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Original Article

Inhibitory Effects of Bee Venom on Growth of A549 Lung Cancer Cells via Induction of Death Receptors※

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[Abstract]

This study was to investigated the effects of the bee venom on inhibition of cell growth via upregulation of death receptor expression in the A549 human lung cancer cells. Bee venom(1-5 ㎍/ml) inhibited the growth of A549 lung cancer cells by the induction of apoptotic cell death in a dose dependent manner. Consistent with apoptotic cell death, expression of TNFR1, Fas, death receptors(DR) 3, 4 and 6 was increased in the cells. Expression of DR downstream pro-apoptotic proteins including caspase-3, -9 and Bax was concomitantly increased, but the expression of Bcl-2, NF-kB were inhibited by treatment with bee venom in A549 cells. Moreover, deletion of DR3, DR4 by small interfering RNA significantly reversed bee venom-induced cell growth inhibitory effect, whereas Apo3L strengthened anti-proliferative effect of bee venom through enhancement of DR3 expression. These results suggest that bee venom should exert anti-tumor effect through induction of apoptotic cell death in lung cancer cells via enhancement of death receptor expression, and that bee venom could be a promising agent for preventing and treating lung cancer.

Key words :
Bee venom;
Lung cancer;
A549;
Apoptosis
Death receptor

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Ⅰ. Introduction

Lung cancer is the leading cause of cancer death in the world for men and women, and its epidemic is closely associated with smoking[1]. The cancer can be divided into two groups, small-cell lung cancer and non-small-cell lung cancer(NSCLC), of which NSCLC accounts for about 80 %[2]. The high mortality rate of this disease is due to the difficulty of early diagnosis and its high potential to invade locally and metastasize to distant organs[3]. Although there are many therapeutic strategies including chemotherapy and radiotherapy, high systemic toxicity and drug resistance hinder the successful outcomes in most cases[4]. Therefore, novel diagnosis, treatment, and prevention approaches are urgently needed for reducing the mortality rates of NSCLC, based upon fully understanding of the molecular mechanisms of apoptosis.

Apoptosis, programmed cell death, plays a critical role in anti-cancer effects of chemotherapeutics, which can be induced by various intracellular and extracellular stimuli via either an intrinsic or extrinsic pathway in different cells[5,6]. While the intrinsic pathway is initiated by a mitochondria mediated death signaling cascade[7], the extrinsic pathway is characterized by the binding of cell surface receptors with their ligands such as death receptor 1(DR1) with tumor necrosis factor(TNF); death receptor 2(DR2) with Fas ligand(FasL); death receptor 3(DR3) with Apo3 ligand(Apo3L); death receptor 4(DR4) and death receptor 5(DR5) with TNF-related apoptosis-inducing ligand(TRAIL), Apo2L[8-15], and subsequent activation of the caspase cascade through formation of the death-inducing signaling complex(DISC) via recruiting the Fas-associated death domain(FADD) protein and procaspase-8[16,17]. Of the TNF cytokine family, TRAIL is currently drawing the attention of researchers and spurring several clinical initiatives to test its efficacy for cancer therapy, because it can selectively induce cell death in various tumors, showing little or no toxicity to normal cells.

According to recent reports[18-22], a combined delivery of TRAIL and chemotherapeutic agents have sensitized cells to TRAIL through mechanisms such as the up-regulation of death receptors, and regulation of Bcl-2 protein expression, overcoming resistance to TRAIL alone. Hence, understanding the molecular mechanisms of TRAIL resistance, and thereby resolving them, are a major challenges hindering the development of effective TRAIL-based therapeutic strategies for NSCLC. Bee venom contains a variety of different peptides, including melittin, phospholipase A2, apamin, adolapin and mast cell-degranulating peptide(MCDP), which has been used as a traditional medicine to treat back pain, rheumatism, and skin diseases due to its antibacterial, antiviral, and anti-inflammatory effects[23]. Moreover, several studies have demonstrated that bee venom either or melittin have anti-proliferative effects on various cancer cells such as prostate, liver, breast, cervical and renal cancer cells through intrinsic or extrinsic apoptosis[24-27]. However, experiments demonstrating the molecular mechanisms of the anti-cancer effects of bee venom in the A549 NSCLC cells have not been reported. In this study, effects of bee venom on cell growth and apoptosis in the A549 NSCLC cells, and its sensitizing mechanism related with TNF cytokine family such as Apo3L, TRAIL were investigated.

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 % phospholipase 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 Bax, Bcl-2, caspase-3, -9, cleaved caspase-3, -9, used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). T4 polynucleotide kinase was obtained from Promega(Madison, WI). Poly(dI-dC), horseradish peroxidase-labeled donkey anti-rabbit secondary antibody, and ECL detection reagent were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Reagents for sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis were purchased from Bio-Rad(Hercules, CA). Caspase inhibitor(Z-VAD-FMK) was from Promega (Madison, WI). DR3, DR4, and DR6 siRNA were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich(St. Louis, MO, USA) unless otherwise stated.

B. Cell culture

The A549 lung cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA), and were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum(FBS) and penicillin/streptomycin(100 U/ml). Cell cultures were then maintained at 37 ℃ in a humidified atmosphere with 5 % CO_2.

C. Cell viability assay

To determine the cell number, A549 lung cancer cells were plated in 24-well plates($5x10^4$ cells/well) with or without Apo3L, and subconfluent cells were subsequently treated with bee venom(1, 2 and 5 ㎍/ml) for 24 hrs. After treatment, cells were trypsinized and pelleted by centrifugation for 5 min at 1,500 rpm, resuspended in 5 ml of phosphate-buffered saline(PBS), and 0.1 ml of 0.2 % trypan blue was added to the cancer cell suspension in each of the solutions(0.9 ml each).

Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. Each assay was carried out in triplicate.

D. Reverse transcription(RT)-PCR

Total RNAs were isolated from cultured cells using RNeasy Plus Mini Kit(Qiagen, Duesseldorf, Germany) according to the manufacturer's manual. The RNA pellet obtained in the final step was dissolved in 30 ㎕ of sterile diethylpyrocarbonate (DEPC)-treated water, and its concentration was determined using a UV spectrophotometer at 260 nm. RNA was kept in DEPC-treated water at -70 ℃ until use. Reverse transcription was performed using High Capacity RNA-to-cDNA Kit(AB, Life Technologies Co, CA, USA).

E. Western blot analysis

The membrane was incubated for 2 hrs at room temperature with specific antibodies: rabbit polyclonal for caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, Bcl-2(1:1,000 dilution, Cell Signaling Technology, Inc, Beverly, MA), Bax(1:500 dilution, Santa Cruz Biotechnology, Inc), goat polyclonal antibody to p50(1:500), p65(1:500), phospho-IkBa (1:200)and mouse polyclonal antibody to kB kinases (1:500). 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). Immuno-reactive proteins were detected with the ECL Western blotting detection system.

F. Transfection assay

A549 lung cancer 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 reagentin OPTI-MEN, according to the manufacturer's specification(WelGENE, Seoul,

Korea). The transfected cells were treated with 2 ㎍/ml bee venom for 24 hrs.

DR3 siRNA seq. 5'- GAAGCCCUAAGUACGGUUAtt
DR4 siRNA seq. 5'- CUCUGAUGCUGUUCUUUGAtt

G. Apoptosis evaluation

A549 Lung cancer cells(2.5×10^5 cells/well) were cultured on 8-chamber slides. The cells were treated with bee venom(1, 2 and 5 ㎍/ml).

The cells were washed twice with PBS and fixed by incubation in 4 % paraformaldehyde in PBS for 1 hr at room temperature. Membrane was permeabilized by exposure to 0.1 % Triton X-100 in phosphate-buffered saline for 5 min at room temperature. TdT-mediated dUTP nick and labeling(TUNEL) assays were performed by using the in situ Cell Death Detection Kit(Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. For 4′-6-Diamidino-2-phenyl indole(DAPI) staining, slides were incubated for 15 min at room temperature in the dark with mounting medium for fluorescence containing DAPI(Vector Laboratories, Inc, Burlingame, CA). The cells were then observed through a fluorescence microscope(Leica Microsystems AG, Wetzlar, Germany).

H. Preperation of nuclear extracts and electromobility shift assays

It was performed according to the manufacturer's recommendations(Promega, Madison, WI). Briefly, 1×10^6 cells/ml was washed twice with 1×PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 g for 1min, and the resulting supernatant was removed. Solution A(50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 ㎍/ml phenylmethyl-sulfonyl fluoride, 1㎍/ml pepstatin A, 1 ㎍/ml leupeptin, 10 ㎍/ml soybean trypsin inhibitor, 10 ㎍/ml aprotinin, and 0.5 % Nonidet P-40) was added to the pellet in a 2:1 ratio(v/v) and allowed to incubate on ice for 10 min. Solution C(solution A + 10 % glycerol and 400mM KCl) was added to the pellet in a 2:1 ratio(v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000 g for 7min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [g-32P] ATP for 10 min at 37 ℃. Gel shift reactions were assembled and allowed to incubate at room temperature for 10min followed by the addition of 1 ㎕(50,000-200,000 cells per medium) of 32P-labeled oligonucleotide and another 20 min of incubation at room temperature. For supershift assays, nuclear extracts from cells treated with bee venom(1~5 ㎍/㎖) were incubated with specific antibodies against the p50, p65 and Rel-A NF-κB isoforms for 1 hr before EMSA. For competition assays, nuclear extracts from cells treated with bee venom(1~5 ㎍/㎖) were incubated with unlabelled NF-κB oligonuclaotide(50×, 100× and 200×) or labeled SP-1(100×) and AP-1(100×) for 30 min before EMSA. Subsequently 1 ㎕ of gel loading buffer was added to each reaction and loaded onto a 6 % nondenaturing gel and electrophoresed until the dye was three-fourths of the way down the gel. The gel was dried at 80℃ for 1 hr and exposed to film overnight at 70 ℃. The relative density of the DNA-protein binding bands was scanned by densitometry using MyImage(SLB, Seoul, Korea), and quantified by Labworks 4.0 software(UVP Inc, Upland, California).

I. Colony formation assay

Approximately 500 mock A549 or stablely transfected A549 lung cancer cells(A549/miR-451 and A549/miR-NC) were placed in a fresh 6-well plate with or without bee venom for another 12 hrs and maintained in RMPI 1640 containing 10 % FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1 % crystal violet in 20 % methanol for 15min.

J. Confocal Immunocytochemistry

To determine whether bee venom could be uptaken into the cells, cells(1×10^5 cells/cm^2) were cultured on the chamber slide(Lab-Tak II chamber slider system, Nalge Nunc Int., Napervill, IL, USA) and then treated by bee venom labeled with superior Alexa Fluor 488 dye(Molecular Probe, Eugene, Oregon, USA). A549 lung cancer cells were incubated for 24 hrs at 37 ℃, and the cells were then fixed in 4 % paraformaldehyde, membrane permeabilized by exposure for 5 min to 0.2 % Triton X-100 in phosphate-buffered saline, and were placed in blocking serum(5 % horse or goat serum in phosphate-buffered saline). Immuno-fluorescence images were acquired using a confocal laser scanning microscope(dual wavelength scan, MRC1024, Bio-Red, Hercules, CA, USA) with a 60 × oil immersion objective.

K. Data analysis

The data were analyzed 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(ANOVA). When the p value in the ANOVA 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. Effects of bee venom on cell growth in lung cancer cells

To assess the inhibitory effect of bee venom on cell growth of A549 lung cancer cells, we analyzed cell viability by direct cell counting. The cells were treated with several concentrations of bee venom(1, 2 and 5 ㎍/ml) for 24 hrs. Bee venom inhibited cell proliferation of lung cancer cells in a concentration -dependent manner. 24 hrs treatment with bee venom inhibited A549 lung cancer cell growth with IC_{50} value of 2.91 ㎍/ml. Morphologic observation showed that the cells were gradually reduced in size and changed into a small round single cell shape by the treatment of bee venom in A549 lung cancer cells(Fig. 1).

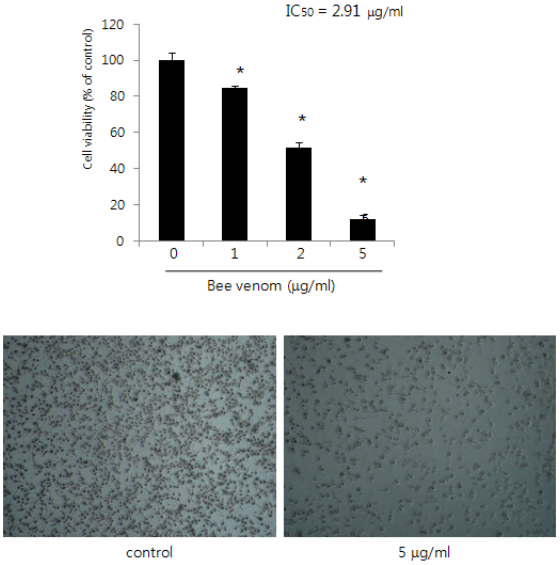

Fig. 1. Effects of bee venom on cell viability in A549 lung cancer cells

Concentration-dependent effect of bee venom was shown on the cell viability assay in A549 lung cancer cells. After treatment of bee venom(1, 2 and 5 ㎍/ml) for 24 hrs, the cells were 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. Morphologic observation with the treatment of bee venom in A549 cells. Columns, means of three experiments, with triplicates of each experiment; *, $p<0.05$, significantly different from untreated control cells.

B. Bee Venom induced apotosis

To determine the inhibition of cell growth by bee venom was due to the induction of apoptotic cell death, we evaluated the changes in the chromatin morphology of cells by using DAPI staining followed by TUNEL staining assays, and then the double labeled cells were analyzed by fluorescence microscope. Conversely well to cell proliferation, DAPI-stained TUNEL-positive cells were significantly

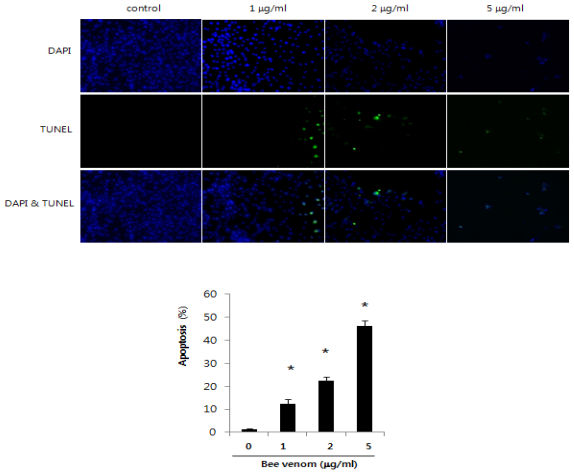

Fig. 2. Effects of bee venom on apoptosis in A549 lung cancer cells

The A549 lung cancer cells were treated with bee venom(1, 2 and 5 μg/ml) for 24 hrs, and then labeled with DAPI and TUNEL solution. Total number of cells in a given area was determined by using DAPI nuclear staining fluorescent microscope. The green color in the fixed cells marks TUNEL-labeled cells. The apoptotic index was determined as the DAPI-stained TUNEL- positive cell number/total DAPI stained cell number (magnification, 200×). Columns, means of three experiments, with triplicates of each experiment; *, $p<0.05$, significantly different from bee venom-untreated control cells.

increased in bee venom treated cells in a concentration dependent way. The treatment of bee venom(5 μg/ml) resulted in about 50 % induction of apoptotic cell death in A549(Fig. 2).

C. Bee venom induced expression of death receptors(DR) in lung cancer cells

Apoptosis can be induced by stimulation of DRs expression. Therefore, to investigate expression of DRs in cancer cells undergoing apoptotic cell death, we performed RT-PCR analysis. RT-PCR analysis showed that bee venom treatment increased TNF-R1, FAS, DR3, DR4 and DR6 mRNA levels in a concentration dependent manner, but TNF-R2 and DR5 expression levels were not changed by bee venom in A549 cells(Fig. 3).

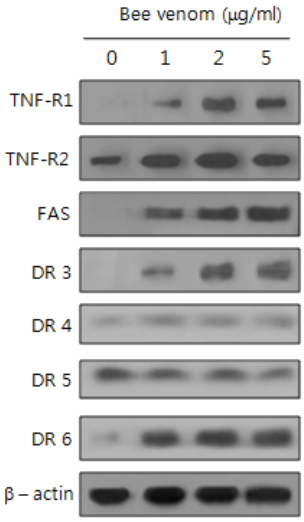

Fig. 3. Effects of bee venom on death recptors expression in A549 lung cancer cells

Cells were treated with bee venom(1, 2, and 5 μg/ml) for 24 hrs, and total RNA were extracted and examined for expressions of TNF-R1, TNF-R2, FAS, DR-3, DR-4, DR-5, DR-6, and GAPDH by RT-PCR. GAPDH was used as an internal control to show equal RNA loading. Each band is representative for three experiments.

D. Effects of bee venom on the expression of apoptotic regulatory proteins

To figure out the relationship between the induction of apoptotic cell death and increase of DR expression, and the expression of their regulatory proteins by bee venom, expression of apoptotic cell death related proteins was investigated by Western blots. The expression of anti-apoptotic protein Bcl-2 was decreased; however, the expression of pro-apoptotic proteins, Bax, caspase-3, caspase-9, cleaved form of caspase-3 and -9 was increased by treatment of bee venom in a concentration dependent manner(Fig. 4).

E. Reversed effects of DR siRNAs on bee venom-induced cell growth inhibition

To determine the relationship between DR expression and cell growth inhibitory effect of bee venom, we transfected A549 cells with DR siRNA

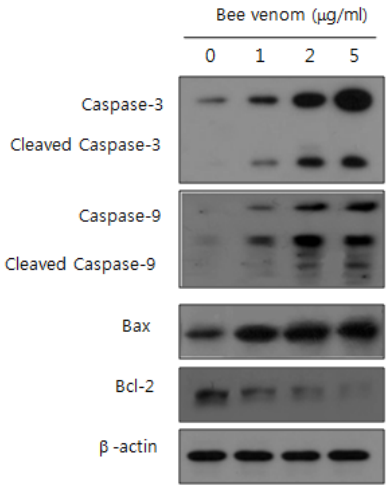

Fig. 4. Effects of bee venom on the expression of apoptotic regulatory proteins

Expression of apoptotic regulatory proteins was determined using Western blot analysis. The lung cancer cells were treated with different concentrations of bee venom(1, 2 and 5 ㎍/ml) for 24 hrs. Equal amounts of total proteins(50㎍/lane) were subjected to 12 % or 8 % SDS-PAGE. Expression of caspase-3, caspase-9, cleaved caspase-3, -9, Bax, Bcl_2, and β-actin were detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. Each band is representative for three experiments.

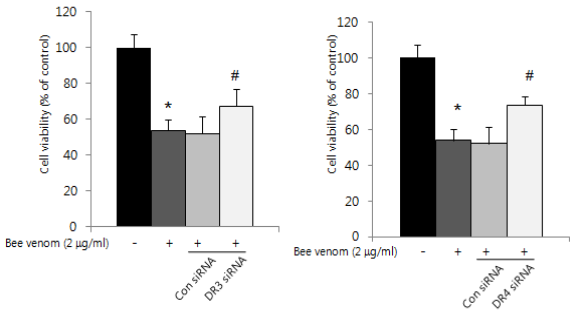

Fig. 5. Effects of siRNA of DRs on bee venom-induced lung cancer cell growth inhibition

The Lung cancer cells were transfected with the DR siRNA(100 nM) for 24 hrs, the cells were then and treated with bee venom(2 ㎍/ml) for another 24 hrs. Total RNA was isolated and RT-PCR was performed to examine gene expression levels of DRs. Each band is representative for three experiments.

using a transfection agent. The cells were transfected with 100 nM siRNA of DRs for 24 hrs, and then treated with bee venom(2 ㎍/ml) for 24 hrs. Expression of the death receptor(DR3, DR4) at mRNA levels was detected by RT-PCR. Knock down of death receptor by transfection of DR3, DR4 and siRNA reversed bee venom-induced cell growth inhibitory effect through apoptosis and DRs expression of DRs(Fig. 5).

F. Effects of bee venom with Apo3L on cell viability and DR3 expression in lung cancer

To determine the mechanism how DR3 is related with inhibition of A549 lung cancer cell growth through apoptosis, we analyzed cell viability by direct cell counting following co-culturing A549 lung cancer cells with Apo3L. The cells were treated with 2 ㎍/ml of bee venom for 24 hrs. Bee venom with Apo3L inhibited cell proliferation of lung cancer cells more significantly than bee venom or Apo3L alone and it also marked the highest in DR3 expression(Fig. 6).

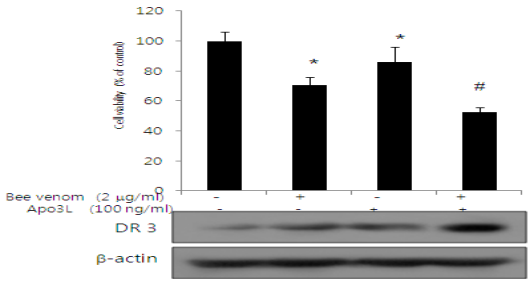

Fig. 6. Synergic effects of bee venom with Apo3L on cell viability and DR3 expression in A549 lung cancer cells

Synergic effects of bee venom with Apo3L was shown on the cell viability assay in A549 lung cancer cells, coincident with the highest DR3 expression. After treatment of bee venom(2 ㎍/ml) with or without Apo3L for 24 hrs, the cells were 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.
Equal amounts of total proteins(50 ㎍/lane) were subjected to 12 % or 8 % SDS-PAGE. Expression of DR3 and β-actin were detected by Western blotting using specific antibodies. β-actin protein was used as an internal control. The above band is representative for three experiments. Columns means are the triplicate experiments of different treatment; *, p <0.05, #, significantly different from TRAIL treated cells.

G. Inhibition of NF-κB

NF-κB is known to be an inhibitory transcription factor of apoptosis. Whether to prevent anti-apoptotic ability of NF-κB is crucial for a agent causing cancer cells go apoptosis. To determine the effects of bee venom on the growth of A549 lung cancer cells through apoptosis, I assessed NF-κB activity in the cells treated for 24 hrs for different concentration with bee venom of EMSA and observed NF-κB signal molecules in the cells by western blot analysis, and NF-κB was highly activated in this cell. however, the activation of NF-κB and its signal molecules were concentration-dependently decreased by the culture in the presence of bee venom in the cells(Fig. 7).

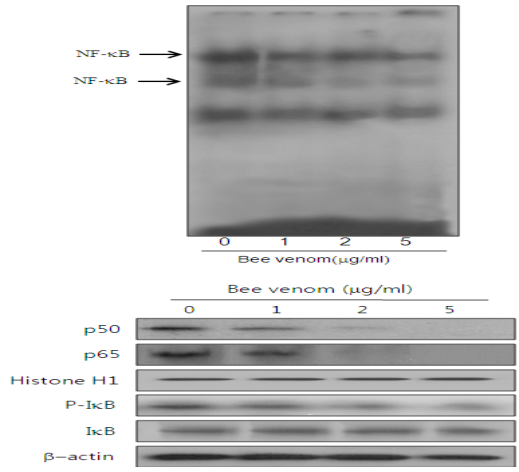

Fig. 7. Inhibition of NF-κB in A549 lung cancer cells by bee venom

Activation of NF-κB was determined by electrophoretic mobility shift assay(EMSA), as described in Materials and Methods. Nuclear extracts from A549 lung cancer cells with bee venom(1, 2 and 5 ㎍/㎖) were incubated in binding reactions of ^{32}P-labeled oligonucleotide containing the B sequence. NF-κB DNA binding activity was determined by EMSA. Equal amounts of total proteins(50 ㎍/lane) were subjected to 12 % or 8 % SDS-PAGE. Expression of p50, p65, IkB, p-IkB and β-actin were detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. Each band is representative for three experiments.

H. Bee venom induced uptake into nucleus of A549 lung cancer cells

It was demonstrated that bee venom could be uptaken into nucleus of A549 lung cancer cells and have little cytotoxic effect on the cells. To investigate whether bee venom can be uptaken into nucleus, and there by case inactivate of NF-κB and express the apoptotic cell death regulatory gene location of bee venom was determined after treatment of cells with fluorescent dye labeled bee venom. The uptake of the labeled bee venom into the cells was shown under a confocal laser scanning microscope. Bee venom was uptaken into the membrane and nucleus of the cells. The translocation into the nucleus was evidenced by the merging of PI staining of nucleus and labeled bee venom(Fig. 8).

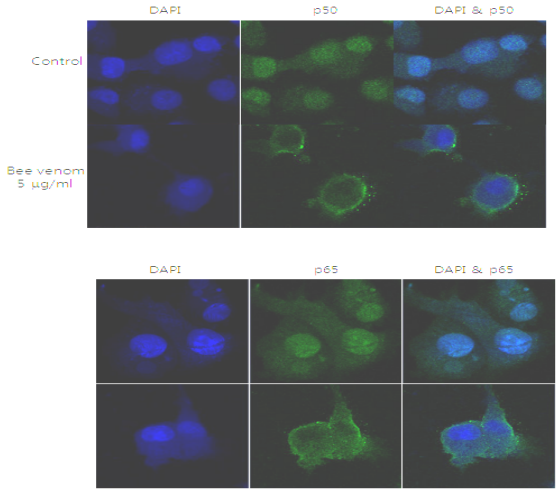

Fig. 8. Uptake of bee venom into A549 lung cancer cells

A549 lung cancer cells were treated with Alexa Fluor 488 dye-labeled bee venom for 24 hrs. Cells were washed, and the uptaken of labeled bee venom was shown by a confocal scanning microscope(magnification, 630×). Double staining(Merge) with fluorescence labeled bee venom and DAPI staining demonstrating the localization of bee venom in the nucleus. Each figure is representative of three similar experiments.

I. Effects of bee venom on the colony formation of A549 lung cancer cells

To determine the sensitivity of bee venom to A549 lung cancer cells, the ability of these 4 cell lines to form colonies on 6-well cell culture plates in the presence or absence of bee venom for 3

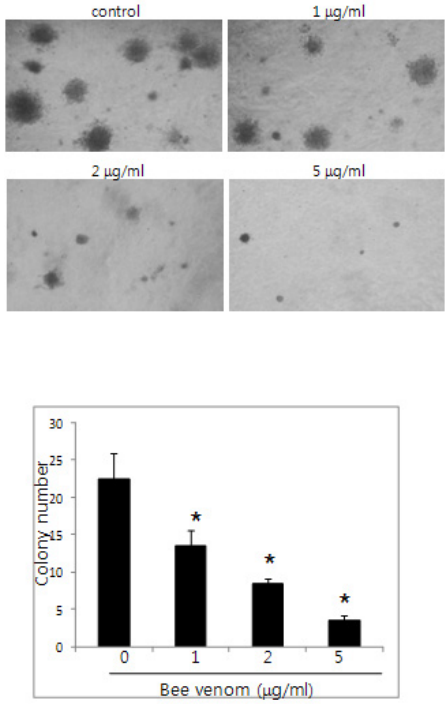

Fig. 9. Effects of bee venom on the colony formation of A549 lung cancer cells

The number of colonies formed from A549/miR-451 cells treated with bee venom was significantly lower than that formed from A549/miR-NC cells. These data obviously showed that upregulation of miR-451 might effectively enhance the sensitivity of A549 cells to bee venom. Columns, means of three experiments, with triplicates of each experiment; *, $p<0.05$, significantly different from untreated control cells.

weeks was studied. The number of colonies formed was reduced in a dose dependent manner. At the highest concentration of bee venom(5 ㎍/㎖), colony formation was reduced over 80 % as compared to the untreated controls(Fig. 9).

Ⅳ. Discussion & Conclusion

The noteworthy findings of the present study was that bee venom inhibited cell growth of human NSCLC, A549 through induction of both intrinsic, mitochondria-mediated, and extrinsic, DR mediated apoptosis via the wide range of interactions with the TNF cytokine family including TNF R1(DR1), Fas(DR2), Apo-3(DR3), DR4, DR5 and DR6 and their apoptosis inducing ligands.

Taken together with the dose dependent decrease of cell viability, proportional increase of caspase active cells and their characteristic apoptotic morphologic change, such as the formation of a plasma membrane bleb or cell shrinkage, it was initially confirmed that a small dose(below 5 ㎍/ml) of bee venom also inhibit cancer cell growth through induction of apoptosis in a concentration-dependent manner with an IC_{50} value of 2.91 ㎍/ml in A549 cells, NSCLC, consistent with previous reports[24-27].

Apoptosis is a tightly controlled physiological process that plays a critical role in developmental modeling, homeostasis maintenance, immune repertoires, and clearance of infected or transformed cells[5]. Apoptosis can be triggered by various extracellular and intracellular stimuli via major apoptotic signaling pathways including the intrinsic mitochondria-mediated pathway and the extrinsic death receptor-induced pathway, and cross-talk between these pathways[6]. Steps in the intrinsic pathway is dependent upon mitochodria membrane permeability regulated by Bax/Bcl-2, which include cytochrome C release from mitochondria, caspase 9 activation, and then activation of effector caspases, including caspase 3[28-32]. Steps in the extrinsic pathway include the Fas-associated death domain (FADD)-dependent recruitment and activation of caspase 8 and/or caspase 10, triggered by the binding of a death receptor ligand to its death receptor including DR1 to TNF; DR2 to FasL; DR3 to Apo3L; DR4 and DR5 to TNF-related apoptosis-inducing ligand(TRAIL, Apo2L), and then activation of the same effector caspases involved in the intrinsic pathway[7,33]. The cytokine TNF is a central mediator of inflammation, immune response and antiviral defence[34]. TNF is a strong activator of transcription factor NF-κB, binding and signaling through two distinct receptors, TNF -R1, -2, members of the TNF receptor superfamily characterized by cysteine-rich pseudorepeats in their extracellular regions[35-37]. Activation of TNFR1 appears to be sufficient to regulate most common

TNF reponses like cytotoxicity and proliferation through activation of NF-κB[38,39]. However, it has been demonstrated that in some cell types, TNFR2 can independently mediate cellular responses like activation of NF-κB[40], proliferation[41] and cell death[42]. The diverse involvement of the two receptors, TNF-R1, -R2 in the large repertoire of cellular TNF responses, brings attention to the molecular mechanisms underlying intracellular events induced by each of them, including cross-talks between these events[43,44].

The Fas signaling-induced pathway begins with the binding of Fas ligands(FasL), Fas and FADD to form the death-inducing signaling complex(DISC), recruiting initiator caspases such as caspase-8 molecules to cleave and activate them[45].

Death-domain-containing receptor, Apo3(also known as DR[3]) was recently discovered to bind to the Apo3 ligand(Apo3L), A249 aminoacid, type-II transmembrane protein with its extracellular sequence showing highest identity to that of TNF[46-50]. Soluble Apo3L induced apoptosis and nuclear factor kB(NF-kB) activation in human cell lines[50]. Caspase inhibitors blocked apoptosis induction by Apo3L, as did a dominant-negative mutant of the cell death adaptor protein, Fas-associated death domain protein(FADD -/MORT1), which is critical for apoptosis induction by TNF[51-54]. Dominant-negative mutants of several factors that play a key role in NF-kB induction by TNF inhibited NF-kB activation by Apo3L. Thus, Apo3L has overlapping signaling functions with TNF, but displays a much wider tissue distribution[47,49,50].

The binding of DR4 and DR5 to TRAIL recruits the FADD and procaspase-8 to form the DISC and subsequently triggers the activation of the caspase cascade[31,32]. More attention was brought to the TRAIL. According to recent studies, systemic administration of TRAIL in mice is physiologically safe, and selectively effective in killing human breast, prostate and colon tumor xenografts. There is even a combined delivery of TRAIL and chemotherapeutic agents are available for cancer therapy due to TRAIL ability to sensitizing target cells through enhancement of death receptors expressions, and regulation of Bcl-2 protein expressions[55,56]. However, human NSCLC, A549 cells are known to be resistant to TRAIL differently from many cancers[57]. In the present study, pro-apoptotic Bax overwhelmed anti-apoptotic Bcl-2, making intrinsic apoptosis going because of loosened mitochondrial outer membrane. Caspase-9 was consecutively triggered and then caspase-3 was activated in a dose dependent manner, implying that bee venom is involved in the induction of intrinsic apoptosis in human NSCLC, A549. In addition, expressions of TNF cytokine family including TNF R1(DR1), Fas(DR2), Apo-3(DR3), DR4, DR5 and DR6 was generally increased, compared to control, of which TNF cytokine family including TNF R1(DR1), Fas(DR2), Apo-3(DR3) was more sensitively enhanced to bee venom than TNF-R2, DR4, DR5 and DR6. This is suggested that bee venom should exert influence upon growth of NSCLC, A549 through dominant extrinsic TNF R1, Fas and Apo-3 signaling apoptosis via con-centration-dependent activation of caspase cascade system and inactivation of NF-kB and its signal molecules, on the other hand, expression of DR4 and DR5 were comparatively low in sensitivity to bee venom and they did not show increase accordingly to concentration, reflecting the above report[57].

Recently, the successful use of small interfering RNA(siRNA) in down-regulating gene expression in several model systems has promoted more and more attempts to explore this methodology in a potentially therapeutic setting[58].

In this study, To determine the relationship between DR expression and cell growth inhibitory effect of bee venom, A549 cells were transfected with DR siRNA using a transfection agent. As a result, it was confirmed that knock down of death receptor by transfection of DR3 and DR4 siRNAs reversed the bee venom-induced cell growth inhibitory effect through extrinsic apoptosis via expression of DR3 and DR4.

Meanwhile, the cytotoxic activity of TNF

cytokine family such as TRAIL, alone may be insufficient for cancer therapy, but its effects may be augmented by co-administration of different chemotherapeutic drugs or other strategies.

From this study to investigating the synergic effect between Apo3L of the most dominant DR3 and bee venom. It was determined that bee venom with Apo3L inhibited cell proliferation of lung cancer cells more significantly than bee venom or Apo3L alone. Several sensitizing agents have been reported in a variety of tumor cell models which sensitized resistant tumor cells to death ligand-induced apoptosis[59-61]. These studies revealed that the development of tumor cell resistance to TRAIL is multi-factorial, spanning from the cell surface through the apoptotic pathways and that the regulation of TRAIL resistance is dependent on the cell tumor system being used. Discovering new methods to improve the apoptotic sensitivity of TRAIL-resistant cancer cells will be a critical question for future clinical applications of TRAIL in cancer therapy.

Consequently, these data provide that bee venom could be a useful candidate compound to enhance the tumor-growth-inhibiting ability of chemotherapeutics through overcoming the resistance via enhancement of DR expression induced apoptosis in the intrinsic as well as extrinsic pathway.

Ⅴ. References

1. Ginsber RJ, Kris MK, Armstrong JG. Cancer of the lung in Principles and Practice of Oncology, 4th ed(DeVita DT Jr, Hellman S, Rosenberg SA eds). Philadelphia PA : Lippincott-Raven Publishers. 1993 ; 673-82.
2. Jemal A, Siegel R, Ward E et al. Cancer statistics. CA Cancer J Clin. 2008 ; 58(2) : 71-96.
3. de Petris L, Crino L, Scagliotti GV et al. Treatment of advanced non-small cell lung cancer. Ann Oncol. 2006 ; 2 : 1136-41.
4. Giaccone G. Clinical perspectives on platinum resistance. Drugs. 2000 ; 59(4) : 9-17.
5. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Int Med. 2005 ; 258(6) : 479-517.
6. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin. 2005 ; 55(3) : 178-94. 71-96.
7. Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst. 2000 ; 92(13) : 1042-53.
8. O'Donovan TR, O'Sullivan GC, McKenna S. Induction of autophagy by drugresistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. Autophagy. 2011 ; 7(5) : 509-24.
9. Kang YJ, Kim IY, Kim EH et al. Paxilline enhances TRAIL-mediated apoptosis of glioma cells via modulation of c-FLIP, survivin and DR5. Exp Mol Med. 2011 ; 43(1) : 24-34.
10. Zhu DM, Shi J, Liu S, Liu Y, Zheng D. HIV infection enhances TRAIL-induced cell death in macrophage by down-regulating decoy receptor expression and generation of reactive oxygen species. PLoS One. 2011 ; 6(4) : e18291.
11. Yoshida T, Horinaka M, Sakai T. "Combination-oriented molecular-targeting prevention" of cancer: a model involving the combination of TRAIL and a DR5 inducer. Environ. Health Prev. Med. 2010 ; 15(4) : 203-10.
12. Inoue N, Matsuda F, Goto Y, Manabe N. Role of cell-death ligand-receptor system of granulosa cells in selective follicular atresia in porcine ovary. J Reprod Dev. 2011 ; 57(2) : 169-75.
13. Ashkenazi A. Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev. 2008 ; 19(3-4) : 325-331.
14. Elrod HA, Sun SY. Modulation of death receptors by cancer therapeutic agents. Cancer Biol Ther. 2008 ; 7(2) : 163-73.
15. Sun SY. Understanding the role of the death receptor 5/FADD/caspase-8 death signaling in cancer metastasis. Mol Cell Pharmacol. 2011 ;

3(1) : 31-4.
16. Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene. 2010 ; 29(34) : 4752-65.
17. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onsetprogression and therapy. Nat Rev Cancer. 2008 ; 8(10) : 782-98.
18. Stegehuis JH, de Wilt LH, de Vries EG, Groen HJ, de Jong S, Kruyt FA. TRAIL receptor targeting therapies for non-small cell lung cancer: current status and perspectives, Drug Resist. Updates. 2010 ; 13(1-2) : 2-5.
19. Van Geelen CM, de Vries EG, de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. Drug Resist Updates. 2004 ; 7(6) : 345-58.
20. Abdulghani J, El-Deiry WS. TRAIL receptor signaling and therapeutics, Exp Opin Therapeut Targets. 2010 ; 14(10) : 1091-108.
21. Jin CY, Park C, Hwang HJ et al. Naringenin up-regulates the expression of death receptor 5 and enhances TRAIL-induced apoptosis in human lung cancer A549 cells, Mol. Nutr Food Res. 2011 ; 55(2) : 300-9.
22. Lu M, Strohecker A, Chen F et al. Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels, Clin. Cancer Res. 2008 ; 14(10) : 3168-76.
23. Park HJ, Lee SH, Son DJ et al. Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-kappaB through interaction with the p50 subunit. Arthritis Rheum. 2004 ; 50(11) : 3504-15.
24. Park JH, Jeong YJ, Park KK et al. Melittin suppresses PMA-induced tumor cell invasion by inhibiting NF-kappaB and AP-1-dependent MMP-9 expression. Mol Cells. 2010 ; 29(2) : 209-15.
25. Park MH, Choi MS, Kwak DH et al. Anti-cancer effect of bee venom in prostate cancer cells through activation of caspase pathway via inactivation of NF-Kβ. Prostate. 2011 ; 71(8) : 801-12.
26. Liu S, Yu M, He Y et al. Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway. Hepatology. 2008 ; 47(6) : 1964-73.
27. Jang MH, Shin MC, Lim S et al. Bee venom induces apoptosis and inhibits expression of cyclooxygenase-2 mRNA in human lung cancer cell line NCI-H1299. J Pharmacol Sci. 2003 ; 91(2) : 95-104.
28. Hengartner MO. The biochemistry of apoptosis. Nature. 2000 ; 407(6805) : 770-6.
29. Reed JC. Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med. 2001 ; 7(7) : 314-19.
30. Li JY, Xu ZJ, Tan MY, Su WK, Gong XG. 3-(4-(Benzo[d]thiazol-2-yl)-1-phenyl-1Hpyrazol-3-yl) phenyl acetate induced HepG2 cell apoptosis through a ROS-mediated pathway. Chem Biol Interact. 2010 ; 183(3) : 341-48.
31. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998 ; 281(5831) : 1309-2.
32. Cain K, Brown DG, Langlais C, Cohen GM. Caspase activation involves the formation of the aposome, a large(approximately 700 kDa) caspase-activating complex. J Biol Chem. 1999 ; 274(32) : 22686-2.
33. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol. 1999 ; 17 : 331-7.
34. Old LJ. Tumor necrosis factor. Sci Am. 1988 ; 258(5) : 59-60.
35. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA, Jr and Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci USA. 1991 ; 88(20) : 9292-6.
36. Beutler B, van Huffel C. Unraveling function in the TNF ligand and receptor families. Science. 1994 ; 264(5159) : 667-8.
37. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral

proteins: activation, costimulation, and death. Cell. 1994 ; 76(6) : 959-62.
38. Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "cidic"c sphingomyelin breakdown. Cell. 1992 ; 71(5) : 765-776.
39. Laegreid A, Medvedev A, Nonstad U et al. Tumor necrosis factor receptor p75 mediates cell-specific activation of nuclear factor kappa B and induction of human cytomegalovirus enhancer. J Biol Chem. 1994 ; 269(10) : 7785-91.
40. Mukhopadhyay A, Suttles J, Stout RD, Aggarwal BB. Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. J Biol Chem 2001 ; 276(34) : 31906-12.
41. Grell M, Becke FM, Wajant H, Mannel DN, Scheurich P. TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. Eur J Immunol. 1998 ; 28(1) : 257-63.
42. Medvedev AE, Espevik T, Ranges G, Sundan A. Distinct roles of the two tumor necrosis factor(TNF) receptors in modulating TNF and lymphotoxin alpha effects. J Biol Chem. 1996 ; 271(16) : 9778-84.
43. Rothe M, Sarma V, Dixit VM, Goeddel DV. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science. 1995 ; 269(5229) : 1424-7.
44. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell. 1994 ; 78(4) : 681-92.
45. Hua F, Cornejo MG, Cardone MH, Stokes CL, Lauffenburger DA. Effects of Bcl-2 levels on Fas signaling-induced Caspase-3 activation: molecular genetic tests of computational model predictions, J Immunol vol. 2005 ; 175(2) : 985-95.
46 Gruss HJ, Dower SK. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood. 1995 ; 85(12) : 3378-404.
47. Vassalli P. The pathophysiology of tumor necrosis factors. Annu Rev Immunol. 1992 ; 10 : 411-52.
48. Nagata S. Apoptosis by death factor. Cell. 1997 ; 88(3) : 355-65.
49. Marsters S, Sheridan J, Donahue C et al. Apo3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-kB. Curr Biol. 1996 ; 6(12) : 1669-76.
50. Chinnaiyan AM, O'ourke K, Yu GL et al. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. Science 1996 ; 274(5289) : 990-2.
51. Kitson J, Raven T, Jiang YP et al. A death-domain-containing receptor that mediates apoptosis. Nature. 1996 ; 384(6607) : 372-5.
52. Bodmer JL, Burns K, Schneider P et al. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis receptor 1 and Fas(Apo-1/CD95). Immunity. 1997 ; 6(1) : 79-88.
53. Screaton G, Xu X, Olsen A et al. LARD: a new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing. Proc Natl Acad Sci USA 1997 ; 94(9) : 4615-9.
54. Golstein P. Cell death: TRAIL and its receptors. Curr Biol. 1997 ; 7(12) : R750-3.
55. Ray S, Almasan A. Apoptosis induction in prostate cancer cells and xenografts by combined treatment with Apo2ligand/tumor necrosis factor-related apoptosis-inducing ligand and CPT-11. Cancer Res. 2003 ; 63(15) : 4713-23.
56. Shankar S, Singh TR, Chen X, Thakkar H, Firnin J, Srivastava RK. The sequential treatment with ionizing radiation followed by TRAIL/Apo-2L reduces tumor growth and induces apoptosis of breast tumor xenografts in nude mice. Int J Oncol. 2004 ; 24(5) : 1133-40.
57. Sheridan JP, Marsters SA, Pitti RM et al.

Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 1997 ; 277(5327) : 818-21.
58. Hannon GJ. RNA interference. Nature. 2002 ; 418(6894) : 244-51.
59. Huerta-Yepez S, Vega M, Jazirehi A et al. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kappa B and inhibition of Bcl-xl expression. Oncogene. 2004 ; 23(9) : 4993-5003.
60. Jazirehi AR, Ng CP, Gan XH, Schiller G, Bonavida B. Adriamycin sensitizes the adriamycin-resistant 8226/Dox40 human multiple myeloma cells to Apo2L/tumor necrosis factor-related apoptosis-inducing ligand(TRAIL)-mediated apoptosis. Clin Cancer Res. 2001 ; 7(12) : 3874-83.
61. Tillman DM, Izeradjene K, Szucs KS, Douglas L, Houghton JA. Rottlerin sensitizes colon carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis via uncoupling of the mitochondria independent of protein kinase C. Cancer Res. 2003 ; 63(16) : 5118-25.