Vitamin C increases the apoptosis via up-regulation p53 during cisplatin treatment in human colon cancer cells

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INTRODUCTION
Colorectal cancer is one of the leading causes of cancer-related deaths in Western countries (1) such as North America (2), and also Korea (3). Despite recent improvements in the treatment of colorectal cancer, the response rate still remains low (4). A key factor in the response of cancer cells to chemotherapeutic drugs is the activation of the apoptotic pathway, which is often impaired in chemoresistant colon cancer cells (5). Many chemotherapeutic drugs including cisplatin induce cytotoxic effects in cancer cells mainly through programmed cell death or apoptosis (6), which is characterized by membrane blebbing, shrinking of cells and their organelles, DNA fragmentation, and finally cell disintegration (7). Cisplatin, a potent DNA damaging agent used in the treatment of several cancers, also exerts its cytotoxic action by induction of apoptosis (8).

The p53 tumor suppressor gene is a nuclear phosphoprotein that serves as a critical regulator of cell survival and proliferation. Its expression level increases in response to DNA damage, and subsequent cell cycle prolongation permits DNA repair or, in severe cases, leads to apoptosis (9). In recent years, cancer therapies using radiotherapy, chemotherapy, and hyperthermia, which can induce p53-mediated apoptosis, have received attention.

Several epidemiological and laboratory studies suggest a relationship between large bowel cancer risk and dietary factors (10-12). Although risk reduction by nutritional intervention may not be sufficient to protect high-risk individuals against colon cancer development, an alternative or complementary effective approach for secondary prevention has been to identify agents with chemopreventive potency and evaluate them in high-risk individuals in combination with nutritional intervention (13-15). Previous studies have reported that vitamin C (VC) protects cells from oxidative stress, which can induce cancer and aging via DNA damage (16-18). Cho et al. (19) and Lee (20) reported the anti-tumor activities of VC in B16F10 murine melanoma cells as well as LNCaP and DU-145 prostate cancer cells, respectively. In addition, it has been reported that VC induces apoptosis of prostate cancer cells (20) and murine melanoma cells in a time-and dose-dependent manner (21, 22). However, the molecular mechanisms of VC in colon cancer cells have not been fully elucidated.

This study was designed to investigate the expression level of p53 involved in the effect of VC on CDDP-induced apoptosis of HCT116, a human colon cancer cell line.

RESULTS AND DISCUSSION
CDDP-induced growth inhibition is potentiated by VC
Cisplatin (CDDP) is a widely used chemotherapeutic agent, but it induces major side effects in normal tissues and organs (23). Previous data indicated that CDDP exerts its cytotoxicity via the formation of mono-, inter-, and intrastrand CDDP-DNA adducts, which can ultimately result in cell cycle arrest at G1, S or G2M phase and in apoptosis (24). Some of DNA damage, such as clastogenesis, can be decreased by pretreating cells with the antioxidants vitamins C and E, which prevent oxida-
Vitamin C increases the apoptosis in colon cancer
Sung Ho An, et al.

Fig. 1. (A) Percentage of surviving HCT116 cells after treatment with CDDP, alone or in combination with VC. HCT-116 cells were treated with (1) 0, (2) 100.0 μg/ml of VC, (3) 0.1 μg/ml of CDDP (4) 0.1 μg/ml of CDDP + 100 μg/ml of VC (5) 1.0 μg/ml of CDDP (6) 1.0 μg/ml of CDDP + 100 μg/ml of VC and incubated for 24 hours, after which cell viability was assessed as described in Materials and Methods. All determinations were made in triplicate. *P < 0.05 compared with control, +P < 0.05 compared with CDDP to CDDP + VC. Experiments were repeated three times. (B) VC augments CDDP-induced DNA fragmentation. HCT-116 grown in 100-mm dishes were treated with 0.1, 1.0, and 10.0 μg/ml of CDDP with and without 100.0 μg/ml of VC for 24 hours. In HCT-116 cells, the percentages of control cells after treatment with 100 μg/ml of VC or 0.1, 1.0, and 10.0 μg/ml of CDDP were 85.0 ± 3.6%, 79.1 ± 4.2%, 64.2 ± 3.5%, and 44.9 ± 2.4%, respectively. Cell viability decreased gradually upon CDDP treatment in a dose-dependent manner. On the other hand, combinations of 0.1, 1.0, and 10.0 μg/ml of CDDP with 100 μg/ml of VC resulted in 68.2 ± 3.8%, 46.8 ± 1.9%, and 38.2 ± 1.2%, respectively. Therefore, the combined treatment of 100 μg/ml of VC with CDDP significantly decreased the cell viability compared to CDDP treatment alone (P < 0.05) (Fig. 1A).

This result indicates that VC augmented the chemotherapeutic response of colon cancer HCT-116 cells.

VC potentiates CDDP-induced cell damage and DNA fragmentation in HCT-116 cells

Apoptosis has been shown to be a significant mode of cell study, treatment with various concentrations of VC resulted in decreased cell growth in a dose-dependent manner; specifically, a dose of 100 μg/ml of VC effectively decreased cell growth (data not shown) and was thus used for all further experiments. We examined the effect of VC during cisplatin-induced apoptosis in the human colon cancer cell line HCT-116. The antiproliferative effects of CDDP and/or VC on HCT-116 cells were determined by measuring the cell count with trypan blue staining. Cells were exposed to varying concentrations of CDDP (0.1, 1.0, and 10.0 μg/ml) and/or VC (100 μg/ml) for 24 hours. In HCT-116 cells, the percentages of control cells after treatment with 100 μg/ml of VC or 0.1, 1.0, and 10.0 μg/ml of CDDP were 85.0 ± 3.6%, 79.1 ± 4.2%, 64.2 ± 3.5%, and 44.9 ± 2.4%, respectively. Cell viability decreased gradually upon CDDP treatment in a dose-dependent manner. On the other hand, combinations of 0.1, 1.0, and 10.0 μg/ml of CDDP with 100 μg/ml of VC resulted in 68.2 ± 3.8%, 46.8 ± 1.9%, and 38.2 ± 1.2%, respectively. Therefore, the combined treatment of 100 μg/ml of VC with CDDP significantly decreased the cell viability compared to CDDP treatment alone (P < 0.05) (Fig. 1A).

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Vitamin C increases the apoptosis in colon cancer
Sung Ho An, et al.

Fig. 3. Cell cycle analysis of HCT116 cells following CDDP and/or VC treatment for 24 hours. HCT116 cells were treated with 0, 100.0 μg/ml of VC, 1.0 μg/ml of CDDP, 1.0 μg/ml of CDDP + 100.0 μg/ml of VC, and stained with PI.

Blockage of cell cycle progression
CDDP, the single most active drug against cervical cancer but not colon cancer, has a maximum additive effect in vitro on
death that is induced by radiation or cytotoxic drugs in a variety of cancer types (28). The degradation of DNA into multiple internucleosomal fragments consisting of 180-200 bp is a distinct biochemical hallmark of apoptosis (29). Internucleosomal DNAase activity in apoptotic cells was studied by DNA fragmentation assay. To investigate whether or not cancer cell death caused by CDDP and VC was due to induction of apoptosis, HCT116 cells were treated with different concentrations of CDDP and/or VC for 24 hours, after which DNA was isolated. Fig. 1B. shows the results of agarose gel electrophoresis of DNA extracted from the cells. Cisplatin induced a 180 bp DNA ladder pattern, characteristic of apoptosis, in a concentration-dependent manner (Fig. 1B: Lane 2-4). A more pronounced DNA ladder was observed in the presence of 100 μg/ml of VC (Fig. 1B: Lane 5-7), indicating enhanced apoptosis.

VC induces morphological features of apoptosis
Morphologically, apoptosis is characterized by chromatin condensation, reduction of cell volume, membrane blebbing, and collapse of the nucleus into small intact fragments (30). In most cells, these alterations seem to be associated with the cleavage of genomic DNA into large fragments (31) and later into oligonucleosomal fragments by a nuclease that is exclusively activated in apoptosis (29). To check whether or not VC plays any role in the regulation of apoptotic morphology, colon cancer cells were treated with 100 μg/ml of VC and/or 1.0 μg/ml of CDDP for 24 hours. When untreated, all of the cells showed normal features (Fig. 2A, C), whereas the growth of VC-treated cells was distinctly inhibited (Fig. 2B, arrow). VC more clearly induced extensive nuclear condensation and fragmentation, whereas the apoptotic bodies were present in CDDP-treated HCT116 cells, as evident by transmission electron microscopy (Fig. 2D). Apoptotic bodies were observed to be comparatively higher in number in cells treated with the combined treatment as compared to the individual treatments (data not shown).

Fig. 4. Effect of VC on expression of p53 protein in HCT-116 cells. p53 protein expression in HCT116 cells treated with CDDP and/or VC for 24 hours. Lane 1: untreated control, Lane 2: 100.0 μg/ml of VC, Lane 3: 0.1 μg/ml of CDDP + 100.0 μg/ml of VC, Lane 4: 1.0 μg/ml of CDDP + 100.0 μg/ml of VC, Lane 5: 1.0 μg/ml of CDDP + 100.0 μg/ml of VC, Lane 6: 0.1 μg/ml of CDDP, Lane 7: 1.0 μg/ml of CDDP, Lane 8: 10.0 μg/ml of CDDP. The results shown are mean ± SD of three individual experiments.
VC pretreatment and also significantly increases apoptosis (32). To determine the percentage of apoptotic cell death, colon cancer cells were cultured for 24 hours in the presence of VC and/or CDDP and subjected to cell cycle analysis by quantifying the apoptotic cell number in the sub-G1 phase, apoptotic region (A0). As shown in Fig. 3, flow cytometric analysis showed that the percentage of apoptosis increased from 4.3% in untreated control to 15.2%, 28.6%, and 47.1% after VC, CDDP, VC plus CDDP treatment, respectively. Combined treatment with 1.0 μg/ml of CDDP and 100 μg/ml of VC for 24 hours resulted in a significant increase in apoptotic cell population compared to the same concentration of CDDP alone. Therefore, these results indicate that VC had the same effect on CDDP-induced apoptosis.

**Vitamin C upregulates expression of p53**

The p53 tumor suppressor gene is a key regulator of DNA repair and cell replication. The p53 family of proteins plays a central role in apoptosis by acting as stress sensors of the cell and triggering the activation of various pro-apoptotic genes. The genetic status of the p53 gene is considered an important candidate for prognosis-predictive assays of cancer (33, 34). Previous studies have reported that VC augments the chemotherapeutic response (CDDP and etoposide) of cervical carcinoma HeLa cells by stabilizing p53 (32), and the level of p53 protein is significantly increased in VC-treated melanoma cells (26), although pretreatment with VC in human fibroblasts does not induce p53 significantly after chromium treatment (35).

In this study, to confirm the effect of VC on expression of p53, Western blot analysis was performed after HCT-116 cells were treated with CDDP doses ranging from 0.1 μg/ml to 10 μg/ml and/or various concentrations of VC for 24 hours. There was an increase in expression of p53 protein upon 100 μg/ml of VC. As shown in Fig. 4, combined treatments of 1.0 and 10.0 μg/ml of CDDP with 100 μg/ml of VC (lane 4-5) markedly induced p53 expression compared to untreated control (lane 1), VC (lane 2), and CDDP control (lane 7-8). Prominent p53 bands were observed in the lanes containing extracts of 10.0 μg/ml of CDDP plus 100 μg/ml of VC. Therefore, our data suggest that VC increases accumulation of p53. Similar observations have been made in other cancer cells, such as B16F10 murine melanoma cells (26), but not colon cancer cells.

Taken together, the results of the present study confirm that a combination of CDDP and 100 μg/ml of VC makes HCT-116 cells more susceptible to apoptosis. This finding strongly supports a new strategy for colon cancer chemotherapy. Combined therapy with CDDP and VC is expected to be effective against colon cancer, although prospective studies are needed to verify its predictive value in chemotherapy for colon cancer patients.

**MATERIALS AND METHODS**

**Materials**

Fetal bovine serum (FBS), Dulbecco’s modified eagle’s medium (DMEM), streptomycin, penicillin, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Gibco BRL, Daejeon Korea. Vitamin C (VC) and general reagents were purchased from Sigma Aldrich, Seoul Korea. The remaining chemicals were purchased from a local company and were of highest purity grade.

**Cell culture and drug treatment**

Human colon cancer cells (HCT116) were obtained from Seoul National university and maintained in DMEM containing 10% fetal bovine serum, 5% L-glutamate, 0.5% sodium pyruvate, and antibiotics (100 μg/ml of streptomycin and 50 U/ml of penicillin) in a humidified atmosphere of 5% CO2 in air at 37°C. Cell were trypsinized and sub-cultured at a 1:3 ratio for routine maintenance and experiments. Cells were plated to a density of 3 x 105 cells per 100 mm plate. An equal number of plates were treated with different concentrations 0.1, 1.0, and 10.0 μg/ml of CDDP and/or 100 μg/ml of VC in culture media. Cells were collected from each plate after 24 hours of incubation for all experiments. Each experiment was repeated three times to confirm reproducibility.

**Dye exclusion assay**

After 24 hours of treatment, cells were harvested with trypsin-EDTA and washed once with phosphate-buffered saline (PBS). Cell number and viability were determined by the trypan blue dye exclusion test.

**Isolation of apoptotic DNA**

HCT-116 cells grown in 100 mm dishes at 80% confluency were treated with or without 100 μg/ml of VC and 0.1, 1, and 10 μg/ml of CDDP for 24 hours. After treatment, all cells were harvested, washed with PBS, and pelleted by centrifugation. The pellets were treated with lysis buffer (50 mM Tris-HCl, pH 7.5 with 1% NP-40 and 20 mM EDTA) and centrifuged for 5 minutes at 1,600 g. The supernatants were collected and the extraction repeated. Combined supernatants were brought to 1% SDS and treated for 2 hours with 5 μg/ml of RNase A at 56°C, followed by digestion for 2 hours with 2.5 μg/ml of protease K at 37°C. After addition of two volumes of 10 M ammonium acetate, DNA was precipitated with 2.5 volumes of ethanol, dissolved in TE buffer, and quantitated by reading OD at 260 nm. Equivalent amounts of DNA from the different treatments were separated by electrophoresis in 1.5% agarose gel with ethidium bromide. The gel was photographed under UV illumination.

**Transmission electron microscopy (TEM)**

The cells were prepared as above, fixed in 2% formaldehyde,
and then washed in 0.1 M PBS. Cells were then fixed with 2.5% glutaraldehyde (0.1 M PBS, pH 7.4). treated with 1% osmium tetroxide (0.1 M PBS, pH 7.4), dehydrated in ethanol, and finally embedded in epoxy resin. Thin sections (60 nm thick) were sliced and then double-stained with uranyl acetate and lead citrate. The sections were examined under a JEM-2000FX electron microscope (JEOL, Tokyo, Japan)

**DNA cell cycle analysis**
The cells were treated with 0.1, 1.0, and 10.0 μg/ml of CDDP and/or 100 μg/ml of VC in media for 24 hours. The cells were then trypsinized, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 1 ml of cold PBS and 4 ml of cold ethanol for 30 minutes at 4°C. The cells were then centrifuged at 1,500 rpm for 5 minutes, after which the pellet was washed twice with cold PBS and incubated in staining solution (20 μg/ml of propidium iodide, 50 μg/ml of RNase, 0.1% Triton X-100, and 0.1 mM EDTA) for 2 hours at 4°C in the dark. The DNA content of the cells was measured using a flow cytometer (Becton Dickinson, FACS Calibur) using Diva software.

**Western blot analysis**
Cells were washed twice in PBS and lysed in lysis buffer. Protein concentrations were determined using a spectrophotometer. Equal amounts of protein were loaded and run on a SDS-PAGE gel, after which the separated proteins were transferred to a nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk in TBS with tween-20 (TBS-T) for 2 hours to a nitrocellulose membrane. The membrane was then blocked with secondary antibody, goat anti-rabbit conjugated with horseradish peroxidase diluted 1:1,000 dilutions for 1 hour. Membranes were subsequently incubated with secondary antibody, goat anti-rabbit conjugated with horseradish peroxidase diluted 1:5,000 for 1 hour. The bands were analyzed and quantified using an image scanner densitometer (Alpha innotech, USA) and normalized to an actin control. The density of the control was taken as 1, and results of the treatment are expressed in relative units (RU) compared to control.

**Statistical analysis**
Statistical analysis was performed using Student’s t-test to determine significant differences between the treatment groups. P values < 0.05 indicate statistically significant differences.

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**REFERENCES**


Vitamin C increases the apoptosis in colon cancer
Sung Ho An, et al.


