in splenocytes. Splenocytes were treated with IOE (0, 0.2, 2, 20 ㎍/㎖), Con A (0.5 ㎍/㎖) or LPS (0.5 ㎍/㎖) for 24 (■) and 48 hr (□), and the supernatant were collected at the indicated times and the released amount of IL-2, IL-10, TNF-α, IFN-g protein measured by the ELISA assay kits. The values are mean ± SD of different experiments.

up-regulated by the treatment with IOE at the concentration of 0.2 ㎍/㎖.

### 6. Changes in protein expression of cytokines in splenocytes

To investigate whether IOE affects the protein expression of inflammatory cytokine at the translational level in mouse splenocytes, the prepared cells from BALB/c mice were treated with various concentrations of IOE (0, 0.2, 2, 20 ㎍/㎖) or LPS (1 ㎍/㎖) for 24 and 48 hr, and the amount of released cytokine expression in the culture media was determined by ELISA kit.(Fig. 6) When cells were treated with various concentrations of IOE, the amount of released IL-2 increased at the concentration of 20 ㎍/㎖ at 48 hr. IL-10 expression also increased significantly under IOE treatment and this increase continued until 48 hr in a dose-dependant manner. After treatment with IOE of 2 and 20 ㎍/㎖ for 24 hr, the concentrations of IL-10 in culture medium were 124 and 284 pg/㎖ respectively compared to 14 pg/㎖ in control. This increase of IL-10 expression by IOE was more dramatic 48 hr after treatment. At the concentration of 2 and 20 ㎍/㎖ IOE, the concentrations of IL-10 in the medium were 251 and 609 pg/㎖ respectively whereas 41 pg/㎖ in control.

In addition, TNF-α expression also increased significantly under IOE treatment. At 24 hr after IOE treatment (0, 0.2, 2, 20 ㎍/㎖), the protein levels of TNF-α were 21.2, 98.2, 136.8 and 149.8 pg/㎖respectively in splenocytes, but were not much different between 24 hr and 48 hr. Furthermore, when the cells were treated with IOE, the amount of IFN-γ expression in culture

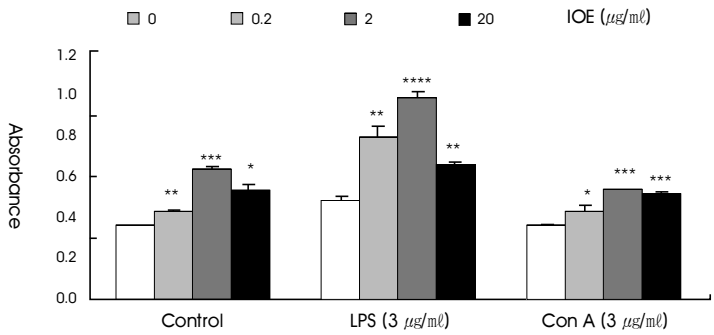


**Fig. 7.** Effect of IOE on splenocyte proliferation. Splenocytes ($1\times10^6$) were seeded into a 96-well plate and treated with IOE (0, 0.2, 2, 20 ㎍/㎖) with or without 3 ㎍/㎖ Con A (T cell mitogen) and 3 ㎍/㎖ LPS (B cell mitogen). The plate was incubated for 72 hr at 37℃ with 5 % $CO_2$. Cellular proliferation was determined using CCK-8. The results were expressed as mean ± SD. Statistically significant value compared with control by T-test (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001).

medium significantly increased at the concentration below 2 ㎍/㎖. At 24 hr after IOE (0.2, 2 ㎍/㎖) treatment, IFN-γ levels increased by 149.7, 660.7 pg/㎖ respectively compared to 13.0 pg/㎖ in control. At 48 hr after IOE (0.2, 2 ㎍/㎖) treatment, IFN-γ levels increased by 672.9, 1499.1 pg/㎖ respectively compared to 91.2 pg/㎖ in control. However at 20 ㎍/㎖ IOE, IFN-γ level was less than that at 2 ㎍/㎖.

### 7. Splenocyte proliferation

To understand whether IOE has other immunomodulatory activity in splenocytes, we investigated the effect of IOE on lymphocyte proliferation. As shown in Fig. 7, IOE remarkably increased splenocyte proliferation at all concentrations when treated with or without Con A and LPS. Especially, when the cells were treated with IOE (0.2, 2, 20 ㎍/㎖) with LPS, splenocyte proliferation increased significantly by 1.7, 2.1, 1.4-fold compared with control.

### 8. Pulmonary metastasis

Since it has been known that IOE is used for many patients suffering from severe cancer, we examined whether it has an ability to reduce cancer metastatic transformation. To do this, the mice were orally administrated with 20 and 200 ㎎/㎏of IOE for 4 days before CT-26 injection while the mice in control group were orally administrated with distilled water. Pulmonary colonization was observed on day 14 after CT-26 inoculation. As shown in Fig. 8-A, tumor metastasis was inhibited significantly (32.4 %, 62.4 % respectively) in the group administered IOE (20, 200 ㎎/㎏), compared with the control group. However, when mice were treated with IOE for a total of 14 days before and after CT-26 injection, pulmonary colonization increased (Fig. 8-B, C) Especially, at 200 ㎎/㎏ of IOE, pulmonary metastasis increased significantly by 330.50, compared with only 84.82 in the control group.

Mice injected intravenously with 10 and 100 ㎎/㎏ of IOE on day 4 before CT-26 injection showed some different results. In the group injected with at the low concentrations of IOE (10 ㎎/㎏), tumor metastasis was inhibited signi-

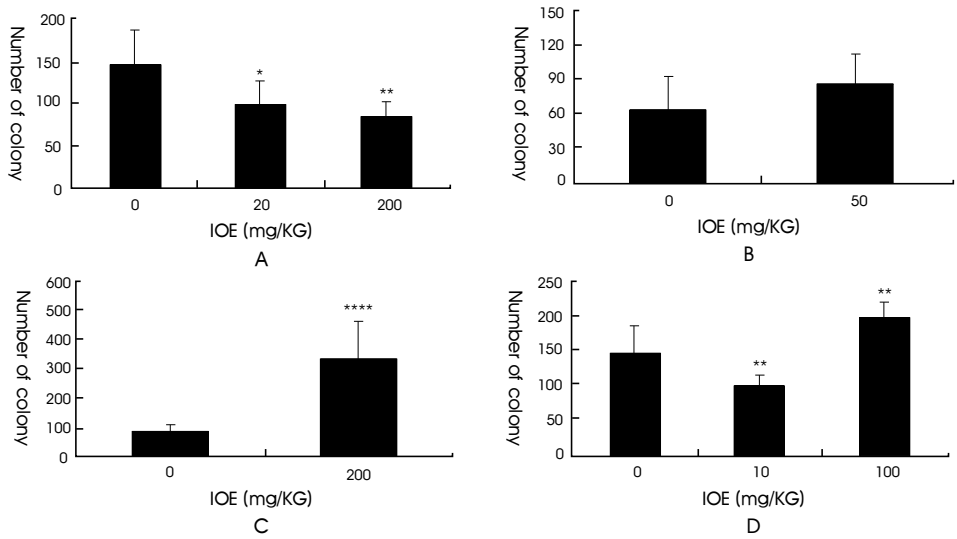


**Fig. 8.** Pulmonary colony assay.
A : Each group was orally administered with water (CON), or IOE (20, 200 ㎎/5㎖/㎏) for 4 days before CT-26 ($2\times10^4$ cells) injection. B : Each group was orally administered with water (CON), or IOE (50 ㎎/5㎖/㎏) for total 14 days before and after of CT-26 ($2\times10^4$ cells) injection. C : Each group was orally administered with water (CON), or IOE (200 ㎎/5㎖/㎏) for total 14 days before and after of CT-26 ($2\times10^4$ cells) injection. D : Each group was injected intravenously with saline (CON) or IOE (10 and 100 ㎎/5㎖/㎏) 4 days before CT-26 ($2\times10^4$ cells). All mices were sacrificed at 14 days after tumor inoculation, and lungs were removed and tumor colonies were counted. The results were expressed as mean ± SD.

ficantly by 33.4 % but at the high concentration of IOE (100 ㎎/㎏), tumor metastasis increased significantly by 35.9 % compared with the control group (Fig. 8-D).

## Discussion

Mushrooms have been recognized as edible and medical resources for many centuries, used for treating a variety of health problems including cancer, immunologic disorder, viral or bacterial infections and even AIDS[18-21].

A number of studies have focused on the medicinal efficacy of mushrooms such as activation of immune cells, modulation of cytokine expression, and anti-tumor activities. It is especially well known that mushroom-polysaccharides potentially have biologic activities to modulate immunological functions[22]. These polysaccharides, often referred to as biological response modifiers (BRMs), or immunopotentiators, have been getting significant attention in recent years because of their immunomodulatory activities, resulting in anti-carcinogenesis, anti-tumor growth and anti-metastasis[23].

*Inonotus obliquus* has been known to have various BRMs, particularly xylogalactoglucan exerting anti-tumor activity[8-10]. Originally, *Inonotus obliquus* was used for anti-helminthic purpose in folk medicine in Russia[22]. It has been reported for its anti-tumor, anti-oxidant activity, anti-mutagenic effect, hypoglycemic activity and even anti-HIV activity over the past few

decades[8-17, 23].

In recent years, clinical studies of medicinal mushrooms or mushroom polysaccharides have succeeded in extending survival of cancer patients and/or improving the quality of life in patients with advanced cancer[21-22, 25]. However, there still lack experimental studies characterizing IOE's bioactivities related with anti-tumor effect and even clinical safety.

Therefore, we here conducted an experimental study about the effects of IOE on immuno-modulating activity and anti-tumor activity before clinical evaluation. In this study we examined the effect of IOE on the cell cytotoxicity, NO production, NK cell activity and several cytokine expressions in macrophage and splenocytes.

To examine the IOE's direct cytotoxic effects on cell growth, we measured the cell death effects on 7250, HT1080, Hep G2, and CT-26 cells. IOE inhibited the growth of selected cancer cells as well as normal cells. IOE showed cytotoxicity on 7250, HT1080, Hep G2, and CT-26 cells dose-dependently, which indicated that IOE has no specific action on the cancer cells.

Activated macrophages have a major role in bactericidal and anti-tumor function by up-regulating the expression of inducible nitric oxide synthase (iNOS) producing nitric oxide (NO)[26-28]. This prompted us to examine the effects of IOE on activation of macrophages by detection of NO release from RAW 264.7 cells treated with IOE. NO production significantly increased at 20 and 200 ㎍/㎖ consistently with the result of iNOS gene expression.

In the effects of IOE on the cytokines gene transcription, IL-1β and IL-10 gene expressions significantly increased in a dose-dependent manner, but TNF-αgene expression was not affected by IOE treatment.

IL-1 is usually secreted by activated macrophages or other antigen presenting cells, and the role of IL-1 released by the macrophages is important for inflammatory response. TNF also acts directly on many other types of immune and inflammatory cells. IL-10 is secreted by tumor cells as generally immunosuppressive mediators and also by immune cells as anti- inflammatory mediators[26-29].

Next, to investigate the effects of IOE on activation of NK cells, we measured cytotoxicity on YAC-1 cells which lost class Ⅰ MHC molecules. IOE showed a significant effect on NK cytotoxic activity compared with control at a ratio of 50 (effector cell) : 1 (target cell). Especially, at ratio of 200:1, the NK cytotoxic activity increased significantly at 20 ㎍/㎖ IOE compared with control. However, NK cytotoxic activity at 200 ㎍/㎖ IOE was lesser than that at below 20 ㎍/㎖ IOE. That might be correlated with general toxicity including NK cells at high concentration as shown in Fig. 1.

Even though IOE up-regulated IL-2, IL-4, IL-10, IL-12, IFN-γ and TGF-β gene expressions dose-dependently in splenocytes, only IL-10, TNF-α and IFN-γ were confirmed to be increased in protein level by ELISA. TGF-β is known to act as an effective tumor suppressor at early stages of carcinogenesis but later during tumor development it might exert oncogenic activity by promoting invasiveness and metastasis[30-32]. TGF-β is also known to act in an anti-inflammatory role.

In assay of splenocyte proliferation, IOE (0.2, 2, 20 ㎍/㎖) promoted the B cell proliferation synergically with LPS treatment rather than T cell with Con A. This result is correlated with

up-regulation of IL-10 above.

Although it is still not conclusive to complete the immune-characterization of IOE in this study because of a shortage of conclusive experiments covering all around cytokines and bioassay, the above results suggest that IOE might have immune properties to up-regulate IL-10 and IFN-γ in macrophages and splenocytes.

We also observed that IOE inhibited or promoted pulmonary colonization of CT-26 cells depending on concentration, administration routes and period of administration. Increase rate of metastatic colonization is thought to be due to cytotoxicity of normal cells at the concentration of 100 ㎍/㎖ in case administrated intravenously because they showed some toxic appearance in liver to the naked eye (data not shown).

From the above results, we cannot conclude that IOE has the cancer-specific property as expected, because we can assume that immune function of IOE is concerned with humoral immunity rather than cell-mediated immunity. Moreover, as it seems that IOE may be able to induce general toxicity to both normal and cancer cells, which suggests that there can be the possibility of getting from IOE serious side effects instead of benefits for cancer patients, even more careful evaluation study yet remains.

In addition, we suggest that IOE would be an anti-inflammation remedy assumed from strong induction of IL-10 very consistently in this study.

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