High-Dose Vitamin C Exerts Its Anti-cancer Effects in a Xenograft Model of Colon Cancer by Suppressing Angiogenesis

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis and is involved in solid tumor carcinogenesis, infiltration, distant metastasis, and tumor angiogenesis. Tumor angiogenesis plays an important role in controlling the progression of cancer. There are different subtypes of VEGF with unique characteristics. VEGFA is the main subtype that efficiently induces the proliferation of endothelial cells and promotes their permeability. VEGFD has a subtype with a strong angiogenic effect in advanced colorectal cancer. VEGF is upregulated under various conditions associated with cancer, including the presence of various growth factors and hypoxia. Transcription enhancement of the VEGF gene by hypoxia stimulation is regulated by the transcription factor hypoxia-inducing factor-1α (HIF-1α). The production of VEGF and other growth factors by a tumor results in the formation of new vasculature in and around the tumor, but the new vascular network is formed structurally and functionally abnormal. Endostatin is a C-terminal fragment of collagen XVIII and an endogenous inhibitor of angiogenesis and tumor growth, which can inhibit the action of VEGF. It has also been reported that endostatin is partially involved in tumor suppression by p53.

Vitamin C (VC) is a water-soluble micronutrient commonly found in the diet and plays an important role as a coenzyme factor in maintaining the body. Also, VC is an important antioxidant that plays a vital role in immune function. It is well known that VC is essential for collagen biosynthesis. Interestingly, under certain conditions VC can act as a pro-oxidant that acts to selectively suppress the growth of cancer cells. The antitumor effect of VC was associated with reactive oxygen species (ROS) by administration of high-dose VC in azoxymethane + dextran sodium sulfate-induced colorectal cancer in our previous study. Furthermore, in melanoma, ROS effect by administration of VC, suggesting an antitumor effect, suggesting that ROS is important for the pathological function of high-dose VC. Expression of p53 has been reported to be involved in increased intracellular ROS development, and VC has been reported to be involved in p53, but it is unclear if VC affect endostatin.

In this study, mice with xenografts of Colon 26 murine colorectal cancer cells were used to investigate the effects of high-dose VC on p53, VEGF and endostatin levels.

MATERIALS AND METHODS

Animals Male BALB/c mice (5 weeks old; Japan SLC, Hamamatsu, Shizuoka, Japan) were purchased. The mice were maintained in a bed net cage (made of stainless steel) under a 12–12 h light and dark cycle at a room temperature of 23 ± 1°C and humidity of 50 ± 10%. The mice were allowed free access to laboratory chow and water. At 6 weeks of age, the mice were randomly assigned to three groups (n = 6 per group): control, Colon 26, and VC + Colon 26. All experimental protocols were approved by the animal care committee in compliance with the regulations of Suzuki University of Medical Science (Approval No. 66). The mice were euthanized by deep anesthesia with sodium pentobarbital.

The Colon 26 murine colorectal carcinoma was purchased from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). A total of 100 µL of tumor cell suspension containing 10⁵ cancer cells was injected subcutaneously into the right hind limbs of the mice.

Tumor volumes were determined using the equation (vol-
ume = LW²/2), where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface.

**VC Treatment** We administered 1, 4, 8, or 12 g/kg of VC (Fuso Pharmaceutical Industries Ltd., Osaka, Japan) solution in distilled water to mice to determine the effective VC dose, and found high and equivalent effectiveness at 4 g/kg and higher doses (data not shown). Thus, the mice in the experimental group received about 4 g/kg of VC in distilled water and the control animals received distilled water. Both were orally administered once daily for 14 d. 16

**Preparation and Staining of Tissue Specimens** On the final day of the study, the lower limb was removed under anesthesia and the tumor tissues were immunohistochemically stained with antibodies against VEGFA (1:100; BioLegend, San Diego, CA, U.S.A.) or VEGFD (1:100; Abcam, Cambridge, U.K.). 22 Briefly, the tissue samples were fixed with phosphate buffered saline (PBS) containing 4% paraformaldehyde and then embedded in Tissue-Tek OCT Compound (Sakura Finetek, Tokyo, Japan), cut into 5 µm thick sections, incubated with the primary antibodies and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit and tetramethylrhodamine isoioxyanate-conjugated anti-rabbit (1:30; Dako Cytomation, Glostrup, Denmark) secondary antibodies for 2 h at 25°C.

**Western Blot Analysis** The lower limb tumor tissues were homogenized in lysis buffer (Kurabo, Osaka, Japan) and centrifuged at 8000 × g for 10 min. The supernatant was then removed and stored at −80°C until further analysis. Western blot analysis was performed as follows with reference to the method of the previous study. 23 To briefly explain, after thawing, equal amounts of protein (12.5 µg/line) were loaded onto a 4–12% BISTRIS Blot Gel (Life Technologies, Carlsbad, CA, U.S.A.) and electrophoresed at 200 V for 20 min. Following separation, the proteins were transferred to a nitrocellulose membrane using the iBlot Western blotting System (Life Technologies). Membranes were treated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Novex, Frederick, MD, U.S.A.), HIF-α (1:1000; Abcam), and β-actin (1:5000; Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) at 25°C for 1 h. Membranes were then treated with a horse-radish peroxidase-conjugated secondary antibody (1:1000; Novex, Frederick, MD, U.S.A.). Immune complexes were detected with ImmunoStar Zeta reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Images were acquired using the Multi-Gauge Software Program ver. 3.0 (FUJIFILM, Greenwood, SC, U.S.A.).

**Quantification of Endostatin and ROS Levels** Blood samples were collected immediately following cardiac puncture. Plasma was separated from blood samples by centrifugation at 3000 × g for 10 min at 4°C, and the supernatant was used for analysis. The plasma levels of endostatin were measured using a commercial enzyme-linked immunosorbent assay kit (Endostatin: Biomedica, Vienna, Austria), according to the manufacturer’s instructions. Plasma and tissue ROS levels were determined using an OxiSelect in vitro ROS/RNS assay kit (STA-347; Cell Bioslabs, Inc., San Diego, CA, U.S.A.), according to the manufacturer’s instructions.

**Statistical Analysis** All data are presented as the mean ± standard deviation (S.D.). For comparisons between test groups, by the Tukey–Kramer test was applied with p < 0.05 considered to be a statistically significant difference.

**RESULTS**

*Effect of VC Administration on Tumor Cell Growth* Tumors were observed in mice xenografted with Colon 26 tumor cells. VC administration suppressed tumor growth. The tumor volume in the Colon 26 + VC group was reduced by 28% compared with that in the Colon 26 alone group (Fig. 1).

*Effect of VC Administration on the Levels of p53, Endostatin, HIF-1α, and ROS* p53 is a gene that drives apoptosis in cancer cells. The levels of p53 the lower limb tumor tissues were increased significantly in the Colon 26 group than in the control group. Treatment with VC further significantly increased the levels in the Colon 26 + VC group compared with that in the Colon 26 group. Plasma endostatin levels were also measured because it is partly associated with tumor suppression by p53. Similar to p53, endostatin levels were significantly increased in the Colon 26 group relative to control and further significantly increased in the Colon 26 + VC group (Figs. 2A, B). Both plasma and intratumoral ROS levels were found to be significantly increased in the Colon 26 group compared with those in the control group, but were significantly decreased in the Colon 26 + VC group compared with those in the Colon 26 group. Similarly, the lower limb tumor tissues HIF-1α levels were significantly increased in the Colon 26 group compared to the control group but were significantly decreased in the Colon 26 + VC group compared to the Colon 26 group (Figs. 2C–E).

*Effect of VC Administration on the Expression of VEGFA and VEGFD* The lower limb tumor tissues were homogenized in lysis buffer (Kurabo, Osaka, Japan) and centrifuged at 8000 × g for 10 min. The supernatant was then removed and stored at −80°C until further analysis. Western blot analysis was performed as follows with reference to the method of the previous study. 23 To briefly explain, after thawing, equal amounts of protein (12.5 µg/line) were loaded onto a 4–12% BISTRIS Blot Gel (Life Technologies, Carlsbad, CA, U.S.A.) and electrophoresed at 200 V for 20 min. Following separation, the proteins were transferred to a nitrocellulose membrane using the iBlot Western blotting System (Life Technologies), which was subsequently blocked with 5% skim milk at 4°C overnight. The next day, the membranes were incubated with antibodies to p53 (1:1000; Cell Signaling Technology, Danvers, MA, U.S.A.), HIF-α (1:1000; Abcam), and β-actin (1:5000; Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) at 25°C for 1 h. Membranes were then treated with a horse-radish peroxidase-conjugated secondary antibody (1:1000; Novex, Frederick, MD, U.S.A.). Immune complexes were detected with ImmunoStar Zeta reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Images were acquired using the Multi-Gauge Software Program ver. 3.0 (FUJIFILM, Greenwood, SC, U.S.A.).

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DISCUSSION

It is well known that p53 has anti-cancer effects, it activates transcription of the α (II) collagen prolyl-4-hydroxylase gene. This enzyme extracellularly releases potent endogenous inhibitors of angiogenesis, such as endostatin and tumstatin, which are derived from collagen types XVIII and IV. These inhibitors suppress the angiogenesis pathway and thereby have anti-cancer effects. As a result of the continuous administration of VC for 14 d in mice with xenografts of Colon 26 tumor cells, tumor sizes were decreased. In addition, the expression levels of p53 and endostatin were increased and the levels of VEGFA and VEGFD were decreased. Taken together, these data suggest that VC may be involved in the p53/endostatin/VEGF pathway. The detailed mechanism of action by which VC causes an increase in endostatin levels is unknown, therefore further research will be needed in the future. Furthermore, VC is involved in the production and maintenance of collagen and may directly affect collagen type XVIII without acting through p53. This aspect is currently under investigation.

VC has also been reported to inhibit the expression of VEGF via HIF-1α. HIF-1α is hydroxylated by the oxygen-dependent enzyme prolyl 4-hydroxylase (PHD), but under hypoxia HIF-1 stabilizes and produces transcriptional activators. After that, VEGF is secreted, endothelial cells proliferate, and new blood vessels are formed. Changes in oxygen concentration also affect ROS. When ROS levels exceed the defense mechanism, ROS cause oxidative stress. Elevated ROS levels have also been reported to contribute to HIF stabilization during hypoxia and reoxygenation. In mice with Colon 26 xenografts, both ROS and HIF-1α levels were elevated, but both were suppressed by VC administration. It has been suggested that the suppression of ROS by VC is involved in the suppression of angiogenesis. However, it is unclear whether this effect of VC is affected by oxygen concentration, so further research is needed.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES
