Original Article

Bee Venom Enhanced Cytotoxic Effect of Natural Killer Cells on Human Lung Cancer Through Inducing Extrinsic Apoptosis※

Jung Hyun Kim and Ho Sueb Song∗

Department of Acupuncture & Moxibustion Medicine, College of Orinetal Medicine, Gachon University

[Abstract]

Objectives : I investigated whether Bee Venom can synergistically strengthen the cytotoxic effects of NK-92 cells, enhancing the inhibition of the growth of Lung Cancer Cells including A549 and NCI-H460 through induction of death receptor dependent extrinsic apoptosis and NO generation in the Nitro-oxide pathway.

Methods : Bee Venom inhibited cell proliferation of A549 or NCI-H460 Human Lung Cancer Cells as well as NK-92 Cells. Moreover, when they were co-punctured with NK cells and concomitantly treated by 3 ㎍/㎖ of Bee Venom, more influence was exerted on inhibition of proliferation of A549 or NCI-H460 Human Lung Cancer Cells than BV or NK cell co-culture alone.

Results : The expression of Fas, TNFR2, DR3, DR6 in A549 Lung Cancer Cells was significantly increased by co-culture of NK-92 cells and treatment of 3 ㎍/㎖ of Bee Venom, compared to co-culture of NK-92 cells alone, whereas the expression of Fas, TNFR2, DR6 in NCI-H460 Lung Cancer Cells was significantly increased by co-culture of NK-92 cells, representing no synergistic effects in the co-culture of NK-92 cell and concomitant treatment of 3 ㎍/㎖ of Bee Venom. Coincidently, caspase-8, a expression of pro-apoptotic proteins in the extrinsic apoptosis pathway demonstrated same results as the above. Meanwhile, In NO generation, there is little change of NO generation in co-culture of NK-92 cells with A549 cells as well as the co-culture of NK-92 cell with them and concomitant treatment of 3 ㎍/㎖ of Bee Venom, whereas increase of NO generation was shown in co-culture of NK-92 cells with NCI-H460 cells as well as the co-culture of NK-92 cell with them and concomitant treatment of 3 ㎍/㎖ of Bee Venom, although synergistic effects by Bee Venom was not found.

Conclusions : These present data provide that Bee Venom could be useful candidate compounds to enhance lung cancer growth inhibiting ability of NK-92 cells through DR expression and the related apoptosis.

Ⅰ. Introduction

Lung cancer is one of common major malignancy, representing high incidence and mortality in the Republic of Korea as well as in the world[1,2]. Although various therapeutic strategies including chemo- therapy, radiotherapy have been tried to overcome it, high systemic toxicity and drug resistance usually cast shadow over successful results in most cases[3]. Therefore, novel therapeutic and preventive agents are urgently required to be developed for minimizing its mortality and increasing 5 year survival rate.

Based on the cumulative experiences of pharmaco- puncture for treating many inflammatory disease such as arthritis, rheumatism, HNPs and Sprains in clinical practice of Korean medicine, the anti-in- flammatory effect of bee venom and its major component, melittin has been investigated and well being established that they exerts anti-inflammatory effects by inhibition of signal molecules' activity in the NF-κB signal pathway[4]. Moreover, some experimental studies have expanded their NF-κB related anti-inflammatory properties to anticancer and demonstrated that bee venom and/or melittin also have anti cancer effects on various cancers including prostate, liver, breast, cervical, renal cancer cells through apoptosis via regulating activities of pro- or anti-apoptotic proteins such as Bax, Bcl-2, caspases, NF-κB[5-7].

Meanwhile, immuno-therapy is also proposed for an alternative to conventional lung cancer therapy, in which a deeper understanding of the immune response against lung cancer should precede innovative clinical approaches. In this regards, previous studies elucidated that natural killer(NK) cells play a critical role in recognizing and killing lung cancer cells in vitro by the balance of activating and inhibitory signals given by different groups of surface receptors, and that the infiltration of them can be considered to indicate a favorable prognosis of survival of patients with lung cancer as the first line of immune surveillance[8-10]. In addition, according to a recent report[11], bee venom inhibited proliferation of lung cancer cells through death receptor(DR) related extrinsic apoptosis as well as mitochondrial intrinsic one.

From the above, in the present study, I investigated anti-proliferative effects of bee venom and NK-92 cells on lung cancer cells including A549 and NCI-H460 invitro. Moreover, I confirmed whether both are synergistically correlated and enhances DR mediated extrinsic apoptosis.

Ⅱ. Materials and methods

A. Materials

Bee venom was purchased from You-Miel Bee Venom Ltd(Hwasoon, Jeonnam, Korea). The composition of the bee venom was as follows: 45~50 % melittin, 2.5~3 % mast cell degranulating peptide, 12 % phos- pholipase A2, 1 % lysophospholipase A, 1~1.5 % histidine, 4~5 % 6-pentyl a-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 TNFR2, caspase-8 and cleaved caspase-8 used in western blot analysis were purchased from Santa Cruz Biotechnology(Santa Cruz, CA). All other reagents were purchased from sigma unless otherwise stated.

B. Cell culture

NK-92 natural killer cell, A549 human lung cancer cell and NCI-H460 human epithelial lung adeno- carcinoma cell were obtained from the American Type Culture Collection(Manassas, VA). 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. A549 and NCI-H460 cells were grown at 37 ℃ in 5 %

CO_2 humidified air in RPMI 1640 medium that contained 10 % fetal bovine serum(FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. In cocultures the cells were grown in a mixed medium(1:1) of A549 and NCI−H460 and NK−92 medium in a trans−well system where the cells were separated by a porous polycarbonate membrane(pore size 0.4 mm ; distance 6.5 mm). A549 and NCI−H460 cells were first seeded at 5×10^4 cells/well, cultured overnight whereafter the inserts with the NK−92 cells were added(5×10^4 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).

C. Cell viability assay

To determine viable cell numbers, the A549 or NCI−H460 human lung cancer cells, and those co−cultured with NK−92 cells were seeded onto 24−well plates(5×10^4 cells/well) and subconfluent cells were subsequently treated with bee venom(3μg/ml) for 24 hr. The cells were trypsinized, pelleted by centrifugation for 5 minutes at 1,500 rpm, resuspended in 10 ㎖ of phosphate−buffered saline(PBS), and 0.1 ㎖ of 0.2 % trypan blue was added to the cancer cell suspension in each solution(0.9 ㎖ each). Subse−quently, 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.

D. 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 l/ml aprotinin, 1 % igapel 630 [Sigma], 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 g)

were separated on SDS−12 % polyacrylamide gels and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). Blots were blocked for 2 hr 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 hr at room temperature with following specific antibodies: rabbit polyclonal antibodies directed against caspase−8, cleaved caspase−8(1 : 1,000 dilutions; Cell Signaling Technology, Beverly, MA), Fas, TNFR1, TNFR2, DR3, DR4, DR5 and DR6(1 : 500 dilutions; Santa Cruz Biotechnology). The blot was then incubated with the corresponding conjugated anti−rabbit and anti−mouse immunoglobulin G−horseradish peroxidase(1 : 2,000 dilutions, Santa Cruz Biotechnology, Inc). Immunoreactive proteins were detected with the ECL western blotting detection system.

E. Reverse transcription(RT−PCR)

Total RNA was extracted by RNeasy(Qiagen, Valencia, CA). The reverse transcription reaction was performed using an RNA to cDNA Kit(Applied Biosystems/Life Technologies Corporation, Carlsbad, CA). The PCR was performed with cDNA as a template using the primers below after an initial 1 min denaturation at 96℃ followed by the indicated cycles of 96 ℃ for 1 min, 60 or 63 ℃ for 1 min and 72 ℃ for 1 min. The PCR primers used were 5′−ACCAAGTGCC ACAAAGGAAC −3′and 5′−CTGCAATTGAAGCACTGG AA−3′ for the human TNFR1; 5′−CTCAGGAG CAT GGGGATAAA−3′and 5′−AGCCAGCCAGTCTGACATC T−3′ for the human TNFR2; 5′−ATGGCGATGGC TGCGTGTCCTG−3′and 5′−AGCGCCTCCTGGGTCTC GGGGTAG−3′ for human DR3; 5′−ACTTTGGTTGT TCCGTTGCTGTTG−3′and 5′−GGCTTTCCATTTG CT GCTCA−3′for human DR4; 5′−TGGAACAACGGGGA CAGAACG−3′ and 5′−GCAGCA GCGCAAGCAGAAA AGGAG−3′for human DR5; 5′−AAGCCGGGGACCAA GGAGACAGAC AAC−3′ and 5′−TGCCGGGGCCCCTT TTTCAGAGT−3′for human DR6; 50−CAAAGCCC AT TTTTCTTCCA−3′and 5′−GACAAAGCCACCCCAAGTT

A−3′for human FAS; and 5′− GAAGGTGAAGGTC
GGAGT−3′and   5′−CTTCTACCACTACCCTAAAG−3′
for glyceraldeh yde−3−phosphate dehydrogenase.

F. Nitric oxide(NO) determination

The nitrite accumulation in the supernatant was
assessed by Griess reaction. Each 50 μl of culture
supernatant was mixed with an equal volume of
Griess reagent [0.1 % N−(1−naphthyl)−ethylene−
diamine, 1 % sulfanilamide in 5 % phosphoric acid]
and incubated at room temperature for 10 min. The
absorbance at 540 nm was measured in a microplate
absorbance reader, and a series of known con−
centrations of sodium nitrite was used as a standard.

G. Statistical analysis

The data were analysed using the GRAPHPAD PRISM
4 ver.4.03 software(GraphPad Software, La Jolla, CA).
Data are presented as mean ± SD. The differences in
all data were assessed by one−way analysis of variance.
When the p−value in the analysis of variance test
indicated statistical significance, the differences were
assessed by the Dunnett's test. A value of $p < 0.05$ was
considered to be statistically significant.

III. Results

A. Synergistic effect of bee venom and NK−92 cells on inhibition of cell growth in A549 human lung cancer cells

To assess whether bee venom enhances inhibitory
effect of NK−92 Cells in A549 human lung cancer
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 bee
venom(3 μg/ml) for 24 hr. As shown in Fig. 1, bee

venom and NK−92 cells inhibited cell proliferation of
A549 human lung cancer cells respectively. Moreover,
when they were co−punctured with NK cells and
concomitantly treated by 3 μg/ml of Bee Venom, more
influence was exerted on inhibition of proliferation
of A549 human lung cancer cells than BV or NK cell
co−culture alone.
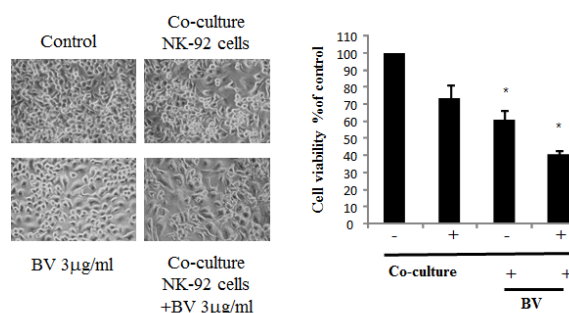

Fig. 1. Bee venom enhances cytotoxic effect of
NK−92 cells on A549 human lung cancer cells
* : p<0.05, significantly different from control cells without
BV treatment and co−culture with NK−92 cells.
The A549 human lung cancer cells, and those co−cultured
with NK−92 cells were seeded onto 24−well plates(5 × 10^4
cells/well) and subconfluent cells were subsequently treated
with bee venom(3 μg/ml) for 24 hr. The cells were then
harvested by trypsinization and stained with 0.2 % trypan
blue. Relative cell survival rate was determined by counting
live and dead cells. The results were expressed as a
percentage of viable cells. Columns, means of three
experiments, with triplicates of each experiment; bars, SD.

B. Synergistic effect of bee venom and NK−92 cells on inhibition of cell growth in NCI−H460 human lung cancer cells

To assess whether bee venom enhances inhibitory
effect of NK−92 Cells in NCI−H460 human lung
cancer 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 bee
venom(3 μg/ml) for 24 hr. As shown in Fig. 2, bee
venom and NK−92 Cells inhibited cell proliferation of
NCI−H460 human lung cancer cells respectively.
Moreover, when they were co−punctured with NK
cells and concomitantly treated by 3 μg/ml of bee
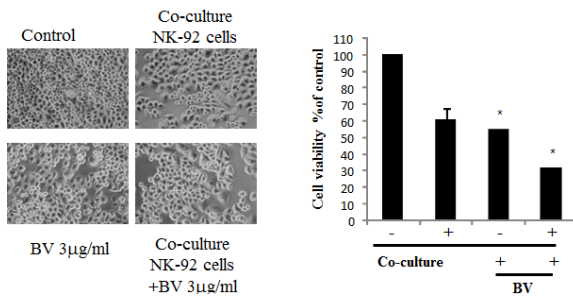venom, more influence was exerted on inhibition of

Fig. 2. Bee venom enhances cytotoxic effect of NK−92 cells on NCI−H460 human lung cancer cells

∗ : $p<0.05$, significantly different from control cells without BV treatment and co−culture with NK−92 cells.
The NCI−H460 human lung cancer cells, and those co−cultured with NK−92 cells were seeded onto 24−well plates(5×10^4 cells/well) and subconfluent cells were subsequently treated with bee venom($3 \ \mu g/ml$) for 24 hr. The cells were then harvested by trypsinization and stained with 0.2 % trypan blue. Relative cell survival rate was determined by counting live and dead cells. The results were expressed as a percentage of viable cells. Columns, means of three experiments, with triplicates of each experiment; bars, SD.

proliferation of NCI−H460 human lung cancer cells than BV or NK cell co−culture alone.

C. Synergistic effect of bee venom and NK−92 cells on the expression of a death receptor and a apoptotic regulatory protein in in A549 human lung cancer cells

To ascertain whether bee venom and NK−92 cells synergistically enhance the expression of a death receptors such as Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and a representative extrinsic apoptotic regulatory protein caspase−8 in A549 human lung cancer cells. The expression of them was investigated by RT−PCR and western blots analysis respectively. As a result, The expression of Fas, TNFR2, DR3, DR6 was significantly increased by co−culture of NK−92 cells and treatment of 3 $\mu g/ml$ of bee venom, compared to co−culture of NK−92 cells alone(Fig. 3). Coincidently, caspase−8, a expression of pro−apoptotic proteins in the extrinsic apoptosis pathway was also more enhanced by co−culture of NK−92 cells and
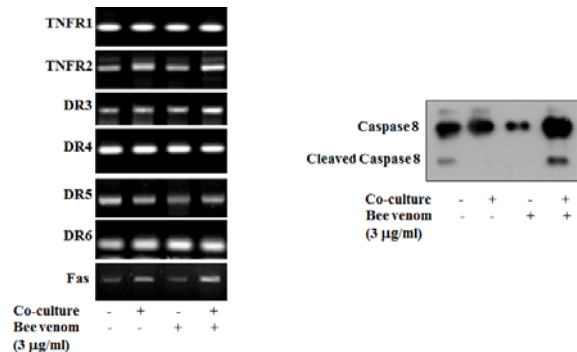

Fig. 3. Bee venom enhances the expression of death receptor and its related apoptotic protein by NK−92 cells in A549 human lung cancer cells
Expression of death receptors such as Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and caspase−8 and cleaved caspase−8, a extrinsic apoptosis regulatory proteins was determined using Western blot analysis. A549 human lung cancer cells were co−cultured with NK−92 cells and subsequently treated with 3 $\mu g/ml$ of bee venom for 24 hr. Equal amounts of total proteins(50 μg/lane) were subjected to 12% or 8% SDS−PAGE. Expression of caspase−8 and β−actin was detected by western blotting using specific antibodies, while expression of Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and GADPH was found by RT−PCR. β−actin or GADPH protein here was used as an internal control respectively. Each band is representative for three experiments.

treatment of 3 $\mu g/ml$ of bee venom than by co−culture of NK−92 cells alone(Fig. 3).

D. Synergistic effect of bee venom and NK−92 Cells on the expression of a death receptor and a apoptotic regulatory protein in in NCI−H460 human lung cancer cells

To ascertain whether bee venom and NK−92 cells synergistically enhance the expression of death receptors such as Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and a representative extrinsic apoptotic regulatory protein caspase−8 in NCI−H460 human lung cancer cells. The expression of them was investigated by RT−PCR and Western blots analysis respectively. As a result, the expression of Fas, TNFR2, DR6 was significantly increased by co−culture of NK−92 cells, whereas concomitant
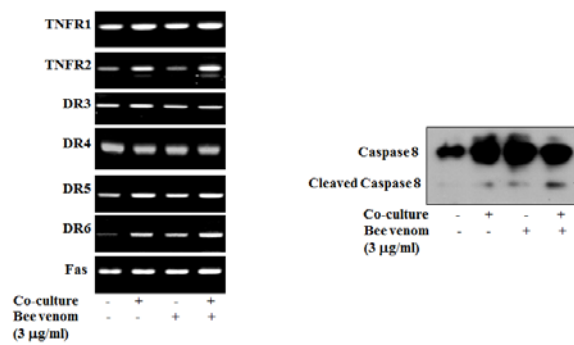
Fig. 4. Bee venom enhances the expression of death receptor and its related apoptotic protein by NK-92 cells in NCI-H460 human lung cancer cells

Expression of death receptors such as Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and caspase-8 and cleaved caspase-8, a extrinsic apoptosis regulatory proteins was determined using western blot analysis. NCI-H460 human lung cancer cells were co-cultured with NK-92 cells and subsequently treated with 3 ㎍/㎖ of bee venom for 24 hr. Equal amounts of total proteins(50 ㎍/lane) were subjected to 12 % or 8 % SDS-PAGE. Expression of caspase-8 and β-actin was detected by western blotting using specific antibodies, while expression of Fas, TNFR1, TNFR2, DR3, DR4, DR5, DR6 and GADPH was found by RT-PCR. β-actin or GADPH protein here was used as an internal control respectively. Each band is representative for three experiments.

co-culture of NK-92 cell and treatment of 3 ㎍/㎖ of bee venom showed little increase of it, compared to co-culture of NK-92 cells alone(Fig. 4). Similarly, caspase-8, a expression of pro-apoptotic proteins in the extrinsic apoptosis pathway was also more enhanced by co-culture of NK-92 cells alone than by co-culture of NK-92 cells and treatment of 3 ㎍/㎖ of bee venom(Fig. 4).

E. Synergistic effect of bee venom and NK-92 Cells on NO in A549 human lung cancer cells

To confirm whether NK-92 cells exert cytotoxic effects on A549 human lung cancer cells through the NO pathway, the nitrite accumulation in the super-natant was assessed by Griess reaction following co-culture of NK-92 cells or/and treatment of 3 ㎍/㎖ of bee venom. Level of NO into the medium was hardly
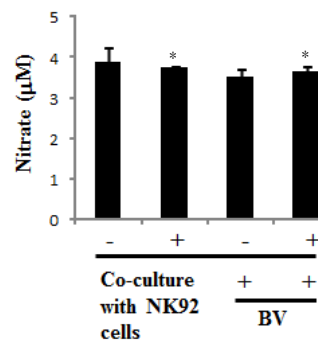

Fig. 5. Effect of co-culture of NK-92 cells on the level of NO in the A549 human lung cancer cells

∗ : $p<0.05$, significantly different from control cells without BV treatment and co-culture with NK-92 cells.
The cells were co-cultured with NK-92 cells and then treated with bee venom(3 ㎍/㎖) for 48 hr. Thereafter, the nitrite release in the supernatant was assessed by Griess reaction as described in materials and methods.
The figures are representatives of three experiments with replicates.
Values are mean ± SD of three experiments with replicates.

increased by co-culture of NK-92 cells compared to control or 3 ㎍/㎖ of bee venom(Fig. 5). Moreover, Synergistic effect of both was not represented either(Fig. 5).

F. Synergistic Effect of bee venom and NK-92 Cells on NO in NCI-H460 human lung cancer cells

To confirm whether NK-92 cells exert cytotoxic effects on NCI-H460 human lung cancer cells through the NO pathway, The nitrite accumulation in the supernatant was assessed by Griess reaction following co-culture of NK-92 cells or/and treatment of 3 ㎍/㎖ of bee venom. Level of NO into the medium was significantly increased by co-culture of NK-92 cells as well as by treatment of 3 ㎍/㎖ of bee venom (Fig. 6). However, synergistic effect of both was not shown compared to co-culture of NK-92 cells or treatment of 3 ㎍/㎖ of bee venom alone(Fig. 6).
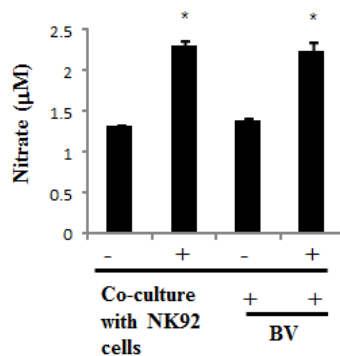
Fig. 6. Effect of co-culture of NK-92 cells on the level of NO in the NCI-H460 human lung cancer cells

* : $p<0.05$, significantly different from control cells without BV treatment and co-culture with NK-92 cells.
The cells were co-cultured with NK-92 cells and then treated with bee venom(3 ㎍/㎖) for 48 hr. Thereafter, the nitrite release in the supernatant was assessed by Griess reaction as described in Materials and methods.
The figures are representatives of three experiments with replicates.
Values are mean ± SD of three experiments with replicates.

Ⅳ. Discussion

Lung cancer is responsible for most cancer mortality worldwide[1,2]. Thus, development of a novel agents and reestablishment of efficacious therapeutic strategies including immuno-therapies are urgently needed. Especially, a thorough understanding of anti-tumour immune responses to non-small cell lung cancer(NSCLC) might pave the way for innovative clinical approaches, in which investigation of the role of NK cells in carcinogenesis is essential[12-16], because the infiltration of NK cells to NSCLC was found to indicate a favorable prognosis of it[8,9], instead of incomplete predictor, tumor-infiltrating CD81 T cells[17,18].

NK cells are innate lymphocytes capable of recognizing and exerting cytotoxic effects on aberrant cells including cancer cells in the absence of prior stimuli in vivo or in vitro[19-21].

Although its underlying molecular mechanisms how to discriminate normal cells from carcinoma cells is being elucidated, it currently become evident that NK cell recognition is dependent upon the dynamic

equilibrium of various activating or inhibiting signals given by surface receptors of target cancer cells[22,23].

In addition, NK cells is closely related to regulation of tumor necrosis factor(TNF) signaling due to their release of TNF family ligands including TNF, fas ligand(FasL) and TNF related apoptosis inducing ligand(TRAIL), which is very complicated and results in DR dependent extrinsic apoptosis[12,24-27]. For instance, Apoptosis is majorly led by TNFR1(DR1), though TNF-mediated processes was also regulated by tumor necrosis factor receptor2(TNFR2)[28,29]. The binding of TNF to DR1 gives rise to the trimerization of DR1 and concomitant recruitment of an adaptor protein, TNFR1-associated death domain(TRADD) into DR forming signaling complex(DISC). Then TRADD serves as a platform to recruit other adapter protein, Fas-associated death domain(FADD), subse-quently activating caspase-8 and initiating a down-stream caspase activations involving caspase-3[30,31]. Besides DR1, there are other death receptors such as FAS(DR2), DR3, DR4, DR5 and DR6 in the TNF receptor(TNFR) superfamily, which are also known as a death domain(DD) -containing receptor, binding to their ligands such as FasL, Apo3L, and TRAIL and therefor, similarly act as DR1 in the extrinsic DR mediated apoptotic pathway[32,33].

Meanwhile, NK cells kill cancer cells at least two mechanisms except for the above DR-ligand pathway[12]. NK cells can use the perforin/ granzyme-containing granule exocytosis pathway and the NO pathway, of which NO is one of the most powerful effector molecules in the cytotoxic function of NK cells against cancer cells in the latter[34-36].

Consistent with the above reports, present study demonstrated that bee venom and NK-92 cells inhibited cell proliferation of A549(Fig. 1) or NCI-H460(Fig. 2) human lung cancer cells respectively. Furthermore, when they were co-punctured with NK cells treated by 3 ㎍/㎖ of bee venom, more influence was exerted on   inhibition of proliferation of A549(Fig. 1) or NCI-H460(Fig. 2) human lung cancer cells than BV or NK cell co-culture alone. And that the expression of Fas, TNFR2, DR3, DR6 in the A549 cells was significantly increased by co-culture of

NK-92 cells and treatment of 3 ㎍/㎖ of bee venom, compared to co-culture of NK-92 cells alone(Fig. 3) and that of caspase-8, pro-apoptotic proteins in the extrinsic apoptosis pathway was also concurrently more enhanced by co-culture of NK-92 cells and treatment of 3 ㎍/㎖ of bee venom. Whereas concomitant co-culture of NK-92 cell and treatment of 3 ㎍/㎖ of bee venom in the NCI-H460 cells showed little synergistic increase of it, compared to co-culture of NK-92 cells alone(Fig. 4). Moreover, that level of NO into the medium was significantly increased by co-culture of NK-92 cells as well as by treatment of 3 ㎍/㎖ of bee venom only in NCI-H460 cells(Fig. 6) and synergistic effect of both was not shown compared to co-culture of NK-92 cells or treatment of 3 ㎍/㎖ of bee venom alone(Fig. 6).

The central and noteworthy finding in this study is that bee venom synergistically enhanced cytotoxicity of NK-92 cells in the lung cancer cells including A549 through increased expression of death receptor and subsequent induction of extricsic apoptosis involving caspase-8 activation, and that bee venom and NK-92 cells increased cytotoxicity through release of NO not in A549 but in NCI-H460 cells respectively, whereas they did not revealed syn-ergistic increase of NO release in A549 and in NCI-H460 cells as well.

Consequently, these present data provide that bee venom could be useful candidate agents to synergis-tically enhance cytotoxicity of NK-92 cells, inhibiting growth of lung cancer cell and they suggest that bee venom should compensate for conventional chemo-therapeutics through overcoming the resistance via enhancement of DR expression.

Ⅴ. References

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