Original Article

Synergistic Effects of Bee Venom and Natural Killer Cells on B16F10 Melanoma Cell Growth Inhibition through IL−4−mediated Apoptosis

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[Abstract]

Objectives : We investigated the synergistic effects of bee venom (BV) and natural killer (NK) cells on B16F10 melanoma cell apoptosis mediated by IL−4.

Methods : We performed a cell viability assay to determine whether BV can enhance the inhibitory effect of NK−92MI cells on the growth of B16F10 melanoma cells, and western blot analysis to detect changes in the expression of IL−4, IL−4Rα, and other apoptosis−related proteins. EMSA was performed to observe the activity of STAT6. To confirm that the inhibitory effect of BV and NK cells was mediated by IL−4, the above tests were repeated after IL−4 silencing by siRNA (50 nM).

Results : B16F10 melanoma cells co−cultured with NK−92MI cells and simultaneously treated by BV (5 μg/ml) showed a higher degree of proliferation inhibition than when treated by BV (5 μg/ml) alone or co−cultured with NK−92MI cells alone. Expression of IL−4, IL−4Rα, and that of other pro−apoptotic proteins was also enhanced after co−culture with NK−92MI cells and simultaneous treatment with BV (5 μg/ml). Furthermore, the expression of anti−apoptotic bcl−2 decreased, and the activity of STAT6, as well as the expression of STAT6 and p−STAT6 were enhanced. IL−4 silencing siRNA (50 nM) in B16F10 cells, the effects of BV treatment and NK−92MI co−culture were reversed.

Conclusion : These results suggest that BV could be an effective alternative therapy for malignant melanoma by enhancing the cytotoxic and apoptotic effect of NK cells through an IL−4−mediated pathway.

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Ⅰ. Introduction

Malignant melanoma is a malignancy of pigment producing cells (melanocytes), which are located primarily in the skin[1]. The incidence of melanoma is increasing worldwide, and the prognosis for patients with high-risk or advanced metastatic melanoma remains poor despite advances in the field[2].

Immunological cytokines, such as interleukin-4 (IL-4), play important roles in the regulation of immune cell function. IL-4 was originally described as a B-cell growth factor, but is since known to activate and differentiate other immune cells as well[3]. IL-4, which exerts its effects by interacting with cell surface receptors, has been used in immunotherapies to enhance the immune response against tumors in both mice and humans[4,5]. IL-4 induces apoptosis of 549 lung adenocarcinoma cells through two pathways: direct activation and mitochondrial pathway[6]. Furthermore, human melanoma cells express the high-affinity IL-4 receptor (IL-4R), suggesting that IL-4 may play a role in host antitumor respones[4].

Natural killer (NK) cells are cells of the innate immune system that play an important role in early responses to viral infection and tumors. NK cells can mediate apoptosis by utilizing two basic mechanisms. The first involves exocytosis of cytotoxic granules containing perforin and granzymes (including granzyme B) from the NK cell. The second pathway involves members of the TNF superfamily (FasL/TNF) and their corresponding ligands (Fas/TNFR)[7].

Bee venom (BV) was used in ancient China for apitherapy and it is mentioned in the Huangdi Neijing. It has been used in oriental medicine for many years to treat inflammatory diseases[8], and in Korean medicine in pharmacopuncture to treat back pain, rheumatism, and many skin diseases due to its antibacterial, antiviral, and anti-inflammatory effects[9,10]. BV consists of a complex mixture of proteins, peptides, and low molecular weight components. BV increases pro-apoptotic proteins by increasing the expression of death receptor (DR) in the cell surface and simultaneously inhibiting the activity of anti-apoptosis factors[11-14]. Several experimental studies have demonstrated that BV and/or melittin have anti-cancer effects[9,10,15]. Additionally, it has been reported that BV enhances the cytotoxic effects of NK cells on human lung cancer and acts synergistically with NK cells to inhibit cancer cell growth[16,17]. However, the anti-cancer effects of BV in malignant melanoma have not been investigated.

Thus, in the present study, I investigated whether BV could act synergistically with and potentiate the inhibitory effect of NK-92MI cells on B16F10 melanoma tumor growth, and whether such effects could be mediated by IL-4.

Ⅱ. Materials and method

1. Materials

BV was purchased from You-Miel BV Ltd. (Hwasoon, Jeonnam, Korea) and it had the following composition: 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 other components, such as protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephrine, and unknown amino acids, with 99.5% purity. The following primary antibodies were used for western blot analysis: caspase-3, caspase-9, caspase-8, Bcl-2 (1:1,000 dilutions; Cell Signaling, Beverly, MA) and Bax, IL-4Rα, STAT6 and p-STAT6 (1:1,000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA) and IL-4 (1:1,000 dilution; Thermo Fisher, Waltham, MA). T4 polynucleotide kinase was obtained from Promega

(Madison, WI). Poly (dI-dC), a 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).

2. Cell culture

B16F10 mouse melanoma cells and NK-92MI human natural killer cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). B16F10 cells were grown in DMEM (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a 5% CO_2 humidified incubator. NK-92MI cells were grown in MEM supplemented with 12.5% FBS, 12.5% horse serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 100 U/ml penicillin, and 100 mg/ml streptomycin. For co-culture experiments, a trans-well system was used, where B16F10 and NK-92MI cells grown in mixed culture medium (1:1) were separated by a porous polycarbonate membrane. First, B16F10 cells were seeded at a density of 5×10^4 cells/well and cultured overnight. Then, the inserts containing NK-92MI cells (5×10^4) were added to the plate and co-cultured with the B16F10 cells.

3. Cell viability assay

To determine cell numbers, B16F10 cells alone or co-cultured with NK-92MI cells, as well as NK-92MI cells alone, were seeded onto 24-well plates (5×10^4 cells/well) and while still subconfluent, they were treated with BV (5 μg/ml) for 24 h. The cells were then trypsinized, pelleted by centrifugation for 5 min at 1500 rpm, and resuspended in 10 ml phosphate-buffered saline (PBS). Following the addition of 0.1 ml 0.2% trypan blue to 0.9 ml cell suspension, a drop of the suspension was placed in a Neubauer chamber and the cells were counted under a microscope. 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 measurement was carried out in triplicate.

4. Western blot analysis

Melanoma tumor tissues containing B16F10 cells were lysed by homogenization in a protein extraction solution (PRO-PREPTM, IntronBiotechnology), followed by a 60 min incubation on ice. The cell lysate was centrifuged at 15,000 rpm for 15 min at 4℃. Equal amounts of protein (20 μg) were separated by SDS on a 12% polyacrylamide gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Water and Process technologies, Trevose, PA, USA). The membranes were blocked for 1 h at room temperature with 5% (w/v) skim milk in Tris-buffered saline Tween-20 (TBST: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20). After a short washing step with TBST, the membranes were immunoblotted with the following primary antibodies: caspase-3, caspase-9, caspase-8, Bcl-2 and Bax, IL-4Rα, STAT6 and p-STAT6 and IL-4. Following incubation with the appropriate secondary antibodies, the signal was developed with a chemiluminescence (ECL) detection system.

5. Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of STAT6 was determined by EMSA according to the manufacturer's instructions (Promega). Nuclear extracts were prepared and processed for EMSA as previously described. The relative densities of the DNA-protein binding bands were determined by densitometry using MyImage (SLB), and quantified with the Lab-

works 4.0 software (UVP, Inc., Upland, CA).

6. Transfection assay

B16F10 cells (1×10^4 cells/well) were plated in 96-well plates and transiently transfected with a short interfering RNA (siRNA) against IL-4, using a mixture of siRNA and the Well Fect-EXPLUS reagent in OPTI-MEN, according to the manufacturer's specification (WelGENE, Seoul, Korea). The transfected cells were treated with BV (5 μg/ml) for 24 h and then used for cell viability and protein expression analyses.

7. Data analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (Graph-Pad Software, La Jolla, CA). Data are presented as mean ± standard deviation (SD) values. The differences between different data groups 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 $p \leq 0.05$ was considered to indicate statistical significance.
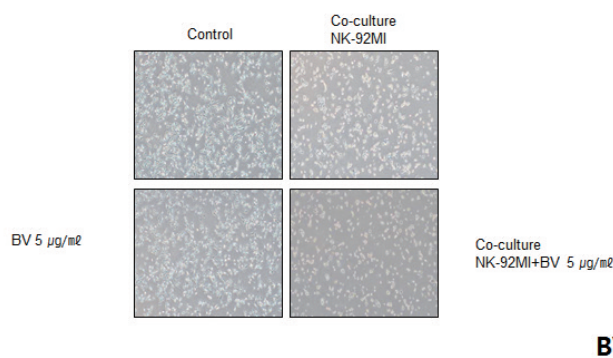
III. Results

1. Effects of BV and NK-92MI cells on the cell viability of B16F10 cells

To assess whether BV enhances the inhibitory effect of NK-92MI cells on B16F10 cell proliferation, I measured cell viability by direct cell counting. B16F10 cells were co-cultured or not with NK-92MI cells and then treated or not with BV (5 μg/ml) for 24 h. As shown in Fig. 1, treatment with BV or co-culture with NK-92MI cells inhibited B16F10 cell proliferation. Moreover, when B16F10 were co-cultured with NK-92MI cells and simultaneously treated with 5 μg/ml of BV, a significantly stronger inhibition of proliferation of B16F10 melanoma cells was observed compared to the one resulting from BV treatment or NK-92MI cell co-culture alone

2. Effects of BV and NK-92MI cells on the expression of apoptotic and cytotoxic regulatory proteins in B16F10 cells

To ascertain whether BV treatment and NK-92MI cells synergistically influence the expression

Fig. 1. BV enhances the cytotoxic effect of NK-92MI cells on B16F10 melanoma cells

B16F10 melanoma cells co-cultured or not with NK-92MI cells, were seeded onto 24-well plates (5×10^4 cells/well), and while subconfluent, they were subsequently treated with BV (5 μg/ml) for 24 h. 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 are expressed as a percentage of viable cells. Each bar represents the mean of triplicate experiments (error bar indicates SD values).

\# : ($p < 0.05$) indicates statistically significant differences from the control group.

* : ($p < 0.05$) indicates statistically significant differences from the BV treated or NK-92MI co-cultured group.
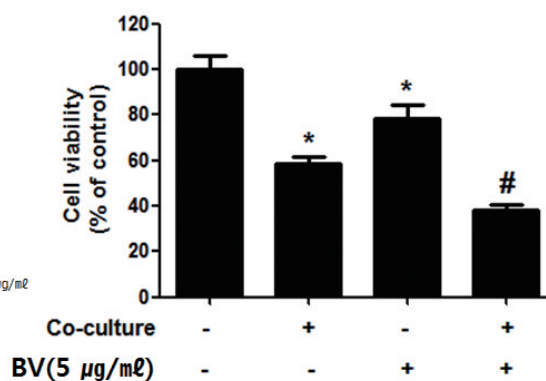
of cytotoxic and apoptotic regulatory proteins in B16F10 cells, the levels of representative proteins, such as IL−4, IL−4Rα, Bcl−2, caspase−3, caspase−9, cleaved caspase−9, and cleaved caspase−8, were analyzed by western blotting. The expression of IL−4 and IL−4Rα, and that of pro−apoptotic proteins, was significantly higher after co−culture with NK−92MI cells and simultaneous treatment with 5 μg/ml BV, compared to that after co−culture with NK−92MI cells or BV treatment alone. Similarly, a significant decrease in the expression of anti−apoptotic bcl−2 was observed following co−culture with NK−92MI cells and simultaneous treatment with 5 μg/ml BV, compared to that after co−culture with NK−92MI cells or BV treatment alone (Fig. 2).

3. Effects of BV on STAT6 activation in B16F10 cells

It is reported that IL−4 induces STAT6 activation, and that snake venom toxin also influences STATs.
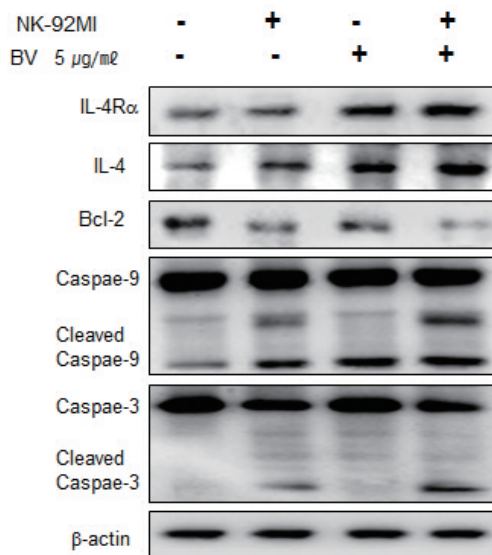

Fig. 2. BV inhibits the growth of B16F10 melanoma synergistically with NK−92MI cells by regulating the expression of cytotoxic and apoptotic proteins

B16F10 melanoma cells were co−cultured with NK−92MI cells and subsequently treated with 5 μg/ml BV for 24 h,. Expression of IL−4, IL−4Rα, bcl−2, caspase−3, caspase−9, cleaved caspase−3, cleaved caspase−9 was then determined. β−actin protein was used an internal control. Each blot is representative of three experiments.

To investigate whether BV activates STAT6 in B16F10 cells, the DNA binding activity of STAT6 was assessed by EMSA. I found that B16F10 cells not treated with BV or co−cultured with NK−92MI cells showed low constituted activation of STAT6. However, BV treatment or co−culture with NK−92MI cells resulted in higher levels of STAT6 activation. Moreover, the DNA binding activity of STAT6 was significantly higher following co−culture with NK−92MI cells and simultaneous treatment with 5 μg/ml BV, compared to that after co−culture with NK−92 cells or BV treatment alone (Fig. 3). The same conditions that resulted in increased DNA binding activity of STAT6 also led to enhanced phosphorylation of nuclear STAT6 in B16F10 cells (Fig. 3).

4. siRNA−mediated IL−4 silencing reverses the inhibitory effects of BV and NK−92MI cells on B16F10 cell growth

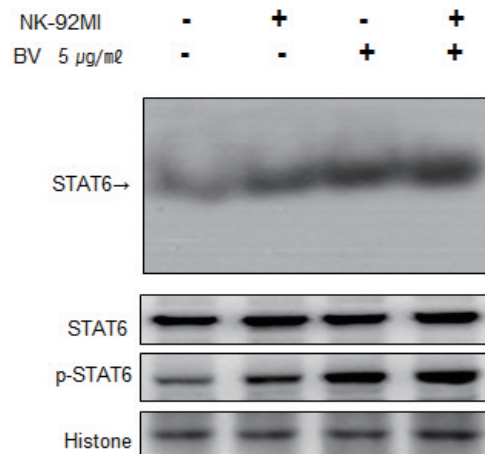To test whether the synergistic inhibitory effect of BV treatment and NK−92MI cells on B16F10 cell

Fig. 3. STAT6 in B16F10 melanoma cells is synergistically activated by BV treatment and co−culture with NK−92MI cells

STAT6 activity was detected by EMSA. The expression of STAT6 and p−STAT6 were detected by western blotting using specific antibodies. The histone protein was used an internal control. Each blot is representative of three experiments.

growth was mediated by IL-4-induced apoptosis and cytotoxicity, I transfected B16F10 cells with 50 nM siRNA against IL-4 for 24 h, followed by treatment with BV (5 μg/ml) for an additional 24 h. Cell growth was then assessed by direct cell counting after trypan blue staining. The results show that siRNA-mediated IL-4 silencing reversed the effect of simultaneous BV treatment and co-culture with NK-92MI cells, resulting in increased B16F10 cell viability (Fig. 4).

5. siRNA-mediated IL-4 silencing reverses the effects of BV and NK-92MI cells on apoptotic and cytotoxic regulatory protein expression in B16F10 cells

To further ascertain whether the synergistic effects of BV treatment and NK-92MI cells on B16F10 cells is mediated by IL-4-induced apoptosis and cytotoxicity, I transfected B16F10 cells with 50 nM siRNA against IL-4 for 24 h, fol-

lowed by treatment with BV (5 μg/ml) for an additional 24 h, and analyzed the expression of apoptotic and cytotoxic regulatory proteins by western blotting. The results show that siRNA-mediated IL-4 silencing reversed the effect of simultaneous BV treatment and co-culture with NK-92MI cells on the expression of IL-4 and IL-4Rα, and of other pro-apoptotic proteins, such as Bax, caspase-3, cleaved caspase-3, caspase-9 and cleaved caspase-9, leading to decreased protein levels. Similarly, the decrease in the expression of anti-apoptotic bcl-2 was also reversed by siRNA-mediated IL-4 silencing in B16F10 cells, leading to increased protein levels (Fig. 5).
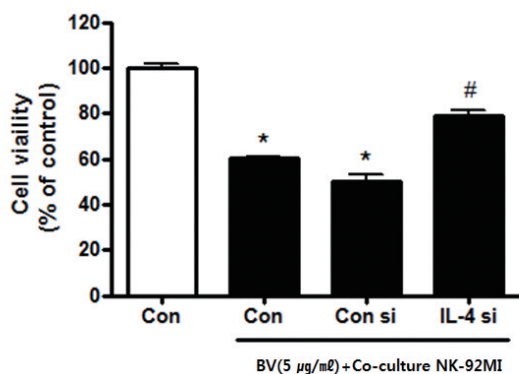

Fig. 4. IL-4 siRNA transfection reverses the synergistic inhibitory effects of BV treatment and co-culture with NK-92MI cells on B16F10 melanoma cell growth

B16F10 melanoma cells were transfected with an IL-4 siRNA (50 nM) for 24 h, and the cells were then treated with BV (5 μg/ml) for another 24 h. A cell viability assay was performed to examine changes in cell growth. Each bar represents the mean of triplicate experiments (error bar indicates SD values). con: control.
: ($p < 0.05$) indicates statistically significant differences from the control group (transfected with siRNA).
* : ($p < 0.05$) indicates statically significant differences from the control group.
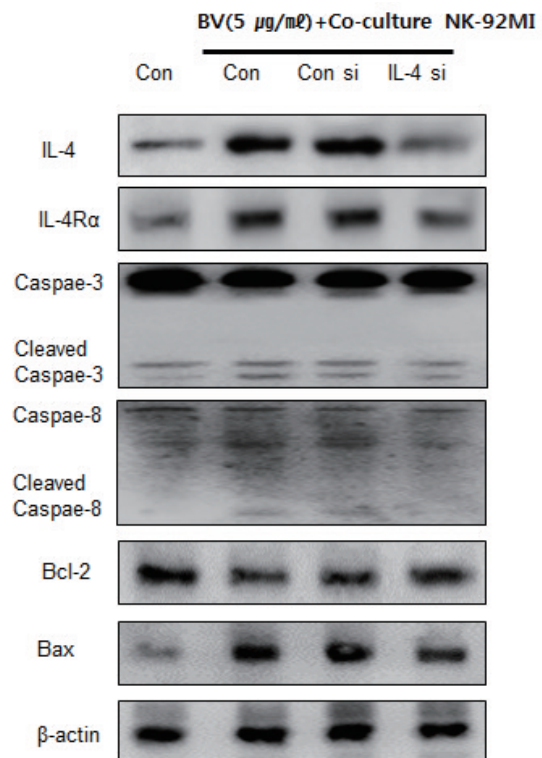

Fig. 5. IL-4 siRNA transfection reverses the synergistic effects of BV treatment and co-culture with NK-92MI cells on the expression of cytotoxic and apoptotic protein in B16F10 melanoma cells

B16F10 melanoma cells were transfected with an IL-4 siRNA (50 nM) for 24 h, and the cells were then treated with BV (5 μg/ml) for another 24 h. Expression of IL-4, IL-4Rα, Bax, bcl-2, caspase-3, caspase-9, cleaved caspase-3, and cleaved caspase-9 was then determined. β-actin protein was used an internal control. Each blot is representative of three experiments.

6. siRNA-mediated IL-4 silencing reverses the inhibitory effects of BV and NK-92MI cells on STAT6 activation in B16F10 cells

To investigate whether BV treatment and NK-92MI cells synergistically influence IL-4-mediated STAT6 activation in B16F10 cells, I transfected B16F10 cells with 50 nM siRNA against IL-4 for 24 h, followed by treatment with BV (5 μg/ml) for an additional 24 h, and assessed the DNA binding activity of STAT6 by EMSA. I found that siRNA-mediated IL-4 silencing reversed the effect of simultaneous BV treatment and co-culture with NK-92MI cells on the DNA binding activity of STAT6, which was instead downregulated (Fig. 6). This decrease in the DNA binding activity of STAT6 was accompanied by a decrease in the phosphorylation of nuclear STAT6 in B16F10 cells upon siRNA-mediated IL-4 silencing (Fig. 6).

IV. Discussion

Apoptosis is induced mainly by the intrinsic mitochondria-mediated pathway or the extrinsic DR-mediated pathway and their interplay[8]. DRs generally bind to their specific ligands or antibodies, which induce receptor trimerization and clustering through the characteristic death domain (DD), recruitment of intracellular adaptor proteins and procaspase-8, and DR inducing signal complex (DISC) formation[19-22]. Once the DISC has been formed, an intracellular cascade of caspase activation is initiated: initiator caspases (caspase-8, -9 and -10) can active effector caspases, such as caspase-3 and -6, initiating programmed cell death[23]. In the case of the intrinsic mitochondrial-mediated pathway, Bax activation and its subsequent translocation to the mitochondria, accompanied by a decrease in Bcl-xL, lead to cytochrome c release, which activates caspase-3 and caspase-9, leading to apoptosis[6,23]. Additionally,
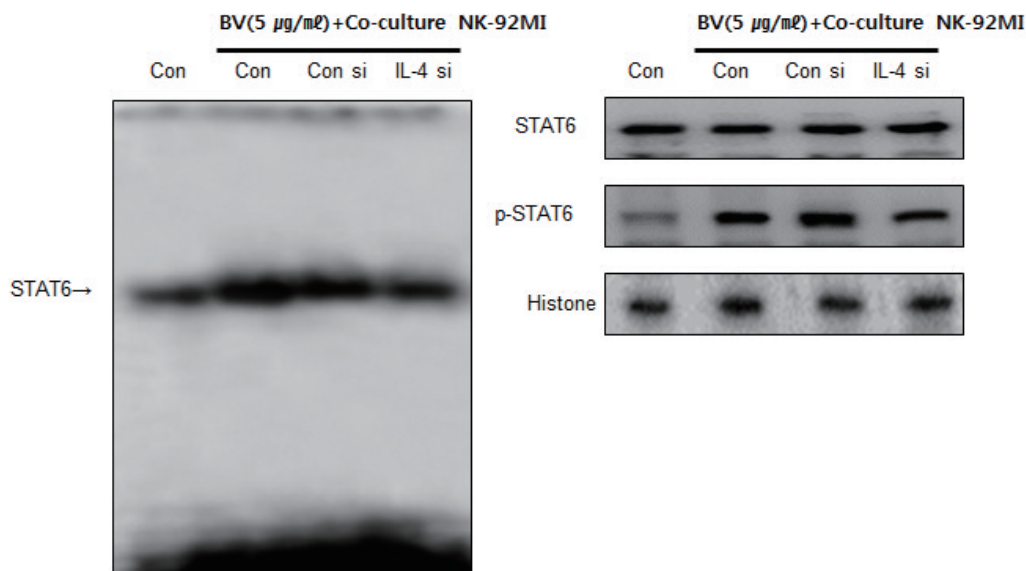

Fig. 6. IL-4 siRNA transfection reverses the synergistic effects of BV treatment and co-culture with NK-92MI cells on STAT6 DNA binding activity

B16F10 melanoma cells were transfected with an IL-4 siRNA (50 nM) for 24 h, and the cells were then treated with BV (5 μg/ml) for another 24 h. STAT6 activity was subsequently detected by EMSA. The expression of STAT6 and p-STAT6 were detected by western blotting using specific antibodies. The histone protein was used an internal control. Each blot is representative of three experiments.

perforin exocytosed from NK cells homopolymerizes in the membrane of the target cell in a calcium-dependent manner, enabling the release of granzyme B into the cytosol. Granzyme B first activates caspase-3, which then cleaves the N-peptide of procaspase-7, rendering it accessible to granzyme B for further maturation. The executioner caspase-7 then cleaves key cytosolic and nuclear substrates. The partial compartmentalization of caspase-3 to mitochondria suggests that it may be activated by caspase-9. Notably, granzyme B may also act independently of the caspase cascade[7].

The results of the present study show that B16F10 cell viability decreased when the cells were co-cultured with NK-92MI cells and then treated with BV (5 μg/ml) to a greater extent than it did after NK-92MI cell co-culture or BV treatment alone, although, compared to the control, cell viability decreased also in the latter two conditions (Fig. 1). The expression of IL-4 and IL-4Rα, and that of pro-apoptotic proteins, in B16F10 cells was increased to a significantly greater extent by co-culture with NK-92MI cells and simultaneous treatment with BV (5 μg/ml) than by co-culture with NK-92MI cells or BV treatment alone. Likewise, anti-apoptotic bcl-2 expression was decreased more by the combined treatment (Fig. 2). These results suggest that the proliferation of B16F10 cells can be inhibited by BV treatment or NK-92MI cells, but the combination of the two leads to synergistic effects. In addition, BV treatment and NK-92MI cells synergistically cause B16F10 apoptosis by influencing the expression of cytotoxic and apoptotic of regulatory proteins, such as IL-4, IL-4Rα, Bcl-2, caspase-3, caspase-9, cleaved caspase-9, and cleaved caspase-8. IL-4 activates two distinct signaling pathways through tyrosine phosphorylation of STAT6 and of the 170 kDa protein 4PS, from which, the STAT6 pathway plays a central role in IL-4-mediated biological responses[24]. STAT6 is synergistically activated by BV treatment and NK-92MI cells (Fig. 3), further confirming the synergistic action of BV and NK-92MI cells in the regulation of IL-4-mediated biological

responses. This study shows that such an action can inhibit proliferation and differentiation of B16F10 melanoma cells through IL-4-mediated apoptosis. Indeed, siRNA-mediated IL-4 silencing in B16F10 followed by BV treatment reversed the effects and cell viability increased (Fig. 4). Similarly, expression of IL-4, IL-4Rα, and other pro-apoptotic proteins was decreased, and that of anti-apoptotic bcl-2 increased upon siRNA-mediated IL-4 silencing in B16F10 cells (Fig. 5). Moreover, STAT6 activation was reversed, as assessed by EMSA and western blot analysis. (Fig. 6) Based on the above results, it can be concluded that the synergistic cytotoxic and apoptotic effects of BV and NK-92MI cells on B16F10 cells is mediated by IL-4.

In summary, BV acts synergistically with NK-92MI cells to inhibit B16F10 melanoma cell growth through IL-4-mediated apoptosis. These results suggest that BV could be an alternative or complementary agent against malignant melanoma.

V. References

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