Administration of High-Dose Vitamin C and Irinotecan Ameliorates Colorectal Cancer Induced by Azoxyemethane and Dextran Sodium Sulfate in Mice

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High-dose vitamin C administration has been reported to exhibit antitumor effect in various mouse models of cancer. However, the underlying mechanism of antitumor effect against colorectal cancer remains to be elucidated. In this study, we investigated the antitumor effect of high-dose vitamin C in a mouse model of chronic inflammation-associated colorectal cancer induced by azoxymethane (AOM) and dextran sodium sulfate (DSS). After cancer induction, the mice were administered vitamin C and/or irinotecan. Because irinotecan is a key drug in colorectal cancer treatment, it was used for comparison in this study. We examined reactive oxygen species (ROS) and interleukin-6 (IL-6) levels in the plasma of mice, as well as collagen type I and caspase-1 expression and neutrophil and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cell counts in the colon tissue. Vitamin C and/or irinotecan administration decreased the plasma level of ROS and IL-6 and increased the expression of collagen type I and caspase-1. Furthermore, it increased neutrophil and TUNEL-positive cell counts. The most significant changes in the parameters analyzed were observed when both vitamin C and irinotecan were administered.

Key words vitamin C; colorectal cancer; azoxymethane; dextran sodium sulfate; irinotecan

In Japan, colorectal cancer is a leading cause of death after lung cancer.15 The treatment of colorectal cancer includes endoscopic therapy, surgery, chemotherapy, and radiation therapy. Although the progression of colorectal cancer is typical, the survival period has been markedly prolonged owing to the advancements in anticancer drug development. The standard therapies for colorectal cancer include the anticancer drugs, such as pyrimidine fluoride, irinotecan, and oxaliplatin, and molecular therapeutic agents, such as anti-vascular endothelial growth factor antibody and anti-epidermal growth factor antibody, 5-Fluorouracil (5-FU), synthesized in 1956 by Duschinsky and Heidelberger, is an anticancer drug that has been intensively examined for use in clinical cancer therapy.59 5-Fluorouracil and its analogues have been shown to be effective for the treatment of human cancers.51,94 Irinotecan is a key drug in the chemotherapy of colorectal cancer, and along with its main active metabolite, SN-38, it inhibits DNA topoisomerase I.51

Vitamin C (l-ascorbic acid) is a water-soluble vitamin present in food. It is an essential micronutrient for human body because it plays a role in the synthesis of collagen, l-carnitine, and various neurotransmitters, in addition to being involved in protein metabolism.67 Several studies have reported the effects of high-dose vitamin C administration on the QOL and survival of cancer patients.69 On the contrary, it has also been reported that vitamin C does not show an antitumor effect in advanced colorectal cancer.10 Vitamin C shows its antitumor effect by exerting direct cytotoxicity through the production of hydrogen peroxide,13 suppression of gene expression related to cell proliferation,12 and activation of the autophagic pathway.11 However, the mechanism underlying the antitumor effect of high-dose vitamin C against colorectal cancer remains to be elucidated.

Therefore, in this study, we investigated the antitumor effect of vitamin C in a mouse model of colorectal cancer induced by azoxymethane (AOM) and dextran sodium sulfate (DSS); this model is used to evaluate chronic inflammation-associated colorectal cancer (CAC). Because long-term inflammation of the intestinal tract caused by inflammation bowel disease is one of the causes by colorectal cancer, we chose to use this model for the study.14,15 Furthermore, we studied the effect of vitamin C when used in combination with irinotecan.

MATERIALS AND METHODS

Animals Male hairless mice (HR-1) aged seven weeks were purchased from Japan SLC, Inc. (Hamamatsu, Japan). 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. The study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Suzuka University of Medical Science.

Research Design At seven weeks of age, the mice were divided into five groups (n=5/group): Group 1 was the control group; Group 2 comprised animals with colorectal cancer induced by AOM and DSS; Group 3 included animals that were administered irinotecan (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) after cancer induction by AOM and DSS; Group 4 consisted of animals that were administered vitamin C (Fuso Pharmaceutical Industries Ltd., Osaka, Japan) after cancer induction by AOM and DSS; Group 5 comprised animals that were administered irinotecan and vitamin C two...
weeks after colorectal cancer induction.

Colorectal cancer was induced using AOM (Sigma Chemical Co., St. Louis, MO, U.S.A.) and DSS (MP Biomedicals, U.S.A.). The groups treated with AOM and DSS received a single intraperitoneal injection of AOM (10 mg/kg). At one week after AOM injection, the animals were given 2% DSS in drinking water for one week with no further treatment for 18 weeks as described previously. After the administration of 2% DSS for one week, we started administering irinotecan and/or vitamin C. According to the standard therapy for colorectal cancer, 60 mg/kg irinotecan was intraperitoneally administered for three weeks once per week and was stopped for two weeks thereafter. Four such courses were performed during the experimental period. Vitamin C (100 mg/mouse) was orally administered five times per week. This treatment schedule is shown in Fig. 1. On the final day of the study, all the mice were euthanized using pentobarbital. Cardiac puncture was immediately performed and 1 mL of blood was collected from all mice. The colon tissue was separated and fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for the histological analysis and was stored in liquid nitrogen for the Western blot analysis.

Staining of Sections The fixed tissue specimens were embedded in Tissue-Tek and cut into 5-µm-thick sections. The sections were stained with hematoxylin and eosin according to a protocol established for histopathological analysis. The other embedded specimens were used to analyze neutrophils and apoptosis. The specimens were washed with PBS and incubated overnight at 4°C with Ly-6C, a neutrophil-specific antigen (100:1; Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). After washing with PBS, the specimens were incubated with a fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (1:30; Dako Cytomation, Glostrup, Denmark) for 2 h at 25±2°C.

Apoptosis was analyzed by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) using an in situ apoptosis detection kit (TaKaRa, Shiga, Japan). Neutrophils and TUNEL-positive cells were evaluated immunopathologically by fluorescence microscopy.

Neutrophil counts were analyzed using the image-processing software, Image J (National Institutes of Health, Bethesda, MD, U.S.A.).

Quantification of the Plasma Level of ROS and IL-6 Blood samples were obtained from the mice on the final day of examination. The plasma IL-6 level was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, U.S.A.) in accordance with the instructions of the manufacturer. The plasma ROS level was determined using an OxiSelect™ In Vitro ROS/RNS Assay kit (Cell Biolabs, Inc., San Diego, CA, U.S.A.) in accordance with the instructions of the manufacturer.

Western Blotting The colon tissue was homogenized with lysis buffer (Kurabo, Osaka, Japan) and centrifuged at 8000×g for 10 min. The supernatant was separated from each sample and stored at −80°C until further analysis. After thawing, the protein sample (12.5 µg/lane) was loaded on 4–12% BIS-TRIS blot gel (Life Technologies, Carlsbad, CA, U.S.A.) and electrophoresed at 200 V for 18 min. After electrophoresis, the proteins were transferred on to a nitrocellulose membrane using the iBlot Western blotting system (Life Technologies). The membranes were blocked with 5% skim milk overnight at 4°C. After blocking, the membranes were incubated with the primary antibody for collagen type I (1:1000; EMD Chemicals Inc., Gibbstown, NJ, U.S.A.) and rabbit anti-caspase-1 antibody (Epitonics, Burlingame, CA, U.S.A.) or β-actin antibody (1:5000; Sigma) at 25°C. Subsequently, the immune complexes on the membrane were treated with horseradish peroxidase-conjugated secondary antibody (Life Technologies) and detected using ImmunoStar Zeta regent (Wako). The images were acquired using the Multi-Gauge software (FUJIFILM, SC, U.S.A.).

Statistical Analysis All data are presented as mean±standard deviation. Differences between groups were evaluated via the one-way ANOVA followed by Tukey’s post hoc test using the SPSS v.20 software (SPSS Inc., Chicago, IL, U.S.A.), and difference with a p value <0.05 was defined as statistically significant.
RESULTS

Effect of High-Dose Vitamin C Administration on Colorectal Cancer Induced by AOM and DSS Treatment

Vitamin C was administered daily for 5d and its administration was stopped for 2d. After 20 weeks of treatment, the colorectal cancer was observed microscopically. Compared with that of the group treated with AOM and DSS only, the groups treated with irinotecan, vitamin C, or irinotecan and vitamin C showed an improvement in the symptoms of colorectal cancer. Moreover, the combination of irinotecan and vitamin C showed the maximum antitumor effect. The values are shown as mean±standard deviation. Tukey’s test was used to compare the groups. *p<0.05. AOM: azoxymethane; DSS: dextran sodium sulfate. (Color figure can be accessed in the online version.)

Effect of High-Dose Vitamin C Administration on the Plasma Level of ROS and IL-6 in the Mice Treated with AOM and DSS

The plasma level of ROS and IL-6 increased in the mice treated with AOM and DSS. The administration of irinotecan, vitamin C, and especially irinotecan and vitamin C, caused the maximum decrease in the plasma level of ROS and IL-6 (Figs. 3A, B).

Effect of High-Dose Vitamin C Administration on the Expression of Collagen Type I in the Colon of the Mice Treated with AOM and DSS

We examined the expression of collagen type I, which forms the supporting tissue of the colon. The mice treated with AOM and DSS had a significantly lower expression of collagen type I than that in the control mice. The expression of collagen type I increased after the administration of irinotecan, vitamin C, or irinotecan and vitamin C. In addition, the increase was the highest in the mice treated with irinotecan and vitamin C (Fig. 4).
Effect of High-Dose Vitamin C Administration on the Expression of Caspase-1 in the Colon of the Mice Treated with AOM and DSS

Further, we examined the expression of caspase-1 in the colon. The control mice and the mice treated with AOM and DSS exhibited low expression of caspase-1. The expression of caspase-1 increased after the administration of irinotecan, vitamin C, or irinotecan and vitamin C. The increase was the highest in the mice treated with irinotecan and vitamin C (Fig. 5).

Effect of High-Dose Vitamin C Administration on Neutrophils in the Colon of the Mice Treated with AOM and DSS

We examined the neutrophil count in the colon. Compared with that of the control mice, the mice treated with AOM and DSS showed increased neutrophil count. In particular, the mice treated with irinotecan and vitamin C showed a significant increase in neutrophil count (Fig. 6).

Effect of High-Dose Vitamin C Administration on TUNEL-Positive Cells in the Colon of the Mice Treated with AOM and DSS

We examined TUNEL-positive cells in the colon. The count of TUNEL-positive cells increased after the administration of irinotecan, vitamin C, or irinotecan and vitamin C. Further, the increase in TUNEL-positive cell count was greater in the mice treated with vitamin C and those treated with irinotecan and vitamin C (Fig. 7).

DISCUSSION

In this study, we found that high-dose vitamin C administration has antitumor effect on mice with colorectal cancer induced by AOM and DSS. In addition, the antitumor effect of vitamin C was reinforced with irinotecan. In the mice treated with high-dose vitamin C, the plasma level of IL-6 and ROS decreased and the expression of caspase-1 and collagen type I and count of TUNEL-positive cells increased. There are
various reports on the antitumor effect of vitamin C in vitro and in vivo and on the underlying mechanism. An examination of human Burkitt’s lymphoma cells showed that the antioxidant action of vitamin C induced lymphoma cell death in a concentration-dependent manner. In a human colon cancer cell line, vitamin C exhibited inhibitory effect on cancer cell proliferation, as its mechanism, signal transduction of ROS, is inhibited by the antioxidant action of vitamin C. Furthermore, it suppressed the expression of various genes. In prostate cancer cell lines, the suppression of cell proliferation by autophagy induction was found in addition to the antioxidant action of vitamin C. Vitamin C administration in mouse models of prostate and ovarian cancers caused the suppression of tumor growth and metastasis. In addition, the administration of vitamin C with the combination of carboplatin and paclitaxel had a synergistic suppressive effect on the proliferation of ovarian cancer cells. Thus, vitamin C has antioxidant effect on various cancers.

Furthermore, we examined the anticancer effect of the ROS scavenger N-acetyl cysteine in the mice treated with AOM and DSS and found negligible anticancer effect (data not shown). Therefore, in the mice with colorectal cancer induced by the...
treatment with AOM and DSS, the antitumor mechanism may not involve antioxidant action. We further examined the mechanisms, except antioxidant action.

In clinical practice, cyclooxygenase-2 (COX-2) inhibitors or aspirin have been shown to inhibit inflammation and prevent the development of colorectal cancer by modulating the prostaglandin (PG) pathway. Detailed regulatory mechanisms have not been elucidated, but in a mouse model of CAC induced by AOM and DSS, PG-prostaglandin E receptor 2 (EP2) signaling has been shown to suppress the development of colorectal cancer. In this study, we have not studied the inhibitory effect of vitamin C on the PG-EP2 pathway. However, unlike the results of other known studies, the neutrophil count increased here, suggesting that it may be affected by inflammatory cytokines other than IL-6. It is thus necessary to further study this point in the future.

We previously reported that the expression of collagen type I decreased in the skin of the mice with colorectal cancer induced by the treatment with AOM and DSS. In this study, the reduction in collagen type I expression in the colon was suppressed by vitamin C administration. It is known that collagen is a major insoluble fibrous protein in the extracellular matrix and connective tissue, and a deficiency of vitamin C makes the tissues such as blood vessels and the skin vulnerable. In addition, collagen is involved in the invasiveness and metastasis of cancer. In this study, the decrease in collagen type I expression due to colon cancer was inhibited by vitamin C administration; therefore, the structure and function of intestinal mucosa might be maintained, and antitumor effect might have been induced.

Furthermore, in this study, we found that vitamin C administration increased the neutrophil count and expression of caspase-1 in colon tissue. Thus, vitamin C treatment increases the count of neutrophils, which secrete active oxygen. Active oxygen activates the nucleotide-binding domain leucine-rich-containing family and pyrin domain-containing (NLRP) 3 inflammasome and produces caspase-1. Caspase-1 induces tumor cell apoptosis, which is considered indicative of antitumor effect. Thus, the antitumor effect of vitamin C may be involved not only in antioxidant action, but also in the retention of colon-supporting tissue by collagen and induction of apoptosis by caspase-1. Wang et al. have reported that neutrophils promote IL6 production and induce tumorigenesis. Our results may be different because of the difference in administration of AOM and DSS. However, the mechanism underlying the increase in neutrophil count due to vitamin C treatment is not known.

In a mouse model of CAC induced by AOM and DSS, in addition, the relation between collagen type I and colorectal cancer is not clear, and future studies are necessary to elucidate the antitumor effect of vitamin C. In this study, the combination of vitamin C and irinotecan showed remarkable effects. Irinotecan inhibits DNA synthesis by inhibiting DNA topoisomerase I and shows an antitumor effect. As high-dose vitamin C exhibits antioxidant effect unlike irinotecan, it appears that it showed a synergistic effect with irinotecan. The results of our study suggest that high-dose vitamin C administration along with chemotherapy in colorectal cancer patients can improve the survival of patients and their QOL. In addition, although anticancer drugs and molecular therapeutic agents are currently used in combination to treat colon cancer, the combined use of these drugs and vitamin C should be an option in the future.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


