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Original Article

Effects of *Ampelopsis Radix* Extracts on Tumor Immunity

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Objectives : This experimental study was carried out to evaluate the immune modulating and anti-tumor activity of *Ampelopsis Radix* extracts (ARE).

Materials and Methods : To elucidate the effects of ARE on the macrophage and NK cell activity, we analyzed NO production, NK cytotoxicity and gene expressions of cytokine related with macrophage and NK cell activity.

Results : ARE activated and promoted macrophages to product NO in part. And, ARE has significant properties to activate macrophages and NK cells by promoting related cytokines like IL-1, IL-12, IFN-γ, iNOS and TNF-α gene expressions. We also observed that ARE promoted protein expression of IFN-γ, and TNF-α in mice splenocytes.

Conclusions : ARE is an effective herbal drug for immune modulating and anti-cancer by promoting activity of macrophages and NK cells.

Key Words: Immune modulating, anti-cancer, macrophages, NK cell

Introduction

It has been well known that host immune status is the most important factor in tumor incidence, growth, metastasis and patient mortality; moreover, this is evident in the course of clinical treatment. For many decades, medicinal agents have been intensively investigated for immunoactivating effects on model systems in vivo and in vitro, and new anti-cancer mechanisms have been identified experimentally. Though enormous effects were devoted to research of tumor immunity, it was paved with disappointments rather than success in clinical use[1,2].

On the other hand, the concern with natural products in medical research for cancer has been growing in recent years. The immune modulating and anti-tumor activities of various herbal plants have been experimented on extensively and reported all over the world. Administration of these Oriental herbs is known to inhibit tumor growth and incidence, and prolong the tumor-bearing rodents' survival in transplanted experimental models. Also, it is known to restore the lowered host defense immunity[3-5].

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Ampelopsis Radix, a well-known traditional Oriental herb, has been used as an anti-inflammatory agent for a long time, such as skin inflammation, pyogenic infection, ulcerous diseases of skin and burns (external use). Also, it has been intermittently prescribed for cancer patients by some Oriental doctors. However, few studies have been conducted in these fields, and little is known about the mechanism of the immunomodulatory and anti-tumor activities[6].

This study is aimed to elucidate the effects of *Ampelopsis Radix* on the immunomodulating system and the value of herbal remedies for cancer. To investigate effects of *Ampelopsis Radix* extract (ARE) on immune modulating and anti-tumor activity, we analyzed nitric oxide (NO) assay, natural killer (NK) cytotoxicity assay and gene expressions of cytokine related with macrophage and NK cell activity.

Materials and Methods

1. Materials

ARE was received from Dunsan Oriental Medical Hospital of Daejeon University. Fifty grams of ARE was mixed with 2 liters of distilled water and left for an hour at room temperature, and then the whole mixture was then boiled for 2 hours. ARE was filtered and then lyophilized. The yield of ARE 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 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). Mice were housed under normal laboratory conditions (23±2℃ and 40-60% relative humidity) with 12 hours light/dark cycle with free access to standard rodent food and water.

3. Cell culture

RAW 264.7 cells were obtained from Korea Research Institute of Bioscience and Biothechnology (Daejeon, Korea). CT-26 cells were obtained from Korean Cell Line Bank(Seoul, Korea). The cells were cultured in DMEM(Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 units streptomycin and 100 units penicillin.

4. NO assay

RAW 264.7 cells were cultured with DMEM containing 10% FBS. RAW 264.7 (5×10^5 cells) were

Tabel 1. Oligonucleotide Sequences of Primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense	5'-AAG CTC TCA CCT CAA TGG A-3'	302
	Antisense	5'-TGC TTG AGA GGT GCT GAT GT-3'	
iNOS	Sense	5'-TGG TGG TGA CAA GCA CAT TT-3'	229
	Antisense	5'-CTG AGT TCG TCC CCT TCT CTC C-3'	
TNF-α	Sense	5'-CTC CCA GGT TCT CTT CAA GG-3'	195
	Antisense	5'-TGG AAG ACT CCT CCC AGG TA-3'	
β-actin	Sense	5'-ACC GTG AAA AGA TGA CCC AG-3'	285
	Antisense	5'-TCT CAG CTG TGG TGG TGA AG-3'	

plated in 24-well plates (BD, NJ, USA) and treated with ARE (0, 2, 20, 200㎍/㎖) and LPS (1㎍/㎖) and incubated at 37℃ with 5% CO_2. NO formation was measured as the stable end product nitrite(NO_2-) in the culture supernatant with Griesse reagent. Briefly, an aliquot of culture supernatant (100㎕) was added to each well of 96-well plates and mixed with the same volume of Griesse reagent (1:1[v/v]; 0.1% N-[1-naphth yl]ethylenediamine dihydrochloride in H2O, 1% sulfanilamide in 5% H_2PO_4), and then the A540 was read with a microplate leader (Molecular device, USA). Nitrite concentration was determined by using dilutions of sodium nitrite in culture medium as standards. By adding ARE to standard solutions of sodium nitrite, it was confirmed that ARE did not interfere with the nitrite assay.

5. Gene expression of IL-1, TNF-α, iNOS in RAW 264.7 cells

RAW 264.7 cells (4×105) were plated into 6 well and treated with various concentration of ARE (0, 2, 20, 200 ㎍/㎖) or LPS (1㎍/㎖) and incubated for 12 and 24 h at 37℃ with 5% CO_2. Total RNA was isolated by the Easyblue 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 1.

6. NK cell ^{51}Cr release assay

^{51}Cr release assay was performed as described previously with modifications. Spleen cell suspensions were prepared in ice-cold DMEM from BALB/c mice. After adjusting to final concentration (1×10^7 cells/㎖), 100㎕ of cell suspension (4×10^6, 2×106 and 1×10^6 cells/well) were plated onto the round bottom 96-well plate (4 well per group) with various concentration of ARE (0.2, 2, 20, 200㎍/㎖) and IL-2 (300 U/㎖). These cells were incubated for 14 hours at 37℃ with 5% CO_2 and prepared as effector cells.

On the other hand, YAC-1 cells (5×10^6) were cultured for using as target cells of NK cell. After labeling the target cells by incubating for 2 hours(37℃, 5% CO_2) with ^{51}Cr (200 ㎌i), washing and lysis of unhealthy cells, the labelled target cells were centrifuged for 5 min at 400×g and adjusted to 2×10^5 cell/㎖. Fifty microliter cell suspension (1×10^4 cells) was added to effector cells and incubated for 4 h. Maximum leased groups were added with 150㎕ of 2% NP-40 and spontaneous leased group with 150㎕ of complete medium. After 4 hours, the cells were

Tabel 2. Oligonucleotide Sequences of Primers

Gene	Primer	Sequence	Product(bp)
IL-1β	Sense	5'-AAG CTC TCA CCT CAA TGG A-3'	302
	Antisense	5'-TGC TTG AGA GGT GCT GAT GT-3'	
IL-2	Sense	5'-TGC TCC TTG TCA ACA GCG-3'	391
	Antisense	5'-TCA TCA TCG AAT TGG CAC TC-3'	
TNF-α	Sense	5'-CTC CCA GGT TCT CTT CAA GG-3'	195
	Antisense	5'-TGG AAG ACT CCT CCC AGG TA-3'	
IFN-γ	Sense	5'-GGA TAT CTG GAG GAA CTG GC-3'	250
	Antisense	5'-GAG CTC ATT GAA TGC TTG GC-3'	
iNOS	Sense	5'-TGG TGG TGA CAA GCA CAT TT-3'	229
	Antisense	5'-CTG AGT TCG TCC CCT TCT CTC C-3'	
β-actin	Sense	5'-ACC GTG AAA AGA TGA CCC AG-3'	285
	Antisense	5'-TCT CAG CTG TGG TGG TGA AG-3'	

concentrated by centrifugation at 500 × g for 10min and cell-free supernatant were 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:

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

7. Gene expression of IL-1, IL-2, IL-12, TNF-α, IFN-γ, iNOS in splenocytes

BALB/c mice were sacrificed and spleen were removed to PBS. After RBC lysis, the cells were washed twice with PBS. The splenocytes(2×10^7 cells) were treated with various concentration of ARE (0, 0.2, 2, 20, 200$\mu g/ml$) or LPS (1$\mu g/ml$) in 6-well plate and incubated for 6 and 12 hours at 37℃ with 5% CO_2. Total RNA was isolated by the Easyblue® reagent (Intron, Korea) 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℃ 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 used primers were described in Table 2.

8. Cytokine expression of IL-2, TNF-α, IFN-γ in splenocytes

BALB/c mice were sacrificed and spleen were removed to PBS. After RBC lysis with lysing buffer, the cells were washed twice with PBS. The splenocyts(5×10^6 cell) were treated with ARE (0, 2, 20, 200$\mu g/ml$), Con A(1$\mu g/ml$) or LPS (1 $\mu g/ml$) in 24 well plate and incubated for 24 and 48 hours at 37℃ with 5% CO_2. Supernatant was harvested and cytokines were determined by ELISA kit(BD, USA).

9. 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 $p<0.05$, $p<0.01$ $p<0.001$ and $p<0.0001$ was regarded as statistically significant.

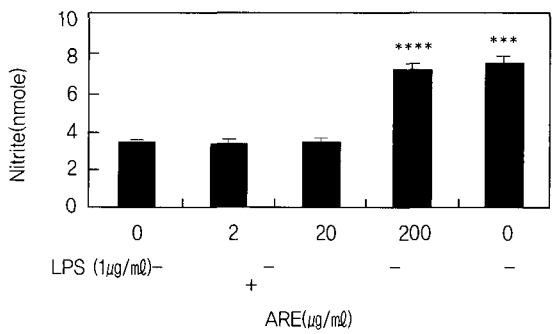

Fig. 1. Effect of ARE on NO production. RAW 264.7 (5×105) were treated with ARE (0, 2, 20, 200$\mu g/ml$) and PBS (control). Each data represents as mean±SD. Statistically significant value was compared with control by T-test. (***: $p<0.001$, ****: $p<0.0001$)

Results

1. NO production

NO production was measured after treatment of various concentration of ARE(0, 2, 20, 200$\mu g/ml$). NO production was significantly increased by 2 fold at 200 $\mu g/ml$ ARE. However, ARE did not affect NO production at low concentration below 20$\mu g/ml$ (Fig. 1).

2. Changes in gene expression in RAW 264.7 cells

To investigate gene expression of cytokines in RAW 264.7, macrophage cell line, RAW 264.7 cells were treated with various concentrations of ARE(0, 2, 20, 200$\mu g/ml$) or LPS (1$\mu g/ml$) for 12 and 24 hours. As shown in Fig. 2, IL-1β and iNOS mRNA expression were up-regulated significantly by ARE treatment on

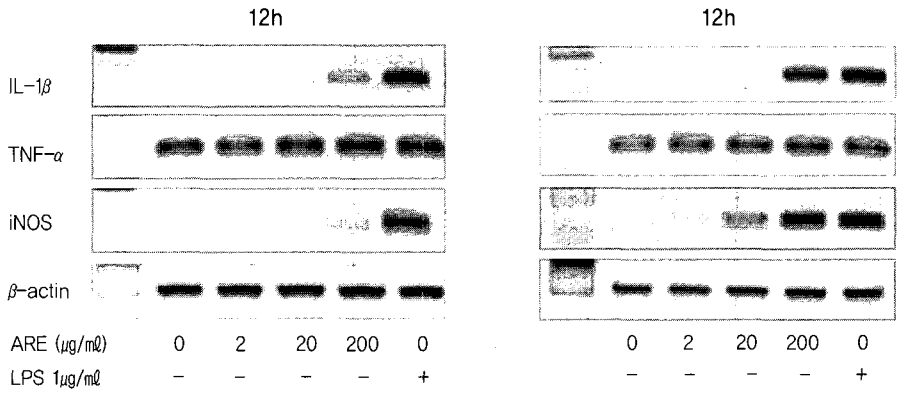

Fig. 2. IL-1β, TNF-α and iNOS gene expression in RAW 264.7 cells. RAW 264.7 cells were treated with ARE (0, 2, 20, 200$\mu g/ml$) or LPS (1$\mu g/ml$) for 12 and 24 hours. Total RNA was isolated and RT-PCR was performed.

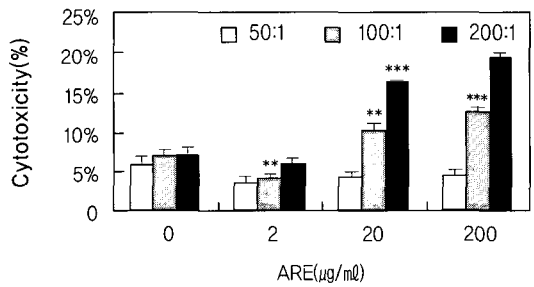

Fig. 3. The Effect of ARE on NK cell activity. Spleen cells (effector cell) were treated with ARE (2, 20, 200$\mu g/ml$) and IL-2 (300U/ml) for 14 h. Yac-1 cells (target cell) labeled with 51Cr were mixed to effector cells for 4 hours. Cell-free supernatant containing released ^{51}Cr was counted by using gamma scintillating counter. Each data represent the mean ±SD. Statistically significant value compared with control by T-test.(*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

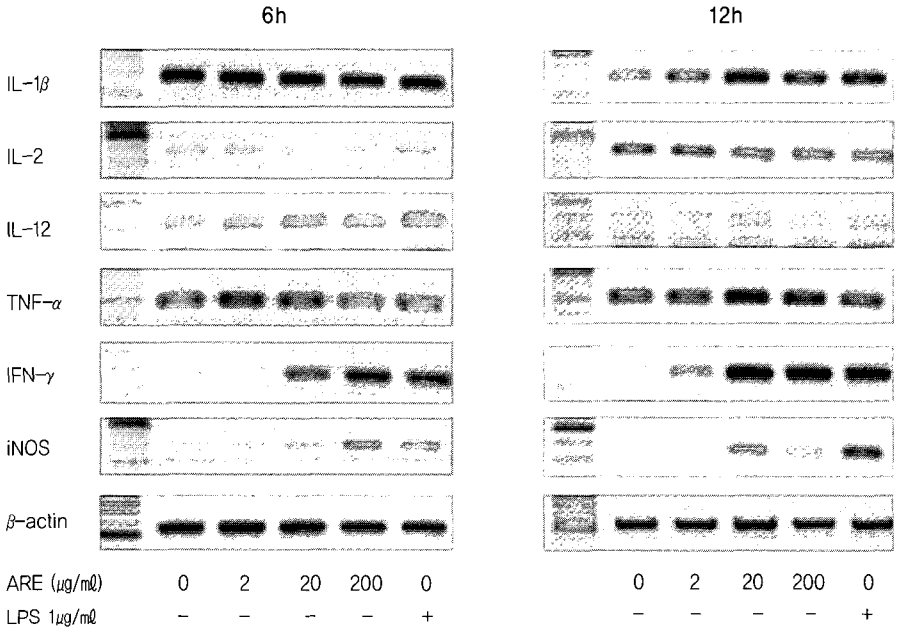

Fig. 4. IL-1β, IL-2, IL-4, IL-10, TNF-α, IFN-γ, TGF-β and iNOS gene expression in splenocytes. Splenocytes were treated with ARE (0, 2, 20, 200$\mu g/ml$) or LPS (1$\mu g/ml$) for 6 and 12 hours.

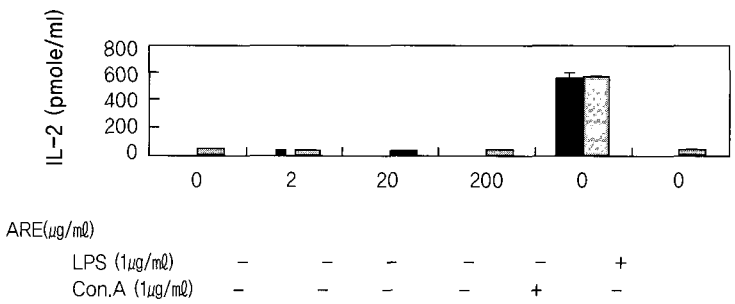

Fig. 5. IL-2 protein expression in splenocytes. Splenocytes were treated with ARE (0, 2, 20, 200$\mu g/ml$), Con A (1$\mu g/ml$) or LPS (1$\mu g/ml$) for 24(■) and 48 hours(□). The supernatant were used for analysis of protein expression. The results were expressed as mean±SD.

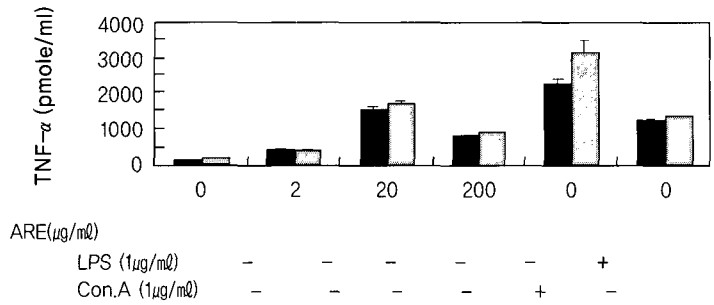

Fig. 6. TNF-α protein expression in splenocytes. Splenocytes were treated with ARE (0, 2, 20, 200$\mu g/ml$), Con A (1$\mu g/ml$) or LPS (1$\mu g/ml$) for 24(■) and 48 hours(□). The supernatant were used for analysis of protein expression. The results were expressed as mean±SD.

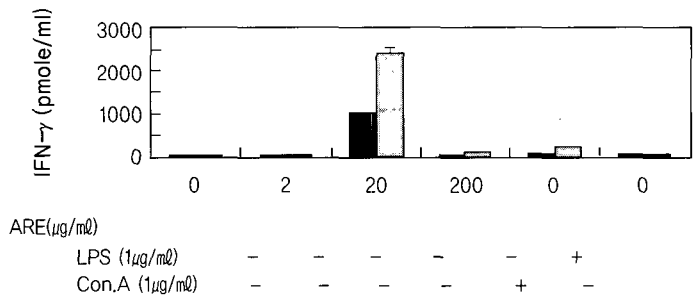

Fig. 7. protein expression in splenocytes. Splenocytes were treated with ARE (0, 2, 20, 200$\mu g/ml$), Con A (1$\mu g/ml$) or LPS (1$\mu g/ml$) for 24(■) and 48 hours(□). The supernatant were used for analysis of protein expression. The results were expressed as mean±SD.

dose-dependent manner. After 24 hours, IL-1β relative mRNA expression level was increased by 128% at 200 $\mu g/ml$ ARE whereas 5% at control. However, ARE did not affect TNF-α gene expression at all concentrations of ARE.

3. NK cell activity

ARE showed a significant effect on NK cytotoxic activity compared with control at 20 and 200$\mu g/ml$ ARE at ratio of 100:1 and 200:1 (effector cell to target cell). AT ratio of 200 (effector cell) : 1 (target cell), ARE (20, 200$\mu g/ml$) increase the NK cytotoxic activity significantly by 16.4% and 19.3% respectively whereas 7.2% in control. However, there is no effect at a ratio of 50:1 at all concentrations of ARE(Fig. 3).

4. Changes in gene expression in splenocytes

To investigate gene expression of cytokines in murine splenocytes, splenocytes were isolated from BABL/c and treated with various concentrations of ARE (0, 2, 20, 200$\mu g/ml$) or LPS (1$\mu g/ml$) for 6 and 12 hours. IL-1 β mRNA expression were up-regulated at over 20$\mu g/ml$ ARE 12 hours after treatment. IL-2 gene expression was not altered by ARE treatment. IL-12 and iNOS were up-regulated slightly on dose-dependent manner. ARE up-regulated IFN-γ gene expression remarkably and dose-dependantly and at 20 and 200$\mu g/ml$ ARE, the gene expression increased by over 20 times of control(Fig. 4).

5. Change in protein levels of cytokines with splenocytes

To investigate protein expression in mouse splenocytes, splenocytes were treated with various concentrations of ARE (0, 2, 20, 200$\mu g/ml$) and LPS (1 $\mu g/ml$) for 24 and 48 hours. Protein expression was determined by ELISA kit. IL-2 expression was not altered by ARE treatment. TNF-α expression also increased by ARE treatment but at 200$\mu g/ml$, TNF-α expression was less than that at 20$\mu g/ml$. IFN-γ expression also increased significantly at 20$\mu g/ml$ ARE(Fig. 5-7).

Discussion

The cancer immunosurveillance is regarded as one of the most important factors in preventing the development of cancer through early destruction of abnormal cells by host's immune system. It has been well known that the specific immune system of the host does not respond to the tumor cell effectively because most tumors have multiple mechanisms for evading immune surveillance. However, nobody can deny that the host immunity is the most important property in tumor incidence, growth, metastasis and patients mortality. Moreover, this is true during clinical treatment[7].

The immune system consists of humoral and cellular immune responses. One can also distinguish between innate immunity and adaptive immunity. According to the characteristic of innate immunity, it can work with less tumor specific antigen dependent manners. And among immune cells related with innate immunity, Macrophages and NK cells are the most important non-specific effector cells[8,9].

They should be widely distributed throughout the body and able to migrate to various tissue sites, also exceedingly responsive to recruiting signals, activation signals and costimulatory triggers. They should possess or produce the mediators killing tumor cells, such as NO or perforin and be prepared to employ, at any given time without previous notice, more than one strategy for elimination of cancer cells[10-12].

In many roles of macrophage and NK cells, production of cytokines is one of the important functions. They can be activated by lymphokines and other cell mediators to kill tumor cells by producing cytokines such as TNF, INF-γ and IL-12[13]. On the other hand, it is virtually impossible to pinpoint a physiological role for these cytokines on the natural mechanism of defense against tumors because of their complex and pleiotypic influences[8,14]. Owing to the complexity of cytokines' interaction, the therapeutic use of these agents is still not obvious. Hence, it might be better to release cytokine not through artificial infusion but through natural production in the human body.

There are many therapeutics and a thousand plants that are candidates for cancer agents in the Oriental medical field. The effects of immune modulating and anti-tumor activity using various herbal plants have been experimented extensively and reported over the world[3-5]. ARE 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 the properties of ARE on enhancing of macrophage and NK cell activities and some related cytokine gene expressions.

Activated macrophage expresses inducible nitric oxide synthase (iNOS), whose product (NO) have major role in bactericidal and anti-tumor function[15-17]. We evaluated the effects of ARE on activation of macrophage by detection of NO release from RAW 264.7 cells treated with ARE. NO production significantly increased by 2 fold at 200$\mu g/ml$ of ARE. Although ARE did not affect NO production in low concentration below 20$\mu g/ml$. ARE also significantly enhanced iNOS mRNA gene expression on dose-dependant manner.

In gene expression of cytokines, TNF-α gene expression was not affected by ARE treatment. But IL-1β mRNA expression was up-regulated significantly on dose-dependent manner. IL-1 is usually secreted by activated macrophage or other antigen presenting cells. TNF also acts directly on many other types of immune and inflammatory cells[15-17]. These results imply that ARE activates cell-mediated response by macrophage.

Next, to investigate the effects of ARE 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, IL-12, iNOS, IFN-γ, TGF-β and TNF-α).

IL-12, produced mainly by monocytes, macrophages and dendritic cells, is a cytokine that regulates the transition from innate to adaptive immunity. It induces secretion of IFN-γ and other cytokines from T and NK cells, and is a central regulator of Th1 cell development and exerts strong anti-tumor effects when administered to mice[18]. 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 destroying tumor cell[19].

ARE increased NK cytotoxic activity compared with control at 20, and 200$\mu g/ml$ ARE at ratio of 100:1 and 200:1 (effector cell to target cell). However, there is not effect at ratio of 50:1. Moreover, it showed a significant increase IL-1 and iNOS in splenocytes same as in RAW 264.7 cells. IL-12, TNF-α and iNOS were up-regulated slightly on dose-dependantly. Especially, ARE up-regulated IFN-γ gene expression remarkably and dose-dependantly. At 20 and 200 $\mu g/ml$ ARE, the gene expression increased by over 20 times of control.

These results indicate that ARE may have antitumor property by promotion of the NK cell activity to lysis the Yac-1 cells and activation of IL-1, iNOS, TNF-α, IL-12 and IFN-γ.

If one looks at the above results, it could be concluded that ARE has significant properties to activate macrophages and NK cells by promoting related cytokines like IL-1, IL-12, IFN-γ, iNOS and TNF-α gene expressions. In conclusion, ARE may present anticancer effects by modulating immune response specific to cancer. It can be a useful anticancer agent and more needs to be learned about their mechanisms of action and therapeutic potential and more clinical trials should be expected.

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