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Original Article

SVCT-2 determines the sensitivity to ascorbate-induced cell death in cholangiocarcinoma cell lines and patient derived xenografts

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ABSTRACT

Cholangiocarcinoma (CC) is a devastating malignancy with late diagnosis and poor response to conventional chemotherapy. Recent studies have revealed anti-cancer effect of vitamin C (L-ascorbic acid, ascorbate) in several types of cancer. However, the effect of L-ascorbic acid (AA) in CC remains elusive. Herein, we demonstrated that AA induced cytotoxicity in CC cells by generating intracellular reactive oxygen species (ROS), and subsequently DNA damage, ATP depletion, mTOR pathway inhibition. Moreover, AA worked synergistically with chemotherapeutic agent cisplatin to impair CC cells growth both in vitro and in vivo. Intriguingly, sodium-dependent vitamin C transporter 2 (SVCT-2) expression was inversely correlated with IC50 values of AA. Knockdown of SVCT-2 dramatically alleviated DNA damage, ATP depletion, and inhibition of mTOR pathway induced by AA. Furthermore, SVCT-2 knockdown endowed CC cells with the resistance to AA treatment. Finally, the inhibitory effects of AA were further confirmed in patient-derived CC xenograft models. Thus, our results unravel therapeutic potential of AA alone or in combination with cisplatin for CC. SVCT2 expression level may serve as a positive outcome predictor for AA treatment in CC.

Introduction

Cholangiocarcinoma (CC) is a malignancy deriving from epithelial cells within the biliary tree expressing markers of cholangiocyte differentiation. Surgical treatment is the preferred option for all subtypes of CC including intrahepatic, perihilar, and distal CC according to anatomical location [1]. As for inoperable CC, traditional chemotherapeutic drugs mainly including gemcitabine and cisplatin have limited efficacy and often cause unexpected side effects [2]. Unfortunately, CC still has low survival rates and obvious therapeutic resistance owing to its highly desmoplastic nature, extensive support by rich microenvironment, and incredible genetic heterogeneity [1]. Therefore, it is urgent to improve treatment options and develop novel therapies for CC.

Vitamin C (L-ascorbic acid, ascorbate) has a controversial history in cancer therapy. Ewan Cameron performed clinical trials showing an effective effect of intravenous ascorbate (AA) on improving the survival of patients with terminal cancer [3–5]. However, AA almost has been denied for cancer treatment after subsequent studies in the Mayo Clinic reporting no effect of AA on patient

Abbreviations: CC, cholangiocarcinoma; AA, ascorbic acid; ROS, reactive oxygen species; SVCT, sodium-dependent vitamin C transporter; NAC, N-acetyl-L-cysteine; CI, combination index; H2AX, histone 2AX; AMPK, adenosine monophosphate (AMP)–activated protein kinase; S6K, p70 Ribosomal S6 Kinase; PDX, patient-derived xenografts; PBS, phosphate-buffered saline; HIF, hypoxia-inducible factor.

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survival with oral administration [6,7]. It was recognized later that the route of AA administration might account for the discrepancy in the results from above two studies. The originally reported studies using intravenous AA produces much higher plasma concentrations than the subsequent double-blind placebo-controlled studies using oral AA [8]. More recently, Chen et al. demonstrated that AA selectively induced death in 75% of the 48 cancer cell lines tested, but had no toxic effect on human normal cells including peripheral white blood cells, fibroblasts, and epithelial cells [9–11]. Furthermore, high-dose AA intravenously (i.v.) or intraperitoneally (i.p.) administration was found to inhibit the growth of various aggressive cancers in xenografts models including glioblastoma, pancreatic cancer, ovarian cancer, colon cancer, and breast cancer [12–17]. These observations have refocused attention on AA treatment for cancer. However, the mechanisms determining the sensitivity of AA-induced cell death in cancer cells remains to be uncovered.

AA is transported into cells by sodium-dependent vitamin C transporters (SVCTs) [18]. SVCT-2 exhibits higher affinity for L-ascorbic acid than SVCT-1 [18] and SVCT-2 plays important role in AA uptake in the liver [19]. So we hypothesize that different expression levels of SVCT-2 may be responsible for the differential sensitivity of CC cells to AA-induced cell death. Moreover, we investigate the mechanisms underlying AA action on CC cells and the combine effect of AA with the conventional chemotherapeutic regimen cisplatin.

Materials and methods

Cell culture and reagents

The CC cell lines CCLP-1, TFK-1, RBE, and HCCC-9810 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and the HuCCT cell line was kindly provided by professor Zhang, Wuhan University. All cell lines were maintained with RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA) and 100 mg/ml penicillin/streptomycin in a 5% CO_2 incubator at 37 °C. L-ascorbate, NAC, and cisplatin were purchased from Sigma (USA). Olaparib was purchased from Selleck (USA). Oligomycin was purchased from Aladdin Bio-Chem Technology (China).

Cell viability assay and colony formation assay

Cell viability assay was performed with the Cell Counting Kit 8 assay (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's protocols. Briefly, ascorbate was added into CC cell lines with a concentration gradient. Optical density (OD) was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm 24 h later.

Inhibitory concentration (IC50) was defined as the dose of that inhibited cell proliferation by 50% relative to the untreated control.

For colony formation assay, cells were plated in 6-well plates in a density of 1000/well. Ascorbate, H_2O_2 and/or NAC was added 4 days later and treated for 24 h. Fourteen days after plating, cells were washed with PBS, fixed with 10% formaldehyde, and stained with 0.05% crystal violet for 15 min at room temperature. The visible colonies were counted manually and representative views were photographed.

ROS level measurements

The cells were treated with 2 mM ascorbate for 1 h and then incubated with 10 μM H2DCF-DA (Molecular Probe, Eugene, OR, USA) for 30 min, washed with PBS for 3 times, trypsinized, and collected. Fluorescence-stained cells were subjected to flow cytometry.

ATP detection

The cells were treated for 0.5, 1.0, 1.5, and 2.0 h with 2 mM ascorbate or PBS. Then, ATP levels were detected with ATP Assay Kit (Beyotime Biotechnology, China) according to manufacturer's protocols.

qRT-PCR

Cell and tissue total RNA was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Then, Trizol prepared RNA was reversely transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, USA) and random primers. SYBR Green-based qRT-PCR was subsequently performed on ABI PRISM 7300HT Sequence Detection System (Applied Biosystems, USA) using cDNA as template. 18s was emplyed as a control for normalization. Primers used in RT-PCR were as follow: SVCT-1, 5′-GCTACCCACAGAGCCTAAGTT-3′ (sense) and 5′-TGACTAACCATGTGCTGGTCG-3′ (antisense); SVCT-2, 5′- CTTCACTCTTCCGGTGGTGAT-3′ (sense) and 5′-TTTCCGTAGTGTAGATCGCCA-3′ (antisense).

Western blot analysis

Total cell proteins (30 μg) were subjected to SDS-PAGE electrophoresis and then transferred to NC membranes. Then, the membranes were blocked with 5% non-fat dry milk in 0.05% Tween-20 (TBS/Tween) for 1 h and incubated with primary antibodies against SVCT-2, phospho-H2AXSer139 (Abclonal Technology, China), PARP, phospho-AMPKThr172, phospho-mTORSer2448, phospho-S6K (Cell Signaling Technology, USA) and β-actin (Cell Signaling Technology, USA) overnight at 4 °C. Subsequently, the membrane was incubated with goat anti-mouse or goat anti-rabbit luorescence-conjugated secondary antibody and scanned with an Odyssey scanner (Li-Cor, Lincoln, NE, USA).

RNA interference

Lentivirus vectors containing short hairpin RNA (shRNA) targeting SVCT-2 (shSVCT-2) and the negative control (shCtrl) were purchased from Genechem Technology (Shanghai, China). The sequences of shSVCT-2 were: sense: 5′-CCUCUCCCGAUUUAUAAAUdTdT-3′; antisense: 5′-AUUUAUAAAUCGGGAGAGGdTdT-3′. Cells were plated at ~50% confluence and transfected with optimal dilutions of lentivirus particles and polybrene. The cells were cultured in DMEM containing puromycin (2 μg/ml) to obtain the stable transfected cells after 48 h transfection.

Immunohistochemistry

Formalin-fixed tumor tissue sections were subjected to two 10-min immersions in xylene, rehydrated through graded alcohols (100%, 100%, 95%, 85%, and 70% alcohols), followed by two 5 min rinses in distilled water. After treated in 3% H_2O_2 for 15 min, the slides were processed for antigen retrieval in 0.05 M citrate buffer (pH 5.6) for 5 min. After blocking with 10% bovine serum for 30 min, the sections were incubated in primary antibodies overnight. Then, the sections were washed 3 times with PBS, followed by incubation in secondary antibodies, and subsequent detection was performed. Finally, the sections were stained with hematoxylin and dehydration in graded alcohols and xylene.

In vivo subcutaneous tumor model

Animal xenograft assays were conducted with 6-week-old male nude mice purchased from Chinese Science Academy (Shanghai, China). Mice were subcutaneously injected with 2×10^6 HuCCT or TFK-1 cells lines resuspended in matrigel on the right flank. Once tumor volume had reached 30 mm^3, treatment commenced with intraperitoneal injection as follows: (i) control, PBS twice daily; (ii) AA, ascorbate at 4 g/kg twice daily; (iii) Cis, cisplatin at 3 mg/kg twice per week; (iv) AA + Cis. Tumor size (length × $width^2$ × 0.5) was measured twice a week. Three to four weeks after treatment, all mice were euthanized and tumors were excised, weighed, and analyzed by immunohistochemistry.

Cholangiocarcinoma patient-derived xenografts (PDX) model

Fresh tumor specimens were procured from previously established PDX models (passage 1) and cutted into small tissue blocks (~50 mm^3). Then, the tumor blocks were engrafted subcutaneously into NOD-SCID mice. After one week, PDXs from patient #1 (N = 12) and patient #2 (N = 10) were treated intraperitoneally twice daily with either ascorbate (4 g/kg) or vehicle (PBS without drug). Tumor size (length × $width^2$ × 0.5) was measured twice a week. Three to four weeks after treatment, all mice were euthanized and tumors were excised, weighed, and analyzed by immunohistochemistry.

Statistical analysis

Statistics were calculated using SPSS 16.0 software (SPSS Inc., USA). The significance of differences between groups was determined by two-tailed student's t test. Pearson's correlation analysis was used to evaluate the correlation between two variables. The data were presented as the mean ± SD. $P < 0.05$ was considered statistically significant.

Study approval

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Ethical Committee of the Second Military Medical University. All clinical samples were approved for analysis by the Ethical Review Committee of the Eastern Hepatobiliary Surgery Hospital with informed patient consent.

Results

Ascorbic acid induces cytotoxicity in cholangiocarcinoma cells

We first determined the effects of AA in pharmacologic concentrations on the survival of CC cells with five cell lines including

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