Enhanced Anti-Cancer Effect of Snake Venom Activated NK Cells on Lung Cancer Cells by Inactivation of NF-κB

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Abstract

In the present study, we investigated anti-cancer effect of snake venom activated NK cells (NK-92MI) in lung cancer cell lines. We used snake venom (4 µg/ml) treated NK-92MI cells to co-culture with lung cancer cells. There was a further decrease in cancer cell growth up to 65% and 70% in A549 and NCI-H460 cell lines respectively, whereas 30-40% was decreased in cancer cell growth by snake venom or NK-92MI alone treatment. We further found that the expression of various apoptotic proteins such as that Bax, and cleaved caspase-3 as well as the expression of various death receptor proteins like DR3, DR4 and Fas was also further increased. Moreover, consistent with cancer cell growth inhibition, the DNA binding activity of NF-κB was also further inhibited after treatment of snake venom activated NK-92MI cells. Thus, the present data showed that activated NK cells could further inhibit lung cancer cell growth.

Key Words: NK-92MI cells, Lung cancer, Snake venom, Death receptor, NF-κB

INTRODUCTION

Lung cancer does not show any symptoms in the initial stages, so it is the most difficult type of cancer to diagnose before it has spread to others organs (Latz et al., 2009; Magne et al., 2012). Thus, lung cancer leads to death more often than any other cancers (Mohammed et al., 2011). Although several anticancer agents have been used to treat lung cancer, chemo-resistance is a major obstacle hindering the successful treatment of lung cancer patients (Triller et al., 2006). Multimodal therapy approaches, such as combining chemotherapy agents with cellular immunotherapy may be effective to prevent toxic effects of chemotherapeutics. Hence we evaluated an anticancer strategy that focuses on overcoming such a barrier by combining snake venom and natural killer cells.

Snake venom is useful biological resource, containing several pharmacologically active components that could be of potential therapeutic value (Son et al., 2007). Particularly snake venom toxin from Vipera lebetina turanica was previously demonstrated as a possible chemotherapeutic against growth of human prostate cancer cell and neuroblastoma cell through induction of apoptosis via modulating the expression of apoptosis regulatory proteins and ROS dependent mechanisms (Son et al., 2007; Park et al., 2009). Previously, we found that snake venom increases DRs expression in colorectal cancer cells (Park et al., 2012a). Thus, it is possible that the snake venom could enhance DRs expression in lung cancer cells, however, the combination effect of snake venom with immune cells like NK-92MI against NSCLC cell lines is not yet reported.

Natural killer (NK) cells are effector lymphocytes of the innate immune system that control several types of tumors by limiting their spread and subsequent tissue damages (Cerwenka and Lanier, 2001; Cooper et al., 2001). NK cells play an important role in adoptive cellular immunotherapy against certain human cancers such as renal-cell carcinoma and malignant melanoma (Hercend et al., 1990; Burke et al., 2010), and are involved in the eradication of experimentally induced and spontaneously developing tumors in mice (Markovic and Murasko, 1991). The use of NK cells in human cancer immu-
notherapy has been proposed and such has recently entered clinical trials (Smyth et al., 2002). NK based cellular immunotherapy can be implemented by using any of the following approaches: administering cytokines or immunomodulatory drugs to activate endogenous NK cells, or by increasing the susceptibility of cancer cells to NK cell mediated cytotoxicity. The susceptibility to natural killer cell-mediated lysis of colon cancer cells is enhanced by treatment with epidermal growth factor receptor inhibitors (Bae et al., 2012). Quercetin enhances susceptibility to NK cell-mediated lysis of tumor cells through induction of NKG2D ligands and suppression of HSP70 in K562, SNU1, and SNU-C4 cells (Bae et al., 2010). NK-92MI cells are highly cytotoxic established natural killer cell line. NK-92MI cells were used in immunotherapy of malignant melanoma in a SCID mouse model (Tam et al., 1999). A synergetic effect on tumor regression by genetic engineered IL18-IL2 fusion protein and natural killer cell cytotoxicity in enhancing IFN-γ production in mice models (Du et al., 2012). Though there are studies to increase cytotoxicity if NK cells by increasing the susceptibility of cancer cells, there is no study of using chemotherapeutics to activate NK cells and enhance the cytotoxicity to give an additive effect. Hence we used the combination of snake venom an established chemotherapeutic agent to activate NK-92MI cells against A549 and NCi-H460.

Apoptosis is the best characterized form of programmed cell death and is an intracellular suicide program possessing morphologic characteristics and biochemical features. Programmed cell death (PCD) has an important role in anti-cancer effects of chemotherapeutics (O’Donovan et al., 2011). Apoptosis can be induced by stimulation of death receptors (DRs) (Kang et al., 2011; Zhang et al., 2011). DRs belong to tumor necrosis factor (TNF) family cell surface receptors, which are activated by TNF family ligands like TNF alpha, Apo3L, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (O’Donovan et al., 2011). NK cells can release several tumor necrosis factor (TNF) family ligands such as TNF, TNF-related apoptosis-inducing ligand (TRAIL), Apo3L and FAS ligand (FASL) to induce cancer cell apoptosis (Smyth et al., 2002; Park et al., 2004; Park et al., 2011; Bae et al., 2012; Jo et al., 2012) by activation of death receptors and subsequent downstream caspases, including caspases-9 and -3, as well as Bax (Eirol and Sun, 2008; Sun, 2011).

The nuclear factor kappa B (NF-κB) enhances proliferation, invasiveness, metastasis and inflammation in various human cancer cells (Nakshatri et al., 1997; Karin and Lin, 2002; Baud and Karin, 2009). NF-κB is involved in proliferative and anti-apoptotic activities that could contribute to the resistance of tumor cells to various therapeutic approaches. In lung cancer cells, the inactivation of NF-κB leads to enhanced therapeutic effect (Karin and Grelten, 2005). In our previous study, we reported the anti-cancer effect of snake venom in ovarian cancer cells by inactivation of NF-κB (Song et al., 2012). Recent studies on the signaling mechanisms of the DR have revealed that members of the NF-κB and caspase families are key regulators of cell death (Sancho-Martinez and Martin-Villalba, 2009).

In the present study we tried to enhance the anti-cancer effect of both snake venom and NK-92MI cells by using both in a combination. The activation of NK-92MI cell lines with snake venom showed an additive effect in cell viability and also increased expression of apoptotic proteins (Bax, and cleaved caspase -3) and death receptor proteins Fas in A549 and DR3 in NCi-H460 cell lines. The combination of snake venom and NK-92MI cells also lead to increased inactivation of NF-κB compared to only snake venom or only NK-92MI cells. Thus in present study we show that a co-culture with snake venom treated NK-92MI cells have higher anti-cancer activity on lung cancer cells mediated by death receptors induced apoptotic pathway and inactivation of NF-κB.

MATERIALS AND METHODS

Chemicals
Snake Venom from Vipera lebetina turanica was purchased from Sigma (St. Louis, MO, USA).

Cell culture
NCI-H460 and A549 human lung cancer cells and NK-92MI human natural killer cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). NCI-H460 and A549 human lung cancer cells were grown in RPMI1640 and DMEM respectively, with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2 humidified air. NK-92MI cells were grown in MEM supplemented with 12.5% FBS, 12.5% horse serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, 1% L-glutamine and 0.1% of 55 mM 2-mercaptoethanol at 37°C in 5% CO2 humidified air. The A549 and NCI-H460 lung cancer cells are grown as adherent cultures in monolayer, however NK-92MI cells were grown as suspension cultures forming aggregates of cells as a cluster.

Treatment and co-culture
The A549 and NCI-H460 cells are plated 24 well or 6 well plates (2.5×104 cells per well). The NK-92MI (5×104 cells per well) cells were cultured in semi-permeable inserts (trans-well, costar) suitable to 24 well or 6 well plates. After 24 h, the NK-92MI cells were pre-treated with 4 µg/ml snake venom for 1 h and transferred to 24 well or 6 well plates containing cancer cells (Park et al., 2012b), and cultured for different time period.

Cell viability assay
At different time points, the cultured cells were trypsinized with TrypLE Express (Invitrogen, Carlsbad, CA, USA) and then the cells were pelleted by centrifugation for 5 min at 1,500 rpm. Cells were resuspended in PBS and 0.2% trypan blue was added to the cancer cell suspension. 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.

Reverse transcription (RT)-PCR
Total RNAs were isolated from cultured cells using RNeasy plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s manual. The RNA pellet obtained in the final step was dissolved in 30 µl 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°C until use. Reverse transcription was performed using High Capacity RNA-to-cDNA Kit (AP, Foster, CA, USA). PCR amplifications were
then carried out with the primers. The PCR primers used were
5'-ACAAGCCTGTAGCCCATGTT-3' and 5'-AAAGAT -
GACCTGCCCAGACT-3' for TNF alpha, 5'-ACCAATGC-
CACAAAGGAAC-3' and 5'-CTG CAATTGAAGCACTGGAA-3'
for the human TNF receptor 1, 5'-CTCAGGAGCATG GG G-
ATAAA-3' and 5'-AGCCAGCCAGTGCAGACTCT-3' for the
human TNF receptor-2, 5'-ATGGCGATGGCTGCGTGTCC-
TG-3' and 5'-AGCGCCTCCTGGCTCGGAGTAG-3' for the
human DR3, 5'-ACTTTGTTTGCCTGTTCTG TG-3' and
5'-GGCTTTCCATTGCTGCTCA-3' for the human DR4, and
5'-CAAAGCCCATTATTTTCTCACA-3' and 5'-GACAAAGGCA-
CCCAAGTTA-3' for human FAS, 5'-TCG CAGAAGTGC A C-
CTAA AG-3' and 5'-AGCCTTCCCCTCATCAAAGT-3' for hu-
man ApoL, 5'-CAGCTCTTCCACCTACAG AAG G-3' and 5'-AAGATT-
GAACACTCCCTGGAG-3' for FasL, 5'-AGACCTCCTGCTGCT-
TATCTG-3' and 5'-TTATTGCGGCCAGAGCC-3' for human
TRAIL, 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-CTTCTAC-
CCTACCCAATAAG-3' for glyceraldehyde-3-phosphate de-
hydrogenase (GAPDH), respectively. The reaction mixture
containing 10 µl premix, 1 µl of each forward and reverse
primers and 1 µl of cDNA are made upto 20
µl. The reverse
transcription PCR is carried out in three steps. Step1: 95°C-3
min, Step2: 95°C-30 sec, 55-65°C (ambient temp)-30 sec and
70°C-1 min and Step3: 72°C-10 min. The Step 2 is repeated
for 30 cycles and lid temp-105°C. The samples are loaded into
horizontal gel electrophoresis and detected under UV light us-
ing MyImage software.

Western blot analysis
Harvested cells were homogenized with lysis buffer [50 mM
Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1
mM PMFS, 10 µ/ml aprotinin, 1% igapel 630 (Sigma Aldrich),
10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA, and 0.5% sodium
deoxycholate] and centrifuged at 23,000×g for 15 min. The
protein concentration was measured by the Bradford method
(Bio-Rad Protein Assay; Bio-Rad Laboratories Inc., Hercules,
CA, USA), and equal amount of proteins (50 µg) were sepa-
rated on a SDS/10%-polyacrylamide gel and then transferred
to a Hybond ECL nitrocellulose membrane (Amersham Phar-
macia Biotech Inc., Piscataway, NJ, USA). Blots were blocked
for 2 h at room temperature with 5% (w/v) non-fat dried milk
in Tris buffered saline [10mM Tris (pH 8.0) and 150 mM NaCl]
solution containing 0.05% tween-20. The membrane was
incubated for 5 h at room temperature with specific antibod-
ies: mouse polyclonal antibodies against Bax, IκB, p- IκB,
p65, histone-H1, TNF-R1 and Fas (1:500 dilution; Santa Cruz
Biotechnology Inc., Santa Cruz, CA, USA); rabbit polyclonal
for p50, TNFR-2, DR3 and DR6 (1:500 dilution, Santa Cruz
Biotechnology Inc.); and for caspase-3, cleaved caspase3,
cleaved caspase-9, inhibitor of apoptosis protein (cIAP) 1 and
2 (1:1,000 dilution; Cell Signaling Technology, Inc., Beverly,
MA, USA). The blot was then incubated with the correspond-
ing conjugated anti-rabbit and anti-mouse immunoglobulin G-
horseradish peroxidase (1:4,000 dilution, Santa Cruz Biotech-
nology Inc.). Immunoreactive proteins were detected with the
**RESULTS**

Effect of snake venom on the cells viability of lung cancer cells (A549 and NCI-H460) and natural killer cells (NK-92MI)

Initially to examine the effect of snake venom on A549 and NCI-H460 human lung cancer cell growth, we analyzed cell growth pattern of the cells after treatment with various concentrations. The \( IC_{50} \) value is found to be 5.2 \( \mu \)g/ml and 3.2 \( \mu \)g/ml respectively in A549 and NCI-H460 cells (Fig. 1). However, the treatment of snake venom (4 \( \mu \)g/ml) does not show any significant effect in NK-92MI cell viability (Supplementary Data, A) as well as expression of granzyme B (Supplementary Data, B) and NO generation which are representative indicators of activation NK cells. Moreover, the occulted cancer cells with activated NK-93MI cell did not further increased NO generation (Supplementary Data, C). These data indicated that granzyme and NO generation did not contribute to anti-cancer bation at room temperature. Subsequently 1 \( \mu l \) of gel loading buffer was added to each reaction and loaded onto a 4% non-denaturing gel and electrophoresis was performed until the dye was three-fourths of the way down the gel. The gel was dried at 80°C for 50 min and exposed to film overnight at -70°C. The relative density of the DNA-protein binding bands was scanned by densitometry using My Image (SLB, Seoul, Korea), and quantified by Lab works 4.0 software (UVP Inc).

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**Fig. 2.** Effect of snake venom activated NK-92MI cells on the cells viability of lung cancer cells (A549 and NCI-H460). The cells were co-culture with NK-92MI cells treated with snake venom (4 \( \mu \)g/ml) and the morphological changes were observed and numbers of viable cells were counted under a microscope (magnification, ×200). Values are mean ± SD of three experiments with replicates. *p<0.05 indicates statistically significant differences from the control group.
Effect of snake venom activated NK-92MI cells on the viability of lung cancer cells (A549 and NCI-H460)

We co-cultured snake venom (4 μg/ml) activated NK-92MI cells with NSCLC cells at a ratio of 1:2 (NSCLC cells: NK-92MI). Morphological features of the A549 and NCI-H460 cells show a reduction in cell size and rounding up of cells in the presence of snake venom (4 μg/ml) activated NK-92MI cells when compared to either snake venom (4 μg/ml) or NK-92MI cells alone. In addition, there is a significant decrease in cell viability (65% and 70% respectively) of A549 and NCI-H460 cells in the presence of snake venom (4 μg/ml) activated NK-92MI cells (Fig. 2).

Effect of snake venom activated NK-92MI cells on the expression of granules and TNF family cytokines in NK-92MI

NK-92MI cells can activate TNF family receptors on cancer cells by the release of various ligands. Hence we performed RT-PCR to find the expression levels of various ligands including TNF alpha and death receptors ligands. The results showed a significant increase in the expression of TNF alpha, Apo3L and FasL (Fig. 3). NK-92MI cells can also release granzyme B and perforin to induce apoptosis in cancer cells. Hence we also performed RT-PCR for the granzyme B and perforin. There is an increase in perforin expression in A549, but not NCI-H460 cells (Fig. 3).

Effect of snake venom activated NK-92MI cells on the expression of TNF family receptors in lung cancer cells (A549 and NCI-H460)

NK-92MI cells can stimulate expression of many death receptors in cancer cells. Hence we performed RT-PCR and western blotting to find the expression levels of various receptors including TNF alpha and death receptors ligands. The results showed a significant increase in the expression of TNFR1, TNFR2, DR3, DR4 and Fas expressions in A549 cell lines and TNFR1, TNFR2, and DR3 in NCI-H460 cells (Fig. 4A). To further investigate the expression of TNF family receptors, we also analyzed the expression levels of various TNF family receptors proteins. The results showed a significant increase in the expression of TNFR2, DR3, and Fas in both lung cancer cells by cocultured with snake venom activated NK-92MI (Fig. 4B).

Effect of snake venom activated NK-92MI cells on the expression of apoptosis regulatory protein in lung cancer cells (A549 and NCI-H460)

NK-92MI cells can stimulate expression of many death receptors in cancer cells. Hence we performed RT-PCR and western blotting to find the expression levels of various receptors including TNF alpha and death receptors ligands. The results showed a significant increase in the expression of TNFR1, TNFR2, DR3, DR4 and Fas expressions in A549 cell lines and TNFR1, TNFR2, and DR3 in NCI-H460 cells (Fig. 4A). To further investigate the expression of TNF family receptors, we also analyzed the expression levels of various TNF family receptors proteins. The results showed a significant increase in the expression of TNFR2, DR3, and Fas in both lung cancer cells by cocultured with snake venom activated NK-92MI (Fig. 4B).
activity was confirmed with supershift assay with anti p50 antibody κ as described in Materials and Methods (A). The NF-κB extract from A549 and NCI-H460 lung cancer cells co-administered

Fig. 6. Effect of snake venom activated NK-92MI cells on the activation of NF-κB in lung cancer cells (A549 and NCI-H460). Nuclear extract from A549 and NCI-H460 lung cancer cells co-administered with snake venom and NK-92MI cells for 1h was incubated in binding reactions of 32P-end-labeled oligonucleotide containing the κB sequence. The activation of NF-κB was investigated using EMSA as described in Materials and Methods (A). The NF-κB binding activity was confirmed with supershift assay with anti p50 antibody (0.5 μg) (B).

Effect of snake venom activated NK-92MI cells on the activation of NF-κB in lung cancer cells (A549 and NCI-H460)

Decrease in the activation of NF-κB has been involved in expression of apoptotic cell death of many cancer cells. Hence we examined the DNA binding activity of NF-κB with EMSA. The results showed that there is a significant inactivation of NF-κB in the lung cancer cells by co-cultured with snake venom activated NK-92MI (Fig. 6A). This band was confirmed as NF-κB by supershift assay with anti-p50 antibody (Fig. 6B).

DISCUSSION

Lung cancer remains the most common cancer diagnosed worldwide and has one of the lowest survival rates of all cancers. Surgery remains the only curative treatment option but because most patients are either diagnosed at advanced stages or are unfit for surgery, less than a third of all lung cancer patients will undergo a surgical resection (Jahangeer et al., 2013). Natural killer (NK) cells play critical roles in host immunity against cancer. In response, cancers develop mechanisms to escape NK cell attack or induce defective NK cells. Currently NK cell-based cancer immunotherapy can aim to overcome NK cell paralysis by the use of chemotherapeutic agents. However a multimodal therapy approach, such as combining chemotherapy agents with cellular immunotherapy, suffers from potential drug-mediated toxicity to immune-effector cells. Overcoming such toxic effects of anticancer cellular products is a potential critical barrier to the development of combined therapeutic approaches. In our present study we evaluated the effect of chemotherapeutic drug snake venom on immunocompetent cells’ (NK-92MI) activation. These immune effector cells efficiently reduced the viability of non-small cell lung cancer cell lines (A549 and NCI-H460), which are also sensitive to snake venom. These data show there is a benefit to using drug-resistant cellular therapy when combined with cytotoxic chemotherapy approaches. NK cells are known to release several cytokines such as granulocyte-macrophage colony-stimulating factor and interferon-γ, which can induce a tumor-killing effect (Yasumura et al., 1994; Glas et al., 2000; Brady et al., 2010). NK cells also kill cancer cells by the release of cytoplasmic granules that contain a number of proteins, such as perforin and granzymes, which lyses target cells.

Besides these cytokines, there are several tumor necrosis factor (TNF) family ligands such as FAS ligand (FASL), TNF and TNF-related apoptosis-inducing ligand (TRAIL), which are also released by NK cells and have been shown to induce cancer cell apoptosis (Cerwenka and Lanier, 2001; Cooper et al., 2001; Smyth et al., 2001; Smyth et al., 2002; Screpani et al., 2005). Previously our studies reported that anti-cancer effect of snake venom in colon (Park et al., 2012a) and ovarian cancer (Song et al., 2012). Several studies also reported the anti-cancer effect of snake venom in multiple myeloma (Al-Sadoon et al., 2013), breast cancer and prostate cancer (Badr et al., 2013). Snake venom induces apoptosis of colon cancer cells through DR4 and DR5 (Park et al., 2012a) and inhibits cell growth through inactivation of NF-κB and STAT3 (Song et al., 2012). This ability of both NK-92MI cells and snake venom to activate death receptor ligands and death receptors respectively is critical when co-administered to lung cancer cells. In present study the snake venom activated NK-92MI cells lead to activation of ligands like Apo3L and FasL on NK-92MI cells. Concomitantly the receptors like DR3, and Fas are activated on lung cancer cells. DRs contain a cysteine-reich cytoplasmic domain, called “death domain”. DRs are activated by interaction of DRs with their ligands (interaction of DR1 with TNF; DR2 with FasL; DR3 with Apo3L; DR4 and DR5 with TRAIL) (Yoshida et al., 2010; Inoue et al., 2011). Activated DRs induce apoptosis through caspase activation (Ashkenazi, 2008). When DRs bind to their ligands, the death domains recruit the intracellular adaptor protein FADD (Fas-associated death domain protein) which results in the activation of caspase-8 that leads to the activation of downstream caspases, including caspases-3, as well as Bax (Elrod and Sun, 2008; Sun, 2011). Similarly, the snake venom activated NK-92MI cells on NSCLC cells lead to the activation of Bax, cleaved caspase-3 and cleaved caspase-8.

The members of the nuclear factor kappa B (NF-κB) family also play an important role in the development and progression of several human malignancies (Nakashatri et al., 1997; Baud and Karin, 2009). Previously our studies showed anti-cancer activity snake venom in ovarian cancer cells through activation of caspase-3 pathway via inactivation of NF-κB (Song et al., 2012). Snake venom also showed anti-arthritis effect by inhibition of inflammation mediator generation by suppression of NF-κB. In the present study, we found an enhanced inactivation ability of snake venom activated NK-92MI cells. Thus in the present study, the administration of snake venom in NK-92MI cells and consecutive co-culture of these NK-92MI cells with NSCLC cells (A549 and NCI-H460) lead to decrease in cell viability of lung cancer cells. This decreased cell viability
is found to be due to activation of TNF-α, Apo3L and Fasl in NK-92MI cells and TNFR2, DR3, and Fas in both A549 and NCI-H460. Furthermore, the result causes by decreasing the activation of NF-κB, and increasing apoptotic proteins in non-small cell lung cancer cells.

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