Original Research Article

Antiproliferative and apoptotic effects of high-dose vitamin C in cholangiocarcinoma cell line

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Abstract

Purpose: To evaluate the antiproliferative and apoptotic effects of high-dose vitamin C in cholangiocarcinoma cell line (CCA).

Methods: Sulforhodamine B colorimetric assay was used to determine cell proliferation in human K100-CCA cell lines. The expression of anti-apoptotic Bcl-2 protein/ pro-apoptotic Bax protein was evaluated by Western blot analysis.

Results: Ascorbic acid inhibited the growth of CCA in a dose-dependent manner. The half-maximal inhibitory concentration (IC50) of ascorbic acid on K100 at 24 h was 55 ± 9.4 mM. It was observed that treatment with ascorbic acid resulted in the reduction of glutathione and increased hydrogen peroxide contents in the cells. Moreover, disturbance of mitochondrial membrane potential occurred in ascorbic acid-treated cells in a dose-dependence manner. The ratio of Bcl2/Bax decreased in CCA treated with ascorbic acid.

Conclusion: The results show that the anti-proliferation effect of ascorbic acid in CCA may be attributable to the modulation of expressions of Bcl-2 and Bax proteins and dissipation of the mitochondrial electrochemical potential gradient, which is an early event leading to apoptosis.

Keywords: Vitamin C, Cholangiocarcinoma, Apoptosis, Glutathione, Mitochondrial membrane potential

INTRODUCTION

Cholangiocarcinoma (CCA) is a rare form of cancerous tumour that originates from the biliary epithelial cells. It is prevalent in the Southeast Asian region and is associated with symptoms like liver fluke infection (caused due to Opisthorchis viverrini and Clonorchis sinensis). This condition leads to other secondary symptoms like cell injury, prolonged inflammation, and reparative biliary epithelial cell proliferation. Due to prolonged inflammation, a local environment is created with the dominance of cytokines and other growth [1] Currently, radical surgery is considered to be the most preferred therapy for CCA as it has high curative potential. However, in most cases, the patients are referred for surgical intervention at an advanced stage of the disease, due to which the surgery may not give the predicted outcome [2]. In addition to surgery, chemotherapy is considered to be another approach for treating...
CCA in inoperable patients. However, the currently-used chemotherapeutic drugs have not shown satisfied results in terms of survival benefit [3]. Hence, there is a need to develop new and advanced chemotherapeutic drugs aiming at high rates of survival with low side effects.

The use of ascorbic acid as a potent chemotherapy agent in treating cancer and its effect in minimising the disease symptoms has a controversial history [4]. Several studies have reported using a high dose of ascorbic acid for treating cancer [5-9]. Furthermore, studies have shown that ascorbic acid treatment led to enhanced prolongation and quality of life. However, later studies showed its disadvantages in treating cancer patients [10,11]. In a previous study, a high dose of pharmacological ascorbic acid concentration was found to induce in vitro cell death and in vivo tumour growth inhibition [12].

Hydrogen peroxide (H2O2) is an oxidative molecule involved in many immune reactions of the body. Hence, high-dose ascorbic acid treatment leads to increased production of H2O2 in human connective tissues. In case of healthy cells, intracellular quenching of H2O2 takes place through natural anti-oxidants present in the cells. However, the cancer cells lack sufficient levels of anti-oxidants to quench H2O2, leading to its accumulation. This causes a condition of increased oxidative stress, and the cancer cells eventually undergo apoptosis [13-15].

Only a few research works have focused on the effect of ascorbic acid on CCA cell line. Thus, the present study aimed at evaluating the antiproliferative and apoptotic effects of high-dose vitamin C or ascorbic acid treatment on K100 CCA cell line.

**EXPERIMENTAL**

**Cell culture**

The study included the human CCA cell line K100 cells, which were provided by Dr. Banchob Sripa of the Department of Pathology, Faculty of Medicine, Khon Kaen University. K100 cells were routinely cultured in Ham's F12 media supplemented with 12.5 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid, pH maintained at 7.3, with 100 U/mL penicillin, 100 unit/mL streptomycin sulphate, and 10 % foetal calf serum. The culture was maintained under an atmosphere of 5 % CO2 at 37 °C [16]. The cells were subcultured every 2-3 days before the cells reached 80% confluency using 0.25 % trypsin–EDTA, and the medium was renewed after an overnight incubation.

**Sulforhodamine B assay**

A 96-well culture plate was used to seed the cultured CCA cells at a density of 5 × 10^3 cells/well. After overnight culture, the cells were pre-treated with ascorbic acid (0.5 - 100 mM) for 24 h. Cell proliferation was determined by sulforhodamine B (SRB) colorimetric assay. Briefly, the cells were washed with phosphate buffer saline (PBS) and fixed with 10 % (w/v) trichloroacetic acid and stained with 0.4 % SRB in 1 % acetic acid for 30 min. The cells were washed with 1 % (v/v) acetic acid to remove excess dye. The protein-bound dye was dissolved in 10 mM Tris base solution (pH 10.5) and incubated for 20 min. A microreader plate was used to determine the absorbance. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. The IC50 concentration (50% inhibition of cell growth) was calculated from the dose-response curves. The experiment was performed in triplicates.

**Measurement of glutathione (GSH) and hydrogen peroxide (H2O2) contents**

The cells were treated with 50 mM ascorbic acid for 24 h. Glutathione (GSH) and H2O2 activities were assessed with GSH assay kit (Item No. 703002, Cayman Chemical Company) and H2O2 assay kit (Item No. ab102500, Abcam, Cambridge, UK).

**Measurement of mitochondrial transmembrane potential**

The dissipation of mitochondrial transmembrane potential leads to an early apoptotic event. To measure the change in ΔΨm, 96 black well plate cell culture, at a density of 10,000 cells/well, was maintained overnight. Then the culture was treated with 50 mM ascorbic acid for 6 h. The assay followed the previously described procedure [17], using the cationic, lipophilic dye, 5,5′,6′-tetrachloro-1,1′,3′,3′ tetrachryl-benzimidazolyl carbocyanine iodide (JC-1) (Clayman Chemical) staining with few modifications. The cultured plate was centrifuged at 1,000 rpm for 5 min at room temperature. The cultured medium was then washed off and loaded with JC-1 dye and left for 20 min for staining. The excess stain was washed by centrifugation, and the culture was again incubated in the assay buffer. Fluorescent microscope was used to analyse ΔΨm with an emission wavelength of 535 nm and
an excitation wavelength of 485 nm. Red fluorescence was observed, which signified J-aggregates formed by JC-1 present in a healthy mitochondrial matrix. In the case of depolarised mitochondria, green fluorescence was observed due to efflux of JC-1 into the cytoplasm, where it exists in the monomeric form. If the fluorescence shifted from red to green, this indicated depolarisation of ΔΨm.

**Western blot analysis of BAX and Bcl-2**

Cells were treated with 50 mM ascorbic acid for 24 h. Then the cells were washed with PBS, lysed with RIPA buffer [150 mMNaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mMTris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4] with the addition of protease inhibitor cocktail (M221: Amresco, OH, USA). Charged separation of the protein samples was done by SDS-PAGE gel electrophoresis (with 8 – 10 % SDS polyacrylamide gel). This electrophoresis process was followed by western blotting. The obtained bands were transferred onto a PVDF membrane. The membranes were blocked with 5 % (w/v) skimmed milk powder in Tris-buffered saline (TBS) containing 0.1 % Tween-20 at room temperature for 1 h. The PVDF membranes were incubated with primary antibody in PBS at 4 °C overnight. After washing with PBS, the blots were incubated with the HRP-conjugated secondary antibodies at room temperature for 1 h. After washing away the unbound antibodies, the blots were incubated in ECL substrate solution (Super Signal West Pico Chemiluminescent Substrate: ThermoScientific, IL, USA). The densities of the specific protein bands were visualised and captured by Image Quant™ 400 (GE HealthCare).

**Statistical analysis**

Data are presented as mean ± SD. Analysis of variance (ANOVA) was used to determine significant differences between each experimental group. An ANOVA on-rank test was also performed for the non-parametric test. The level of significance was set at p < 0.05 using SigmaStat software version 4 (Systat Software, Inc. California, USA).

**RESULTS**

**Effect of ascorbic acid on cancer cell proliferation**

The effect of ascorbic acid on cell proliferation was evaluated by SRB assay in K100 CCA cell line (Figure 1). Based on these measurements, the IC50 value of ascorbic acid on K100 at 24 h was determined to be 55 ± 9.4 mM. The antiproliferation effect of ascorbic acid depended on dose and time. These results confirmed the anti-proliferative effects of ascorbic acid on CCA cell line.

**Effect of ascorbic acid on GSH and \( \text{H}_2\text{O}_2 \) contents**

The basal GSH content was decreased (Figure 2), whereas the \( \text{H}_2\text{O}_2 \) content in cell was increased in cells treated with ascorbic acid (VIT C) compared with control (p < 0.05) (Figure 3).

**Effect of ascorbic acid on dissipation of mitochondrial membrane potential**

Due to the accumulation of J-aggregates in the
Figure 3: Effect of ascorbic acid on H₂O₂ content in CCA. K100 cells were treated with varying concentrations of ascorbic acid (range, 5-50 mM) for 24 h. Values are mean ± SD (n = 3). *Significantly different compared with control (p < 0.05).

Figure 4: Effect of ascorbic acid on mitochondrial membrane potential. K100 was treated with THC for 6 h. ΔΨm change was examined using JC-1 staining method. Fluorescence microscopy was performed with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The experiments were performed in duplicate.

Expression of Bcl2 and BAX

The pro-apoptotic activity of ascorbic acid is attributed to its ability to modulate the expression of Bcl2 and Bax proteins. In the present study, there was a dramatic increase in the expression of Bax protein, whereas a significant decrease in synthesis of Bcl2 protein was marked when the cells were treated with ascorbic acid. The ratio of Bcl2/Bax is shown in Figure 5.

DISCUSSION

We observed that the treatment with ascorbic acid resulted in a reduction of GSH and increased H₂O₂ contents in cells. Although the mechanism and physiological relevance remain to be fully understood, the presence of ascorbic acid leads to the production of H₂O₂ [13,15]. It is well known that H₂O₂ is involved in the redox control of several physiological processes including cell proliferation and apoptosis [13,18].

Since ascorbic acid produced H₂O₂ and causes the reduction of GSH, this imbalance of redox state may disrupt the membrane potential of mitochondria present inside the cells. In our study, we found that disturbance of the mitochondrial membrane potential (ΔΨm) was detectable in ascorbic acid-treated cells.

Apoptosis is regulated and executed by different interplays of many genes responsive to various stimuli. The major apoptotic regulatory genes are Bcl-2 and p53 [20]. The p53 gene induces...
apotosis by direct or indirect modulation of the expressions of Bcl-2 family proteins (Bax and Bcl-2) [21]. The Bcl-2 proteins play a crucial role in controlling apoptosis through the regulation of permeability of the mitochondrial membrane. Bax is a pro-apoptotic protein found in the cytoplasm. Along with other pro-apoptotic proteins, Bax protein promotes apoptosis through the formation of homodimeric and heterodimeric complexes. This results in the formation of channels in the mitochondrial membrane, facilitating the release of cytochrome c and apoptosis-inducing factors (AIFs) into the cytoplasm. In contrast, the anti-apoptotic Bcl-2 protein localises the outer mitochondrial membrane and mediates its anti-apoptotic effects by stabilising the integrity of the mitochondrial membrane [20-23]. In this study, we also found that the pro-apoptotic effect of ascorbic acid is attributed to its ability to modulate the expression of Bcl2 and Bax proteins.

CONCLUSION

In conclusion, our results showed that the mechanism of anti-proliferation effect of ascorbic acid in CCA cells may be attributable to the modulatory expression of Bcl-2 and Bax proteins and dissipation of the mitochondrial electrochemical potential gradient, which is an early stage of cell death.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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