

Synergistic Effect of Natural Killer Cells and Bee Venom on Inhibition of NCI-H157 Cell Growth

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[Abstract]

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Objectives : This study examined the effects of Bee venom on apoptosis in NCI-H157 human lung cancer cells and for promoting the apoptosis effects of Natural killer cell.

Methods : Bee venom and Natural killer-92 cells were cultured either separately from or together with NCI-H157 cells for 24 hours. To figure out whether Bee venom enhances the cytotoxic effect of Natural Killer-92 cells, a cell viability assay was conducted. To observe the changes in Death receptors, apoptotic regulatory proteins and Nuclear factor- κ B, western blot analysis was conducted. To observe the effect of Bee venom through an extrinsic mechanism, a transfection assay was conducted.

Results :

1. Natural killer-92 cells and Bee venom significantly inhibited the growth of NCI-H157 cells and co-culture had more inhibitory effect than the separate culture.
2. Expressions of Fas, DR3, DR6, Bax, caspase-3, caspase-8, cleaved caspase-3, cleaved caspase-8 were increased, and expressions of Bcl-2 and cIAP were decreased. More efficacy was observed in co-culture than in separate culture.
3. Nuclear Factor- κ B activation was clearly decreased. And co-culture showed much less activation than separate culture.
4. As a result of treatment for DR-siRNA, the reduced cell viability of NCI-H157 cells and the activity of Nuclear factor- κ B were increased. With this, it can be seen that Bee venom and Natural killer-92 cells have an effect on the cancer cells through the extrinsic mechanism.

Conclusion : Bee venom is effective in inhibiting the growth of human lung cancer cells. Furthermore Bee venom effectively enhances the functions of Natural killer cells.

Key words :

Bee Venom ; Lung Cancer ; Natural Killer cell ; Death Receptor ; Nuclear Factor- κ B ; Apoptosis

Received : 2016. 02. 20.

Revised : 2016. 03. 01.

Accepted : 2016. 03. 02.

On-line : 2016. 03. 18.

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I. Introduction

Because of population aging, interest in cancer has been increasing steadily. Life expectancy of South Korea's population is more than 81.5 years old, about 10 years older than the world average in 2013, thus it is conceivable that cancer incidence or prevalence is higher than other countries^{1,2}.

73,759 people of South Korea died of cancer in 2012. Among them, mortality rate of lung cancer is located on the first place (33.1 people per 100,000)^{3,4}. Because lung cancer is not detected at an early stage and lung cancer in its early stage can easily pass by the diagnostic test due to the invasion or metastasis to surrounding tissues is easy⁵. Therefore, it is known that the mortality rate of lung cancer is so high. Many medical scientists stress the gravity of early detection of lung cancer and have developed a variety of diagnostic techniques and tried various methods (for example, multi-modality therapy or chemotherapy, radiation therapy, etc) to cure lung cancer^{6,7}. Recently, a cancer therapy by targeting the gene has been studied and new radiotherapy techniques for reducing damage to normal tissue surrounding lung cancer or mitigating side effects of the radiation damage are emerging in a stream⁵.

In addition, treatment with immune cells have been spotlighted recently as new cancer therapies. Controlling the environment around cancer cell and the host's immune system, immunotherapy aims at maximizing the effectiveness of radiation therapy and chemotherapy. In this regard, various treatment researchs such as a cancer vaccine therapy and cytokine therapy are in progress^{8,9}. Cell-immune therapy using immune cells such as T lymphocytes, dendritic cells has also been studied. In the various body's immune cells, the Natural Killer cell (NK-cell) which is mainly involved in cancer-related immune is focused especially. NK-cell, as one of the innate immune cells, is known that it has a selective cytotoxicity against cancer cells, and directly involved in the generation, pro-

liferation and metastasis¹⁰⁻¹².

In Korean medicine, A lot of research regarding the treatment of cancer with herb medication¹³⁻¹⁷ and pharmacopuncture¹⁸ has been conducted and many Korean medicine doctor have made a great effort to find a cure for cancer through a variety of case report. In other medicine fields, studies using herbal medicine extract have been made for human immune¹⁹⁻²³.

Pharmacopuncture is now being used in many clinic and its efficacy for disease such as various arthritis, HIVD, strains has been proven^{24,25}. Among them, as anti-cancer effects of melittin, one of the components of Bee Venom (BV), has emerged, various cancer researches using BV to cervical cancer²⁶, prostate cancer^{27,28} and lung cancer²⁹⁻³¹ have been conducted until now. According to these researches, BV is known to act on the apoptosis pathway of cancer cells, as a result, it promotes apoptosis in cancer cells³⁰. In addition, BV rises the cytotoxic effect of human natural killer cell and affects expression of The Nuclear Factor- κ B (NF- κ B) family^{27,28,30}, Death receptors (DR)^{26,29,31} in the cancer cells.

By extension, based on these researches, the author puts together the targets which are affected by BV, NK-cell and confirms the synergistic effect of the BV and NK-cell by comparing each efficacy of the BV and NK-cell. Whereupon the author obtains a significant result.

II. Materials and method

1. Materials

Bee Venom (BV) was purchased from You-Miel Bee Venom Ltd (Hwasoon, Jeonnam, Korea). The composition of the BV was as follows : 45-50 % melittin, 2.5-3 % mast cell degranulating peptide, 12 % phospholipase A2, 1 % lysophospholipase A, 1-1.5 % histidine, 4-5 % 6-pentyl α -pyrone lipids,

0.5 % secarpin, 0.1 % tertiapin, 0.1 % procamine, 1.5–2 % hyaluronidase, 2–3 % amine, 4–5 % carbohydrate, and 19–27 % of others, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephrine, and unknown amino acids, with 99.5 % purity. All of the secondary antibodies such as Bax, Bcl-2, caspase-3, caspase-8, cleaved caspase-3, cleaved caspase-8, cIAP, Fas, DR3, DR6 used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). T4 polynucleotide kinase was obtained from Promega (Madison, WI, USA). Poly (dI-dC), horseradish peroxidase-labeled donkey anti-rabbit secondary antibody, and ECL detection reagent were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA).

2. Cell culture

NK-92 natural killer cell, NCI-H157 cell were obtained from the American Type Culture Collection (Manassas, VA, USA). NK-92 cells were grown at same conditions in MEM alpha medium that contained 20 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml IL-2. NCI-H157 cells were grown at 37 °C in 5 % CO₂ humidified air in RPMI 1640 medium that contained 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. In co-cultures the cells were grown in a mixed medium (1 : 1) of NCI-H157 and NK-92 medium in a trans-well system where the cells were separated by a porous polycarbonate membrane (pore size 0.4 µm ; distance 6.5 mm). NCI-H157 cells were first seeded at 5×10⁴ cells/well, cultured overnight whereafter the inserts with the NK-92 cells were added (5×10⁴ cells/well). All cells were cultured in 24-well plates from Costar. RPMI 1640, MEMalpha, penicillin, streptomycin, and FBS were purchased from Gibco Life Technologies (GrandIsland, NY, USA).

3. Cell viability assay

To determine the viable cell number, NCI-H157 cells, NK-92 cells and those co-cultured with NK-92 cells were seeded onto 24-well plates (5×10⁴ cells/well) and subconfluent cells were subsequently treated with BV (3 µg/ml) for 24 hrs. The cells were trypsinized, pelleted by centrifugation for 5 min, at 1500 rpm, resuspended in 10 ml of phosphate-buffered saline (PBS), and 0.1 ml of 0.2 % trypan blue was added to the cancer cell suspension in each solution (0.9 ml each). Subsequently, a drop of suspension was placed in a Neubauer chamber, and the living cancer cells were counted. Cells that showed signs of trypan blue uptake were considered to be dead, whereas those that excluded trypan blue were considered to be viable. Each assay was carried out in triplicate.

4. Western blot analysis

Cells were homogenized with lysis buffer (50 µM Tris, pH 8.0, 150 µM NaCl, 0.02 % NaN₃, 0.2 % SDS, 1 µM phenylmethylsulfonyl fluoride, 10 µl/ml aprotinin, 1 % igapel 630 (Sigma aldrich co., St Louis, MO, USA), 10 µM NaF, 0.5 µM EDTA, 0.1 µM EGTA, and 0.5 % sodium deoxycholate) and centrifuged at 23,000 g for 1 hr. Equal amounts of proteins (80 µg) were separated on SDS 12 % PAGE and then transferred to a nitrocellulose membrane (Hybond ECL ; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were blocked for 2 hours at room temperature with 5 % (w/v) nonfat dried milk in Tris buffered saline (10 µM Tris, pH 8.0, 150 µM NaCl) containing 0.05 % Tween 20. The membrane was incubated for 5 hrs at room temperature with specific antibodies : rabbit polyclonal antibodies for Bcl-2, Bax, cIAP, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, Fas, DR3, DR6 (1 : 1,000 dilution, Cell Signaling Technology, Inc., Beverly, MA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse immunoglobulin G-horseradish per-

oxidase (1 : 2,000 dilutions, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive proteins were detected with the ECL Western blotting detection system.

5. Transfection assay

NCI-H157 cells (3×10^4 cells/well) were plated in 24-well plates and transiently transfected with siRNA, using a mixture of siRNA and the WelFect-EXPLUS reagent OPTI-MEN, according to the manufacturer's specification (WelGENE, Seoul, Korea). The transfected cells were treated with $2 \mu\text{g}/\text{ml}$ Bee Venom for 24 hrs. Thereafter, cell viability assay was performed as described above.

6. Data analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (GraphPad Software, La Jolla, CA, USA). Data are presented as mean \pm SD. The differences in all data were assessed by one-way analysis of variance (ANOVA). When the P value test indicated statistical significance, the dif-

ferences were assessed by the Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

III. Experimental results

1. Effect of Bee Venom and NK-92 Cells on Cell Viability in NCI-H157 Cells

To assess whether BV enhances inhibitory effect of NK-92 Cells in NCI-H157 Cells, we analyzed cell viability by direct cell counting. The cells were co-cultured or not with NK-92 cells and then treated or not with BV ($3 \mu\text{g}/\text{ml}$) for 24 hrs. As shown in Figure 1, BV and NK-92 Cells inhibited cell proliferation of NCI-H157 Cells respectively. Moreover, when they were co-punctured with NK-92 cells and concomitantly treated by $3 \mu\text{g}/\text{ml}$ of BV, more influence was exerted on inhibition of proliferation of NCI-H157 Cells than BV or NK-92 cells co-culture alone (Fig. 1).

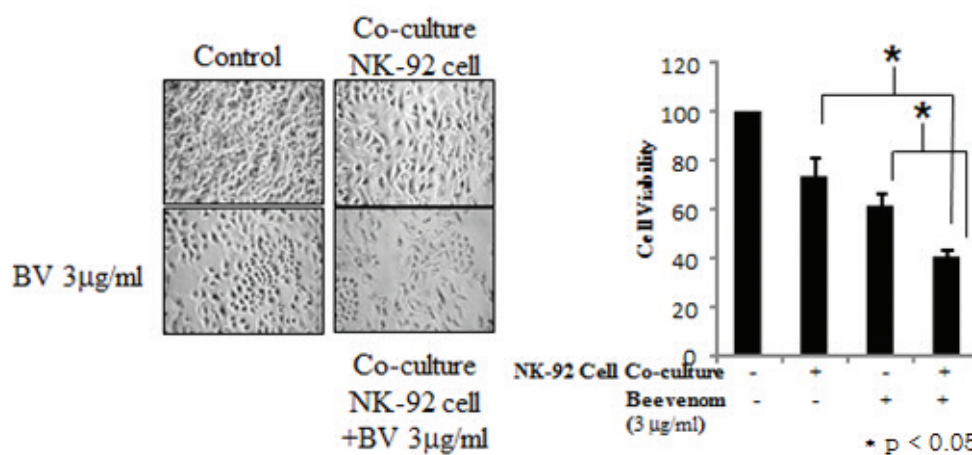


Fig. 1. Bee Venom enhanced cytotoxic effect of NK-92 cells on NCI-H158 human lung cancer cells

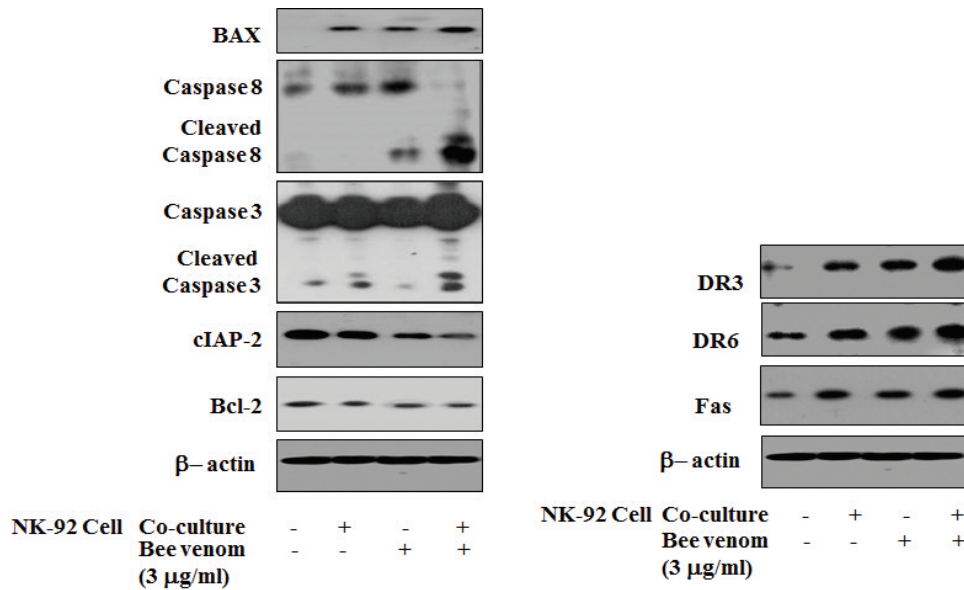


Fig. 2. Bee Venom enhanced the expression of death receptors and its related apoptotic proteins by NK-92 cells in NCI-H157 human lung cancer cells

2. Effect of Bee Venom and NK-92 Cells on the Expression of a Death receptor and a Apoptotic regulatory proteins in NCI-H157 Cells

To ascertain whether BV and NK-92 Cells synergistically enhance the expression of DRs such as Fas, DR3, DR6 and representative apoptotic regulatory proteins such as Bax, Bcl-2, cIAP, caspase-3, caspase-8, cleaved caspase-3 and cleaved caspase-8 in NCI-H157 cells. The expression of them was investigated by Western blots analysis. As a result, the expression of Fas, DR3, DR6 was significantly increased by co-culture of NK-92 cells and BV, compared to co-culture of NK-92 cells or BV treated cells alone (Fig. 2). Coincidentally, expression of pro-apoptotic proteins such as Bax, caspase-3, caspase-8, cleaved caspase-3 and cleaved caspase-8 was more increased and that of anti-apoptotic proteins such as Bcl-2, cIAP was more

decreased by co-culture of NK-92 cells and BV than by co-culture of NK-92 cells or BV treated cells alone (Fig. 2).

3. Effect of Bee Venom and NK-92 cells on NF-κB activity

To determine the synergistic inhibitory effect of BV and NK-92 cells on the growth of NCI-H157 cells, it was assessed NF-κB activity in the related cells including NCI-H157 cells, NK-92 cells, NK-92 cells co-treated NCI-H157 cells and 3 µg/ml of BV and NK-92 co-treated NCI-H157 cells for 24 hrs by EMSA. As represented in Fig. 3, NF-κB was highly activated in NCI-H157 cells, however, the activity was decreased in BV treated or NK-92 cells co-treated cells. Moreover, it was significantly decreased in BV and NK-92 cells co-treated NCI-H157 cells compared with the others (Fig. 3).

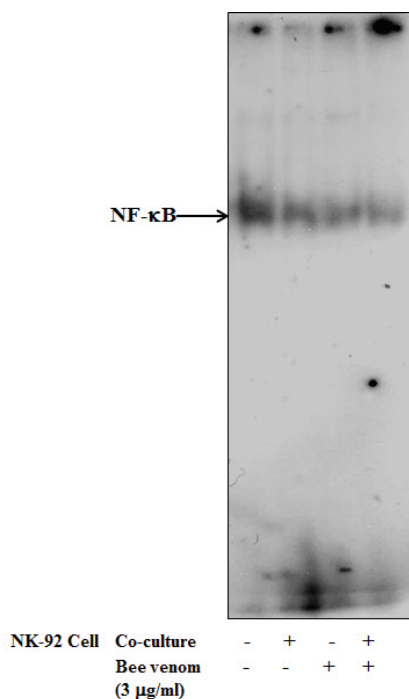


Fig. 3. Effect of Bee venom and NK-92 cells on NF- κ B activity

4. Reversed effects of Death Receptor siRNAs on Bee Venom-induced inhibition in NCI-H157 cells

To reconfirm whether BV and NK-92 cells synergistically inhibited cell growth of NCI-H157 cells through DR-mediated apoptosis, NCI-H157 cells were transfected with DR siRNA by using a transfection agent. The cells were transfected with 100 nM siRNA of DRs including Fas, DR3 and DR6 for 24 hrs, and then treated with BV (3 μ g/ml) for 24 hrs. Thereafter, cancer cell growth was measured by direct counting after trypan blue staining and EMSA was additionally performed. As results, siRNA transfected Fas, DR3 and DR6 reversely increased the cell viability and enhanced NF- κ B activity (Fig. 4).

IV. Discussion

Apoptosis performs a very important role in controlling the homeostasis of the cell. It also acts to remove harmful cells³². If the disorder of the gene in nucleus that causes apoptosis were incurred, it would be to destroy the homeostasis of the cells causing the development and growth of tumors. Namely to control the apoptosis in cancer therapy is the ultimate goal for today, and most anticancer drugs are currently being used control mechanism that trigger apoptosis induction^{33,34}.

Apoptosis occurs through two mechanisms that are expressed in the mitochondria³⁵ and from the cell surface³⁰. In the cell surface, receptors such as Fas, TNFR2, DR3, DR6 are situated and activated by tumor necrosis factors such as TNF- α , Fas ligand, Apo3L, TRAIL³³. And then it occurs the action of substances such as Death domain, caspase-6, caspase-8 causing apoptosis^{26,31}. Also, TNF secretes Cytochrome C by stimulating mitochondrial, thereby promotes apoptosis by increasing the activity of caspase-9³².

NF- κ B is a factor that plays an important role in some cancer cells, viz, 5 types of the transfer protein such as RelA, RelB, c-Rel, p50/p105, p52. These factors perform six functions, that is, life extension, survival, angiogenesis, metastasis, inflammation, and apoptosis inhibition of cancer cells. In particular, the factors are to extend the life of the cancer cells by inducing anti-apoptotic substance such as cIAP1, XIAP, Bcl-2, and then perform a major role in resistance to various treatment applied³⁶⁻³⁸. In other words, the regulation of NF- κ B can be a key to the treatment in the cancer cells. Currently, the substances that may inhibit or reduce the activity of NF- κ B such as flavonoids, brucine, gemcitabine, acacetin, tectochrysin³⁴, sulindac sulfide³³ have been detected and studied. In Korean medicine, the studies of herb medication and pharmacopuncture for inducing apoptosis have been actively conducted^{13,16-18}, and lately, there are many studies that BV has the effect of regulation of NF- κ B^{24,26-31,35,38,39}.

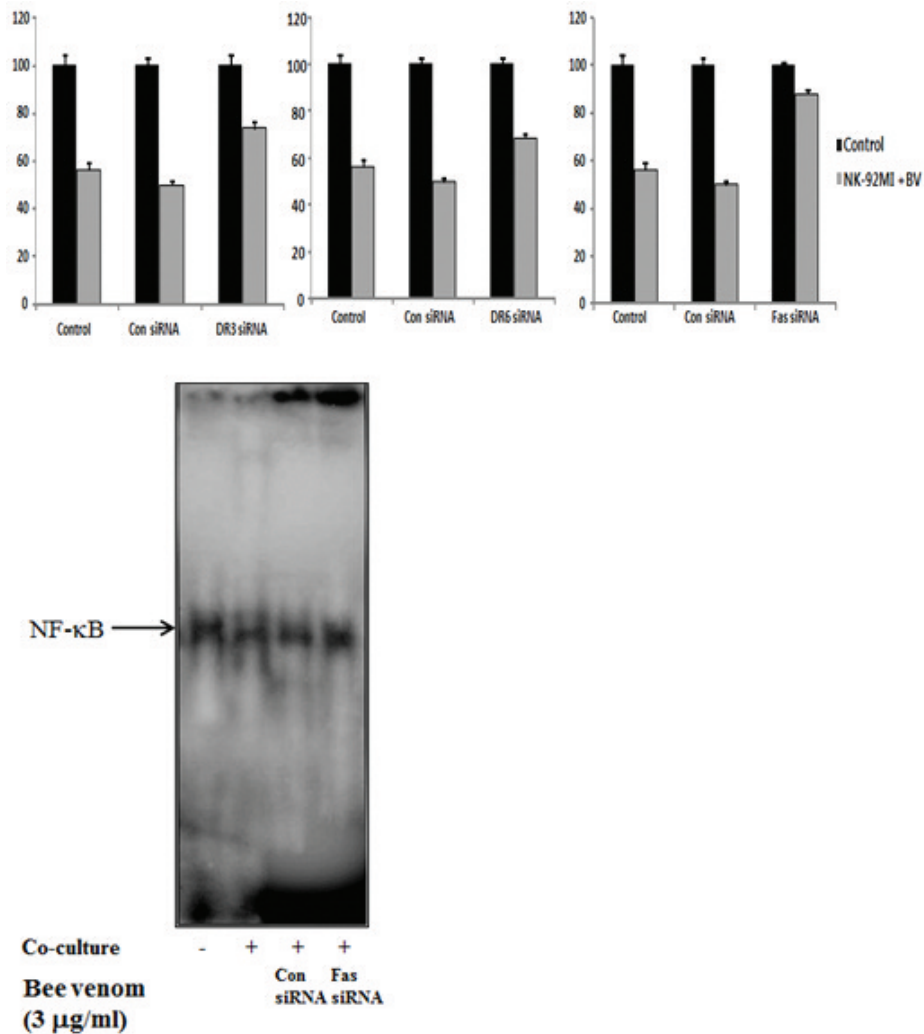


Fig. 4. Effects of siRNA of Death receptors on Bee Venom-induced anti-proliferation in the NCI-H157 human lung cancer cells

BV, as a composite material that contains various protein complexes, melittin, phospholipase, dopamine, norepinephrine, histidine, pyrone lipids, hyaluronidase, amine and mast cell degranulating peptides^{25,38,40} has been used a variety of clinical symptoms that is sprains, rheumatism, HIVD, etc²⁴. Especially, through a number of studies, it was revealed that the melittin which occupies about 50 % of the composition of BV is a very effective anti-inflammation, and further studies about its anti cancer effect have been made steady progress^{40,41}. According to Ahn²⁵, the melittin reduces the expression of Bcl genes and proteins involved in apoptosis control, enables the caspase by expressing the Bax gene involved in apoptosis in-

duction, and lowers survival ability of cancer cells by reducing the expression of PGE-2 and COX-2 proteins as inflammatory substance. In addition, it also carries out that serves to prevent cancer metastasis by reducing the Rac1-dependent activity associated with cancer metastasis.

In addition, according to studies using entire BV, it demonstrates that BV not only reduces the size of the cancer cells but do not affect the size and number of normal cells^{39,40}, further it shows that the appropriate targeted therapy to cancer cells is possible. And rather than causing the apoptosis directly, BV increases the expression of DRs in the cell surface (Fas, DR3, DR4, DR6, and so on), thereby it increases the amount of the pro-apop-

otic proteins (Caspase-3, caspase-9, Bax, etc.). Moreover, BV that inhibits the activity of the anti-apoptosis factor, NF- κ B, consequently promotes the apoptosis³⁶⁻³⁹.

NK-cell is the body's innate immune system and the body's critical immune factor that is involved in infection defense, autoimmune, hematopoietic stem cells and reproduction^{9,10}. Other characteristic point of NK-cell against other immune cell such as T-cell is that it can function the immune system without previous infection and MHC restriction¹². In other words, NK-cell is primarily responsible for the major surface in the treatment of cancer research, because it is responsible for immunity to neoplasms such as cancer¹⁰⁻¹². Through the study of Kim³⁰, it demonstrates that BV enhances the cytotoxicity of NK-cell to tumor cells. Based on this report, the author tries to compare the effect of co-action of BV and NK-cell with the effect of each BV or NK-cell on the expression of apoptotic factors (DR, pro-apoptotic proteins, anti-apoptotic factor) in human lung cancer cells, and so to examine the synergistic effect of BV toward NK-cell.

In the figure of the viability of cancer cells, as the results of culturing each BV or NK-92 cell to human lung cancer cells, both statistically significant decrease in cancer cell population was observed, in addition, it showed that the survival rate of cancer cells group co-culturing NK-92 cell and BV was significantly less than the survival rate of cancer cells group culturing each BV or NK-92 cell (Fig. 1).

Examining the expression results of the DRs and apoptotic factors, the expression of Fas, DR3, DR6 of each BV and NK-92 cell culture groups was stronger than the control group, and the expression of DRs of the group culturing with both BV and NK-92 cell was strongest than any other groups. The more activity of DRs increases, the easier action of TNF is made, which suggests that apoptosis pathway operates easily.

Looking at the expression of pro-apoptotic proteins (Bax, caspase-8, caspase-3, Cleaved caspase-8, Cleaved caspase-3), it can be seen that the groups culturing each BV and NK-92 cell have no-

ticeable appearance of pro-apoptotic proteins than the control group. And like on, the expression of pro-apoptotic protein in the group co-culturing BV and NK-92 cell was observed higher than any other groups. This suggests that BV can activate function of the substances that are involved in apoptosis.

About the expression of anti-apoptotic agents such as CLAP-2, Bcl-2, NF- κ B, it was obtained the result that both BV and NK-92 cell can effectively inhibit the expression of anti-apoptotic agents. And especially, it was observed that the expression of anti-apoptotic agents is significantly prevented in group co-culturing BV and NK-92 cell.

Integrating this results, BV and NK-cell facilitate activity of TNF increasing the expression of DRs, function to induce apoptosis while activating the pro-apoptotic proteins that appear in the apoptosis pathway and inhibiting the anti-apoptotic agents, and furthermore, the effect can be most powerful when BV and NK-cell work at the same time.

Finally, for reliably demonstrating the effect of the BV and NK-cell to DRs, the author observed the change of cancer cell viability and NF- κ B siRNA by treating the DR-siRNA on cancer cells group which has reduction of the cell viability. The siRNA acts as inhibitor of the protein production attaching to the RNA for the protein production. In this study, it plays the role as inhibiting the production of DRs. As a result of treating to DR3, DR6, Fas-siRNA to group of co-culturing BV and NK-92 cell, it was confirmed that the survival of cancer cells rises significantly. By this means, it can be re-confirmed that BV and NK-92 cell are involved in the inhibition of cancer cell DRs and NF- κ B (Fig 4).

In conclusion, the author presents the possibility that BV could be a method of cancer treatment. As the therapeutic mechanism, by enhancing the expression of the DRs helping the immune function of NK-92 cell, activating the pro-apoptotic proteins, inhibiting the expression of anti-apoptotic factors, BV can induce the apoptosis of cancer cells. In order to emerge as anti-cancer therapies, it

needs that future research about the anti-cancer effects of BV should be done. Based on this research, the author expects that many a study of synergistic effect between several chemotherapy, immuno-therapy and BV are to be made.

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