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Enhancement of NK Cytotoxicity, Antimetastasis and Elongation Effect of Survival Time in B16-F10 Melanoma Cells by Oregonin

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We investigated the antitumor activity of oregonin, a diarylheptanoid derivative purified from *Alnus hirsuta Turcz, Betulaceae*. Oregonin is a potential novel immunomodulator, which augments the activation of natural killer (NK) cells, and thereby leads to a powerful antitumor activity. To evaluate the cytotoxicity of oregonin against tumor cells, we examined the effectiveness of NK cells and determined that oregonin could increase NK cell cytotoxicity. This was confirmed by MTT assay. In addition, the survival time of C57BL/6 mice were measured by inoculating B16-F10 melanoma cells to mice via intra muscular (i.m.) injection. Oregonin treatment after 10 hours of inoculation at 10 mg/kg dosage showed a significant extension of survival time by up to 51.32%, when compared to the control group. Moreover, oregonin significantly reduced the incidence of pulmonary metastasis, which may be developed from B16-F10 melanoma cells. These findings suggest that oregonin may be classified as a new and novel immunomodulator due to its potential antitumor activity.

Key words: Oregonin, NK cells, Antimetastasis, Survival time elongation

INTRODUCTION

Today, many countries and pharmaceutical companies who recognize that there is a limitation of R&D in the current conservative chemical synthesis are turning their interest to galenical derivatives. Indeed, the taxol extracted from Japanese yew has become a prominent future substitute for cancer therapy due to its antitumor potencies. In contrast, although existing antitumor agents mainly demonstrate the effectiveness of their toxic function against tumor cells, such conservative agents have long been a clinical dilemma due to their toxic effect on both normal and immune cells (Jurgen *et al.*, 1990). However, galenicals, which are known to have a promising antitumor effect (Chihara *et al.*, 1970, Komatsu *et al.*, 1969, Ohno *et al.*, 1984 , have been reported to reduce such a side effect (Sangbae *et al.*, 1999, Myoungyun *et al.*, 2001). This is an advantage of galenicals over chemical antitumor agents. Particularly, many compounds extracted from various plants have recently attracted attention in terms of their anti-tumor effects. Of these, tannin is one of the most actively studied compounds due to its various actions. In view of this research attention, we tried to investigate the antitumor effect of oregonin, a derivative of diarylheptanoid. Oregonin, which belongs to *Betulaceae*, is horizontally distributed nationwide excluding Kyungbuk and Chungnam provinces, and geographically distributed in Japan, Manchuria, Saghalien, Kamchaka and Russia.

Many studies on the efficacy of oregonin have been conducted over the past 10 years. In 1992 Kiuchi *et al.* elucidated that diarylheptanoid extracted from *Alpinia officinarum* blocked the semi-synthesis process of prostaglandin and leukotriene (Kiuchi *et al.*, 1992). In 1998, Doug *et al.* also demonstrated that diarylheptanoid extracted from Alpinia blepharocalyx strongly blocked the platelet aggregation of human blood induced by collagen, arachidonic acid and adenosine diphosphate (Doug *et al.*, 1998). Additionally, in 1998 Yamazaki *et al.* confirmed that YPE-01, a new diarylheptanoid, selectively inhibited 5-lipoxygenase and showed anti-inflammatory effect (Yamazaki *et al.*, 1998), and in 1999 Prasain *et al.* reported that diarylheptanoid might inhibit NO secretion (Prasain *et al.*, 1998). In addition, several researchers have reported that

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diarylheptanoids may have anti-oxidant effect with various other physiological activations as well as antitumor effect (Sheth *et al.*, 1973, Kawai *et al.*, 1990, Saxena *et al.*, 1995).

The natural killer (NK) cell was accidentally found by immunologists when taking tumor-specific, cytotoxic T lymphocyte (CTL) from mice (Halary *et al.*, 1997), which comprises about 5-10% of total lymphocytes in the blood. Importantly, NK cells play a strong role in building a defense barrier against viruses and cancer (Christine *et al.*, 1997).

On the other hand, although the biological role of NK cells is still unclear, there is some evidence that NK cells are associated with the rejection against implanted tumor cells, the prevention of metastasis and the rejection against bone marrow implantation (BMI) (Killion *et al.*, 1998). However, NK cells are known to not affect anything small sized metastatic cancer masses that are strongly restrained on their function. The antitumor effect of NK cells may be a hallmark of the first defense line against tumor lesion prior to the acquired immune response. Furthermore, the potency of NK cells in peripheral blood may be decreased in progressive cancers as occurs with other immune responses, and may even not be detected in proliferous malignant diseases.

Despite the metastasis of tumor being a very important factor in controlling patient prognosis, the mechanism of metastasis as well as the appropriate treatment have not been well elucidated to date, mainly because there have been no appropriate experimental trials in tumor metastasis. The closest trials have been conducted by Sugiura in Lewis lung cancer cell line in 1995 and by Satow in lung metastasis. Lewis lung cancer is a metastatic lung cancer, which naturally occurs from the subcutis of foot to the lung, and has been broadly used in research up to now. Fidler succeeded in isolating a high metastatic mutant, B16-F10, in 1973 (Fidler, 1973). Following this isolation, it was proved that cancer is a group having a different property from metastasis, and based on this knowledge, many high metastatic cell lines have been isolated to date. In studying metastasis, we may consider experimental or artificial metastasis and natural metastasis.

In this study, we investigated the effect of oregonin and the extension of survival time through the effectiveness of NK cells, and accordingly we examined the antitumor and inhibitory effects against B16-F10 melanoma.

MATERIALS AND METHODS

Materials

The study was performed by using 5 week-old, male C57BL/6 mice (18~22 g) and 6 week-old, male ICR mice (20~25 g. Oregonin, a derivative of *Alnus hirsuta Turcz, Betulaceae*, was provided by the Department of Herbal Medicine, Chungang University. Equipment used were centrifuge, clean bench, CO_2 incubator, autoclave, water bath (all Vision Sci. Co., Korea), deep freezer (Sanyo, Japan), microscope (Olympus, Japan), electronic balance, pH meter (both Mettler Toledo, Switzerland), ELISA reader (BIO-RAD, Japan), and LN_2 tank (Minnesota Valley Engineering, Inc., U.S.A.). All other apparatuses used in this study were sterilized under high pressure for 20 minutes at 121°C, 15lb/in^2. Materials/Reagents used were BSA, DMSO, Griess reagent, LPS (*E. coli* serotype 0111:B4), MTT, RPMI 1640, sodium nitrite, trypan blue (all Sigma Chem. Co., U.S.A.), FBS, penicillin-streptomycin, trypsin-EDTA (all Gibco BRL, U.S.A.), nylon wool (Wako, Japan), ficoll, percoll (both Pharmacia, U.S.A.), recombinant IL-2 (Genzyme, U.S.A.), recombinant INF-γ (R&D, U.S.A.), and thioglycollate broth (Difco, U.S.A). Other materials used during the study such as buffers and other reagents were supplied as first or super class and formulated in our lab as appropriate. In this study, we used B-16 melanoma cell, adopted from the Department of Immunology, School of Pharmacy, Chonnam University, and mouse lymphoma cell, Yac-1, adopted from the Department of Immunology, School of Pharmacy, SungGyunKwan University.

Tumor cell culture

The B16-F10 mouse melanoma cells used in this study were sub-cultured 2~3 times a week in RPMI-1640 media containing 10% FBS, penicillin 100 U/ml and streptomycin 100 μg/ml, and then cultured in CO_2-incubator (37°C, 5% CO_2). The cultured tumor cells, reaching to log phases, were diluted with a favored amount of concentration 20 hours before study. The FBS used in this procedure was inactivated by heating for 30 minutes at 56°C

Formulation of cell suspension

Tumor cells reaching to log phase were pre-cultured 24 hours before study at $2\sim3 \times 10^5$cells/ml in a 75 cm^2 screw-capped culture flask (Spinner culture). The number of cells in culture media generally reached up to $0.8\sim1.0 \times 10^6$cells/ml after 24 hours. We set the cell suspension of $1\sim5 \times 10^5$cells/ml as the final concentration by diluting a fresh medium (Run bottle).

NK cell separation

High-density NK cells were purified by sedimentation on a discontinuous Percoll density gradient by modifying the procedures created by Timonen and colleagues (Timonen, et al., 1981, Gallagher, et al., 1992). The 6 week-old, male (20~25 g) ICR mouse was killed by a cervical dislocation,

and the spleen removed. It was then moved on to a 60 mm petri dish containing HBSS 5 ml, broken and suspended with ACK buffer 5 ml added to eliminate red blood cells from the spleen. These were centrifuged for 20 minutes at 500 g and washed, and a regular spleen cell suspension formed by diluting an appropriate PBS. We isolated lymphocyte over human lymphocyte ficoll isopaque in the spleen cell suspension and isolated T lymphocyte over T cell isolation nylon wool column in the same way. We controlled the T lymphocyte concentration to be 8×10^7cells/ml and isolated NK cells by percoll's discontinuous density gradient. The T lymphocyte suspension was maintained for 4 hours at 4°C to protect voluntary aggregation. One-hundred percent percoll was diluted to 70, 65, 60, 57.5, 55, and 50% by RPMI 1640 containing 10%-FBS in percolls discontinuous density gradient. First, 15 ml of 70% solution was added and followed as it for the rests. Of these, the top floor was the 50% solution. Cell suspension passing the nylon wool was put in the discontinuous density gradient top floor and centrifuged for 30 minutes at 550 g. NK cells were abundant in the second (55%) and third (57.5%) floors, and thus we took this part gently. We added then into the medium and centrifuged for 10 minutes at 200 g to form the re-suspension, which was used in this study by diluting to an appropriate concentration.

MTT assay

We sterilized 5 mg/ml of MTT stock solution (Sigma) in pH 7.5 PBS, filtered undissolved small amounts of substances with a 0.22 μm filter and added 10 μl MTT (5 mg/ml) to either 100 μl cell suspension or cell monolayer of micro titer well and cultured them in an incubator installed with humidifier for 3 hours at 37°C. We then mixed the blue and non-aqueous formazan crystal with 0.04 M HCl 100 μl melted by propan-2-ol until completely melted and finally read the plates with ELISA reader at test wavelength 570nm (reference wavelength 630nm).

NK cell antitumor activity *in vivo*

By preparing five, 6 week-old, male ICR mice (20~25 g) at each concentration group, we injected D-PBS buffer to the control group and 1, 5, and 10 mg/kg of oregonin to the active groups via peritoneal with an adjustment of the substances to 0.1 ml. We injected the active substances into mouse peritoneal, isolated NK cells from the peritoneal after 20 hours of injection, and distributed 1×10^5cells on to the 96 well plates in order to use them as effecter cells. For this, we cultured for 20 hours in a CO_2 incubator to maintain a proportion of 20:1 between the effecter cells and the target cells (Yac-1). Yac-1 cells were pre-cultured. MTT assay was carried out to measure the macrophage antitumor activity.

NK cell antitumor activity *in vitro*

We distributed 1×10^5 NK cells, isolated from the spleen of a 6 week-old, male (20~25 g) ICR mouse, on to the 96 well plates and used them as effecter cells. For this, we maintained a proportion of 20:1 between the effecter cells and the target cells (Yac-1). Yac-1 cells were pre-cultured. We treated D-PBS buffer in the control group, 1, 10, and 100 μg/ml of oregonin in the active groups, and IL-2 1000U/ml in the positive control group. We set the total volume in each well at 200 μl, and cultured for 20 hours in a CO_2 incubator. Following these procedures, MTT assay was carried out to measure the NK cell activity *in vitro*.

Elongation of survival time

We prepared five, male C57BL/6 mice (5 weeks) in each group and B16-F10 mouse melanoma cells for the transplantation. B16-F10 mouse melanoma cells were used and the proportion between culture media and fresh media was set at 1:1 (volume: volume)36~48 hours before study. We measured the weight of the C57BL/6 mice and shaved the transplantation site (right thigh) 24 hours before transplantation. We injected optimized B16-F10 cells to the shaved transplantation site via intra muscular (i.m.) injection. We set the cell suspension at 1×10^4cells/ml as the final concentration by diluting PBS. The total volume was set at 0.05 ml. Ten hours after i.m. oregonin injection, we injected D-PBS buffer 0.05 ml to the control group and 1, 5, and 10 mg/kg of oregonin to the active groups per 48 hours. Similarly, the total volume was set at 0.05 ml and we measured the mouse survival time in B16-F10 melanoma cells by comparing the control group with the active groups.

Inhibitory effects on melanoma metastasis

We prepared five, male C57BL/6 mice (5 weeks) in each group and B16-F10 mouse melanoma cells for transplantation. B16-F10 mouse melanoma cells were used and the proportion between the culture media and the fresh media was set at 1:1(volume: volume) 36~48 hours before study. We measured the weight of the C57BL/6 mice and injected optimized B16-F10 cells to the transplantation site (right forefoot). We set the cell suspension at 5×10^5 cells/mouse as the final concentration by diluting PBS, and set the total volume of cells at 0.05 ml. After transplantation, when the melanoma size reached 7~8 mm (commonly 18~20 days), we cut the ankle of the right forefoot. After that, we injected all doses to the peritoneal cavity, and injected D-PBS buffer 0.1 ml to the control group and 1, 5, and 10 mg/kg of oregonin to the active groups per 72 hours for 3 weeks.

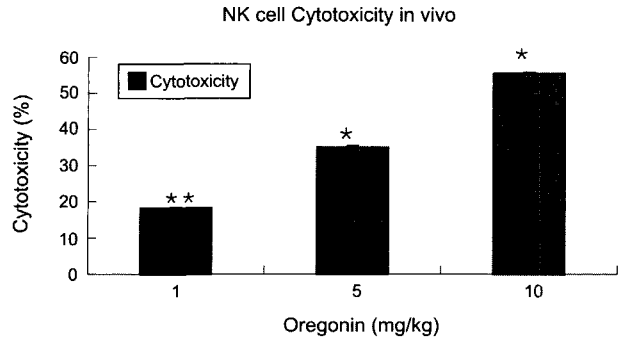

Fig. 1. *In vivo* effect of oregonin on NK cell antitumor activity.
Oregonin was i.p. injected to mice 20 hours prior to the removal of mice spleens. (Ed- confirm use of 'and' in both the following underlined section) Oregonin -treated NK cells and effector cells were cocultured for 20 hrs with Yac-1 and target cells (effector/target ratio=20:1). Tumoricidal activity was evaluated by cell viability as measured by MTT-assay. The Y-axis percentages indicate cytotoxicity in comparison with the control group (**p < 0.01, *p < 0.05; the percentage of the control group is 0%.).

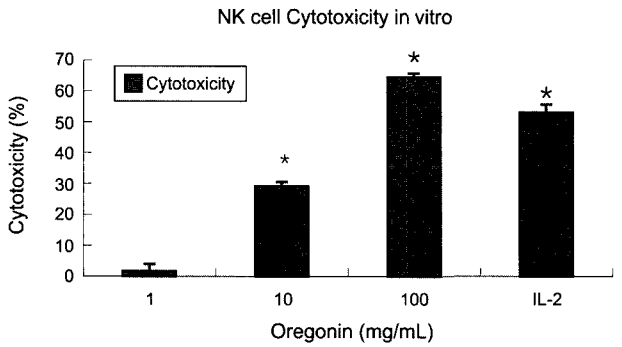

Fig. 2. *In vitro* effect of oregonin on NK cell antitumor activity.
NK cells and effector cells originating from the mouse spleen were cocultured for 20 hrs with Yac-1 and target cells in the presence of oregonin (effector/target ratio=20:1). Tumoricidal activity was evaluated by cell viability as measured by MTT-assay. The Y-axis percentages indicate the cytotoxicity in comparison with the control group (**p < 0.01, *p < 0.05; the percentage of the control group is 0%.).

The total volume was set at 0.1 ml. Three days after the last injection, we measured the weight of the C57BL/6 mice, opened the breast, conducted a pneumonectomy and lastly measured the number of colonies of metastasized melanoma cells in the lung.

Statistical treatment

We calculated mean values and standard deviations, and applied Student's T-test to verify the significance of the differences between the control and active groups. Significance was assigned at P-value > 0.5

RESULTS

The NK cell, which relates to the natural immune response, plays a very important role in killing tumor cells (immunosurveillance) and in deleting the virus-infected cells in the early infection phase. In addition, NK cells have an early defense property against microbes, which may degrade the autologous monocyte having intracellular bacteria (Manoussaka *et al.*, 1997). Early NK cell activation plays an important role in T cell immune response, which appears 7~10 days after infection. As for these NK cells, we measured their antitumor activity with oregonins in MTT assay.

NK cell antitumor activity

To determine the impact for NK cells *in vivo* by oregonin, we measured the antitumor activity by MTT assay at 1, 5, and 10 mg/kg doses. As a result, the cytotoxicity of the active groups was significantly increased by up to 18.10%, 34.91%, and 55.39% (Fig. 1).

In vivo, it was thought that oregonin may directly activate NK cells and also be activated by other cytokines secreted by immune cells. Additionally, IL-1 and interferon (IFN), which have been known to be secreted by NK cells, activate macrophage, which secretes IFN-α, IFN-β and induces NK cell activation, reciprocally. Therefore, we measured MTT in order to observe the antitumor effects represented by the direct action to NK cells *in vitro*. As a result, the cytotoxicity of the active groups was significantly increased by up to 29.10 and 64.61% at doses of 10 and 100 μg/ml, respectively (Fig. 2). These results may be interpreted to indicate/confirm that oregonin directly affects NK cells and thus increases their antitumor activity.

Cytotoxicity (%) =|value of control group-value of active groups| × 100 / value of control group

Elongation of survival time

We investigated the survival days in mice by injecting oregonin every 48 hours after transplanting B16-F10 melanoma cells into the right thigh of C57BL/6 mice. As a result, the active groups administered 5 mg/kg and 10 mg/kg showed 35.96% and 51.32% life span increase compared to the control group, respectively, which represented a significant level of elongation (Fig. 3).

Inhibitory effects on melanoma metastasis

We measured the number of tumor cell colonies presented through the lung metastasis by administering oregonin for 3 weeks at 72 hour intervals after cutting the mouse ankle when the tumor size reached 7~8 mm. This was done after transplanting B16-F10 melanoma cells

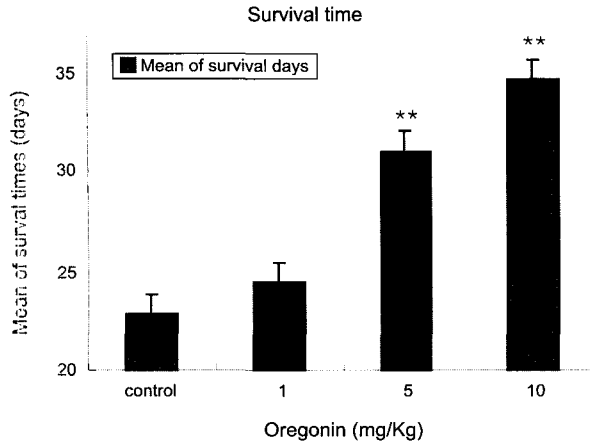

Fig 3. Life-extending effect of oregonin on B16-F10 melanoma
Ten hrs after inoculation with 5×10^5 cells/mouse, male C57BL/6 mice (5weeks) were treated with i.m. injection of oregonin at the indicated doses every 48hours. The control group was exposed to PBS. Oregonin extended the survival time of the mice in a dose-dependent manner (** $p < 0.01$, * $p < 0.05$).

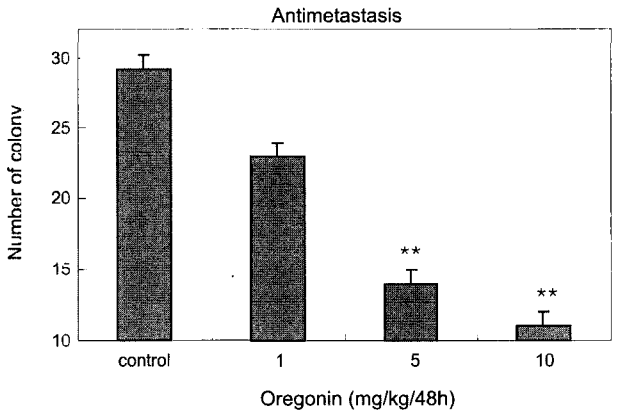

Fig 4. The effect of oregonin on the inhibition of melanoma metastasis
Ten hrs after inoculation with 5×10^5 cells/mouse, male C57BL/6 mice (5weeks) were treated with i.m. injection of oregonin at the indicated doses every 48hrs. The control group was exposed to PBS. Oregonin showed remarkable inhibitory effects on melanoma metastasis (**$p < 0.01$).

into the right forefoot of C57BL/6 mice. As a result, the active groups showed a significant inhibition force of metastasis by 52.05% and 62.33%, at 5 and 10 mg/kg doses, respectively, compared to the control group (Fig. 4). This result, in conjunction with the result showing the effect of life span elongation, suggests that oregonin may inhibit the metastasis of B16-F10 and thus elongate the life span by inhibiting the movement (metastasis) of solid tumor cells to other organs.

DISCUSSION

To investigate the antitumor effect of oregonin, NK cell, an immune cell, was used. Commonly, NK cells eliminate tumor cells and virus infected cells by a similar process of CTL-mediated lysis (Poccia *et al.*, 1997). These activations are seen in early response and help to defend during Tc cell activation, proliferation and differentiation into the functional CTL.

In general, immune responses of NK cells play an important role in eliminating the locally growing and circulating tumor cells. The antitumor effects of NK cells were increased dose-dependently both *in vivo and in vitro*. In the 100 μg/ml group, *in vitro,* the enhanced cytotoxicity effect was greater than that of the positive control group (IL-2 group). In accordance with these results, oregonin is thought to increase its potencies against local tumor cells by stimulating NK cells that are responsible for the early immune response.

On the other hand, metastasis is the major cause of treatment failure for cancer patients. In our study of survival time and metastasis of C57BL/6 mouse & B16-F10 mouse melanoma cells, we found that there was an excellent outcome in a dose-dependent manner. Based on the present results indicating that NK cells activated by oregonin destroyed circulating tumor cells and prevented subsequent metastasis including micrometastasis, we suggest that immunological factors play a dominant role in resistance to tumor metastasis. In chemotherapy, adriamycin slightly influences circulating tumor cells, although it markedly inhibits locally growing tumor cells. Accordingly, circulating tumor cells were arrested in target organs and formed micrometastasis under chemotherapy alone. This finding supports the previous concept that locally growing and metastatic tumors presumably show different chemosensitivities (Furukawa *et al.,* 1993). Cancer cells that are rapidly proliferating in a local area are known to be good target cells of DNA-working anticancer drugs including adriamycin (Fritzer-Szekeres *et al.,* 1998). However, metastatic cells circulating through the blood stream and lymph nodes are only slightly affected by DNA-working anticancer drugs. Resistance of metastatic cells to adriamycin was previously reported in a colon cancer-bearing mice model and breast cancer patients (Anand *et al.,* 1995, Ohishi *et al.,* 1996) It was also reported that 5-fluorouracil and mitomycin C significantly suppressed tumor growth, but not metastasis, in the immunochemotherapy of human stomach cancer with OK-432 and anticancer drugs (Furukawa *et al.,* 1993) The exact reasons that metastatic cells are resistant to anticancer drugs have not been elucidated yet. For further study on the resistance mechanisms to anticancer drugs, we investigated the characteristics of highly metastatic B16F10 melanoma and determined that oregonin reduced lung metastasis. This is potential evidence that oregonin may be effective in inhibiting tumor cells.

In conclusion, we confirmed the antitumor effect of

oregonin which augments NK cell activities against cancer cells, by showing a direct cytotoxicity. Our results of an inhibition of metastasis for B16-F10 mouse melanoma cells in addition to an extended survival time effect, both *in vitro* and *in vivo*, demonstrate that oregonin may activate the inner immune cells and may also have an antitumor effect.

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