Regular Article

High-Dose Vitamin C Administration Inhibits the Invasion and Proliferation of Melanoma Cells in Mice Ovary

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Several studies have been conducted to explore the anticancer effects of vitamin C (VC). However, the effect of high-dose VC administration on melanoma is still unknown. Therefore, in this study, we investigated the effects of high-dose VC (4 g/kg) on the invasion and proliferation of melanoma cells in various organs of mice. B16 melanoma cells (1×10^6 cells/100 μ L) were intravenously injected into the tails of female mice, and VC solution (4 g/kg) was orally administered once a day for 14 d. On the 15th day, samples from the liver, lungs, jejunum, and ovaries were collected and analyzed for invasion and proliferation of melanoma cells. Oral VC administration decreased the number of dihydroxyphenylalanine (DOPA)-positive cells and gp100-positive melanoma cells in the ovaries and suppressed the invasion and proliferation of melanoma. Compared to melanoma-administered mice, macrophage inflammatory protein-2 levels and number of neutrophils were increased in the VC + melanoma-administered mice. Furthermore, the concentrations of VC, iron, and hydrogen peroxide, and the number of terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells were significantly increased in the ovaries of VC + melanoma-administered mice compared to those of melanoma-administered mice. These results suggest that VC can reduce the invasion and proliferation of melanoma cells in the ovaries, and neutrophils in the ovaries play an important role in achieving this melanoma-suppressive effect.

Key words vitamin C; melanoma; ovary; neutrophil

INTRODUCTION

Melanoma is a malignant tumor of melanocyte cells, and it is thought to be caused by carcinogenesis of melanocytes in the basal layer of the epidermis.¹⁾ Although most melanomas occur in the skin, they have also been found in the mouth, intestines, and eye, owing to the distribution of melanocytes.²⁾ Though the morbidity of melanoma is 1 to 2 people per 100,000 in Japan, the number has been increasing in recent years.³⁾ The exact cause of melanoma is still unknown, but it is assumed to be triggered by genetic predisposition and environmental factors.⁴⁾ For example, the melanoma incidence in Caucasians is several times higher than Hispanics and blacks, and the incidence in Whites living in areas of high UV intensity is higher than those living in areas of low UV intensity, suggesting the involvement of UV radiation in the etiology of melanoma.⁵⁾ In Japan, the majority of melanomas arise at sites which are less exposed to UV radiation, such as the trunk and proximal limbs.⁶⁾ In rare cases, melanoma is also found in the ovaries.^{7–9)}

Standard treatments for melanoma include surgical resection, chemotherapy, radiation therapy, biological therapy, and targeted therapy.¹⁰⁾ However, the prognosis of malignant melanoma in the gynecological field is poor and depends on how well it can be resected by surgery.¹¹⁾

Furthermore, vitamin C (VC) is a widely used general purpose medication and OTC drug.¹²⁾ VC is involved in collagen formation, prevention of anemia, and inhibition of melanin production. In addition, VC is also an antioxidant which removes reactive oxygen species (ROS), however, in the presence of ferric iron, VC can also act as a pro-oxidant, gen-

erating ROS such as hydroxyl radical and hydrogen peroxide (H_2O_2).^{13,14)} In recent years, the use of VC infusion therapy in cancer treatment has attracted attention, as it does not exhibit adverse drug reactions. This treatment is already undergoing clinical trials in the United States and being widely tested as a personalized treatment modality in Japan, the cost of which is borne by the patients themselves.^{15,16)} A treatment combining high-dose VC and anticancer drugs uses the mechanism of action of pro-oxidants. Compared to normal cells, cancer cells have lower levels of antioxidant enzymes such as catalase, therefore, ROS generated by VC are thought to act selectively on cancer cells and cause apoptosis.¹⁷⁾ However, the effect of high-dose VC on the invasion and proliferation of melanoma cells is unknown.

Thus, in this study, we investigated the effect of high-dose VC on the invasion and proliferation of melanoma cells using C57BL/6J and gp91^{phox}, a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit, knockout (gp91^{phox-/-}) mice. In particular, we examined the organ-specific action of VC.

MATERIALS AND METHODS

Animal Experiments Female C57BL/6J mice (7 weeks old; Japan SLC, Hamamatsu, Shizuoka, Japan) and gp91^{phox-/-} mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were reared at a constant temperature of $23 \pm 1^\circ\text{C}$ with $50 \pm 10\%$ relative humidity under a 12-h light/12-h dark cycle. At 8 weeks of age, mice were randomly assigned to four groups ($n=6$ per group): control, high-dose VC, melanoma, and VC + melanoma. B16 melanoma cells lines (Cell Resource

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Fig. 1. Effect of High-Dose Vitamin C on the Invasion and Proliferation of Melanoma Cells in the Ovary

The number of DOPA-positive cells (indicated by arrows indicate in the images) (A), the expression of gp100-positive melanoma cells (B), MIP-2 levels (C), and the number of neutrophils (D) in the experimental and control groups. The data are represented as mean $\pm$ standard deviation (S.D.). Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Scale bar = 100 μ m. Abbreviation: Vit C, vitamin C. (Color figure can be accessed in the online version.)

Center for Biomedical Research, Tohoku University, Sendai, Japan) were used at passages 5 to 15, and the melanoma cells were cultivated as previously described¹⁸⁾ and intravenously injected (1×10^6 cells/100 μ L) into the tail of the mice to induce melanoma. This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Suzuka University of Medical Science (Approval No. 66). All surgeries were performed under pentobarbital anesthesia and every effort was made to minimize animal suffering.

VC Treatment To determine the effective VC dose, we examined the effect of 1, 4, 8, or 12 g/kg of VC (Fuso Pharmaceutical Industries Ltd., Osaka, Japan) solution in distilled water, and found high and equivalent effectiveness at 4 g/kg and higher doses (data not shown). Thus, mice in experimental groups were orally administered approximately 4 g/kg of VC in distilled water for 14 d. The control animals were administered distilled water.¹⁹⁾

Preparation and Staining of Tissue Specimens On the 15th day, we extracted lung, liver, jejunum, and ovary samples under anesthesia. The tissue samples were fixed with phosphate buffered saline (PBS) containing 4% paraformaldehyde

(Wako Pure Chemical Industries, Ltd., Osaka, Japan) and then embedded in Tissue-Tek OCT Compound (Sakura Finetek, Tokyo, Japan) to cut into 5 μ m thick sections. The dihydroxy-phenylalanine (DOPA)-positive cells, which are markers of melanocytes rich in tyrosinase in each tissue, were stained as described previously.²⁰⁾ In addition, the sections were immunohistochemically stained for neutrophils²¹⁾ using antibodies against neutrophil-specific antigen Ly-6C (100:1; Bio-Rad laboratories Inc., Hercules, CA, U.S.A.). Briefly, the sections were incubated with antibodies overnight at 4°C washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (1:30; Dako Cytomation, Glostrup, Denmark) for 2 h at 25°C. Further apoptosis was analyzed using the *in situ* apoptosis detection kit (TaKaRa, Shiga, Japan). The numbers of DOPA-positive cells, neutrophils, and terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells were determined using an analysis software Image J v20 (National Institutes of Health, Bethesda, MD, U.S.A.). Five observation fields (1 mm²) were randomly analyzed from the observed images and the average number of cells per field was determined using the image processing software Image J v20.

Fig. 2. Effect of High-Dose Vitamin C on the Invasion and Proliferation of Melanoma Cells in the Lungs

The number of DOPA-positive cells, gp100-positive melanoma cells, and neutrophils in the experimental and control groups. The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Abbreviation: Vit C, vitamin C.

Fig. 3. Effect of High-Dose Vitamin C on the Invasion and Proliferation of Melanoma Cells in the Liver

The number of DOPA-positive cells, gp100-positive melanoma cells, and neutrophils in the experimental and control groups. The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Abbreviation: Vit C, vitamin C.

Fig. 4. Effect of High-Dose Vitamin C on the Invasion and Proliferation of Melanoma Cells in the Jejunum

The number of DOPA-positive cells, gp100-positive melanoma cells, and neutrophils in the experimental and control groups. The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Abbreviation: Vit C, vitamin C.

Western Blotting The ovary samples were homogenized with lysis buffer (Kurabo, Osaka, Japan) and centrifuged at $8000 \times g$ for 10 min. Then, the supernatant was separated and stored at -80°C until further analysis. Western blotting was performed as previously described.²²⁾ Briefly, the samples were separated by electrophoresis and the membranes were incubated with primary antibodies gp100, a melanoma marker, (1:100; Abcam, Cambridge, U.K.) macrophage inflammatory protein-2 (MIP-2) (1:100; R&D Systems, Minneapolis, MN, U.S.A.), and β -actin (1:5000; Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) at 25°C for 1 h. Immune complexes on the membranes were visualized with horseradish peroxidase-conjugated secondary antibody (1 : 1000; Novex, Frederick, MD, U.S.A.) and detected with ImmunoStar Zeta reagent (Wako Pure Chemical Industries, Ltd.). The images were acquired using the Multi-Gauge Software Program ver. 3.0 (FUJIFILM, Greenwood, SC, U.S.A.).

Neutrophil Separation Method from Ovary Homogenate Neutrophils in the supernatant of the ovary homogenate were separated using an isolation kit (Neutrophil isolation kit;

Cayman, Ann Arbor, MI, U.S.A.), and then the number of neutrophils in 1 mm^2 was calculated using a hemocytometer (Sunlead Glass Corp., Koshigaya, Japan).

VC Concentration in the Ovaries and Separated Neutrophils The VC concentration in the ovary supernatant, and separated neutrophils was measured using the VC measurement kit (Ascorbate assay kit; Cayman). The VC concentration in neutrophils was calculated as the concentration per fixed number of cells, based on the number of cells calculated as described above.

Measurement of H_2O_2 and Iron in Ovary The concentrations of H_2O_2 and iron in the ovaries were measured using the OxiSelect™ STA-347 *in vivo* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA, U.S.A.) and metalloassay kit (Metallogenics, Chiba, Japan), respectively in accordance with the manufacturer's instructions.

Statistical Analysis The results were analyzed using the Microsoft Excel 2010 software (Microsoft Corp., Redmond, WA, U.S.A.). Differences between groups were evaluated using one-way ANOVA followed by Tukey's *post-hoc* test in

the Statistical Package for the Social Sciences (SPSS) v20.0 software (SPSS, Inc., Chicago, IL, U.S.A.). Data are presented as mean $\pm$ standard deviation (S.D.). The results were considered significant at $p < 0.05$.

RESULTS

Effect of High-Dose VC Administration on the Number of Neutrophils and the Invasion and Proliferation of Melanoma Cells in the Ovary In the ovaries of melanoma-administered mice, the numbers of DOPA-positive cells and gp100-positive melanoma cells were increased (Figs. 1A, B). However, in the VC + melanoma-administered mice group, the numbers of both cell types were significantly decreased ($p < 0.05$) compared with those in melanoma-administered mice (Figs. 1A, B). Further, the number of neutrophils and MIP-2 levels were highest in the ovaries of VC + melanoma-administered mice (Figs. 1C, D).

Effect of High-Dose VC Administration on the Number

Fig. 5. Effects of High-Dose Vitamin C Administration on the VC Concentration in the Ovary Homogenate (A) and in the Neutrophils (B) in the Ovary Samples of the Experimental and Control Groups

The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Abbreviation: Vit C, vitamin C.

Fig. 6. Effect of High Dose VC Administration on the Concentrations of H₂O₂ (A) and Iron (B), and Number of TUNEL-Positive Cells (C) in the Ovary Samples of the Experimental and Control Groups

The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Scale bar = 100 μm. Abbreviation: Vit C, vitamin C. (Color figure can be accessed in the online version.)

of Neutrophils and the Invasion and Proliferation of Melanoma Cells in the Lungs, Liver, and Jejunum The numbers of DOPA-positive cells, gp100-positive melanoma cells, and neutrophils were significantly increased ($p < 0.05$) in the lung, liver, and jejunum in melanoma-administered mice, but were not significantly different when compared to those of VC + melanoma-administered mice (Figs. 2–4) this, further analysis investigating the effects of high-dose VC administration focused on ovary.

VC Concentration in the Ovary Homogenate and Separated Neutrophils VC concentration in the ovary homogenate and separated neutrophils was significantly lower ($p < 0.05$) in melanoma-administered mice than that in VC-administered mice, but was highest in VC + melanoma-administered mice (Figs. 5A, B).

Effect of High-Dose VC Administration on the Concentrations of H₂O₂ and Iron, and TUNEL-Positive Cells in the Ovaries Next, we measured the concentration of H₂O₂ and iron in the ovaries, which is closely related to VC administration.

The iron and H₂O₂ concentration, and the number of TUNEL-positive cells in the ovaries were significantly increased ($p < 0.05$) in VC + melanoma-administered mice than those in melanoma-administered mice (Figs. 6A–C).

Effect of High-Dose VC Administration on the Number of Neutrophils and the Invasion and Proliferation of Melanoma Cells in the Ovaries of gp91^{phox}−/− Mice In the ovaries of gp91^{phox}−/− mice, the numbers of DOPA-positive cells and gp100-positive melanoma cells increased in the melanoma group and VC + melanoma group, but did not vary greatly between both these groups (Figs. 7A, B). Similarly the number of neutrophils was not significantly different between the melanoma group and the VC + melanoma-administered group (Fig. 7C).

Fig. 7. Effect of High Dose VC Administration on the Invasion and Proliferation of Melanoma Cells and the Number of Neutrophils in the Ovaries of gp91^{phox-/-} Mice

The number of DOPA-positive cells (A), gp100-positive melanoma cells (B), and neutrophils (C) in the experimental and control groups. The data are represented as mean $\pm$ S.D. Tukey's *post-hoc* test was used to compare the differences between the groups. * $p < 0.05$. Scale bar = 100 μ m. Abbreviation: Vit C, vitamin C. (Color figure can be accessed in the online version.)

DISCUSSION

The results obtained from each organ (lung, liver, jejunum, and ovary) revealed that VC administration significantly reduced the invasion of melanoma in the ovaries. The number of neutrophils was increased in the VC + melanoma-administered mice. Moreover, H₂O₂ and iron concentrations in the ovaries were significantly increased in the VC + melanoma-administered mice. However, in the gp91^{phox-/-} mice, the numbers of DOPA-positive cells, gp100-positive melanoma cells, and neutrophils were not different between the melanoma group and the VC + melanoma group.

In our previous study, in a model of sodium dextran sulfate (DSS)-induced ulcerative colitis, administration of high-dose VC regulated neutrophil and cell apoptosis and improved ulcerative colitis.²³⁾ In addition, irinotecan-induced skin dryness was improved by administering high-dose VC *via* ROS.²⁴⁾ Furthermore, neutrophils and ROS were involved in azoxymethane + DSS-induced colorectal cancer.¹⁹⁾ In this study,

neutrophils also play an important role in melanoma, suggesting that neutrophils are crucial in the pathological function of high-dose VC. In this study, the effect of VC on the invasion and proliferation of melanoma cells was found to be organ-specific. In addition, neutrophils in the ovaries were increased upon VC administration. Neutrophils are involved in the ovulation and maintenance of the sexual cycle,²⁵⁾ and it is thought that neutrophils rich in VC accumulate in the ovaries because they take up VC. However, the detailed mechanism is unknown. Nevertheless, when neutrophils infiltrate the ovary, they release β -endorphins, an analgesic peptides, to maintain the sexual cycle.^{26,27)} β -Endorphin reportedly acts on the theca and granulosa cells surrounding the follicle to regulate the production of steroid hormones.^{28,29)} Therefore, this may explain how increased neutrophilic infiltration in the ovaries help to maintain the sexual cycle. Moreover, VC has also shown to stimulate the production^{30–33)} and function^{34,35)} of leukocytes, especially neutrophils. In addition to the increase in neutrophil count in the ovaries, MIP-2, which is a neutro-

phil chemotactic factor, was also elevated by VC administration. Surprisingly, VC did not induce neutrophil production in the other organs examined. Thus, VC can promote cancer cells death by increasing the number of neutrophils and stimulating their phagocytic activity. Interestingly, VC administration in normal mice did not change in leukocyte production or function. This suggests that VC may be more effective when immune cells are damaged; however, further research is warranted.

Neutrophils release ROS to kill cancer cells, neutrophils themselves tend to be damaged.³⁶⁾ However, Neutrophils generally store high concentrations of VC³⁷⁾ and protect themselves against oxidative damage.^{38,39)} VC is protected from neutrophil autooxidation by its antioxidant function.^{40,41)} As melanoma of the ovary increases, so does the neutrophils infiltration. In this study, a large concentration of VC was stored in the neutrophils, and it may because a high-dose of VC assisted in defending the autooxidation mechanism.

Furthermore, high VC concentration causes a Fenton reaction with iron around cancer cells to generate H₂O₂.⁴²⁾ Normal cells are not affected because they contain catalase which neutralizes H₂O₂, while cancer cells are affected because they are catalase-deficient.⁴³⁾ In the present study, ovaries of the VC + melanoma-administered mice showed increased H₂O₂ and iron concentrations, and apoptotic cells, suggesting increased melanoma cell death. These results indicate that VC can safely and effectively reduce the levels of melanoma cells in the ovary, and it may be promising as a therapeutic agent for melanoma.

Finally, to validate the effect of neutrophils, we used gp91^{phox-/-} mice. The effect of VC was attenuated in gp91^{phox-/-} mice, suggesting that H₂O₂ generated by gp91^{phox} (NADPH oxidase 2) expressed in neutrophils caused cancer cell death. Therefore, it is speculated that gp91^{phox} plays an important role in suppressing cancer cells as well as the action of neutrophils themselves.

CONCLUSION

High-dose VC showed organ-specific effects and suppressed the invasion and proliferation of melanoma in the ovaries. VC administration increased the neutrophil count in the ovaries compared to other organs, and promoted cancer cell death by stimulating their phagocytic function. Moreover, gp91^{phox} in neutrophils plays an important role in suppressing cancer cells. Therefore, it can be expected that VC can safely and effectively treat melanoma cells in the ovaries. However, because this study was performed in mice, the results need to be validated using human trials.

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Conflict of Interest The authors declare no conflict of interest.

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