Free Radical Biology and Medicine 53 (2012) 1607–1615

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Enhanced antitumor activity of vitamin C via p53 in Cancer cells

Jinsun Kim [a], Soon-Duck Lee [a], Boogi Chang [a], Dong-Hoon Jin [b], Sam-Il Jung [a], Mee-Young Park [a], Youngsoo Han [a], Young Yang [a], Keun Il Kim [a], Jong-Seok Lim [a], Young-Sook Kang [c], Myeong-Sok Lee [a,*]

[a] Research Center for Women's Diseases, Department of Biological Sciences, Korea
[b] Institute of Innovative Cancer Research, ASAN Medical Center, Seoul, Korea
[c] College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

ARTICLE INFO

Available online 4 August 2012

Keywords:
Ascorbate
Oxidative stress
p53
Reactive oxygen species
Transcriptional network
Free radicals

ABSTRACT

Ascorbate is an important natural antioxidant that can selectively kill cancer cells at pharmacological concentrations. Despite its benefit, it is quite difficult to predict the antitumor effects of ascorbate, because the relative cytotoxicity of ascorbate differs between cancer cell lines. Therefore, it is essential to examine the basis for this fundamental disagreement. Because p53 is activated by DNA-damaging stress and then regulates various cellular conditions, we hypothesized that p53 can sensitize cancer cells to ascorbate. Using isogenic cancer cells, we observed that the presence of p53 can affect ascorbate cytotoxicity, and also reactivation of p53 can make cancer cells sensitive to ascorbate. p53-dependent enhancement of ascorbate cytotoxicity is caused by increased reactive oxygen species generation via a differentially regulated p53 transcriptional network. We also found that transcriptionally activated p53 was derived from MDM2 ubiquitination by ascorbate and subsequently its signaling network renders cancer cells more susceptible to oxidative stress. Similar to the p53 effect on *in vitro* ascorbate cytotoxicity, inhibition of tumor growth is also stronger in p53-expressing tumors than in p53-deficient ones *in vivo*. This is the first observation that ascorbate cytotoxicity is positively related to p53 expression, activating its transcriptional network to worsen intracellular oxidative stress and consequently enhancing its cytotoxicity. Based on our study, reactivation of p53 may help to achieve more consistent cytotoxic effects of ascorbate in cancer therapies.

Introduction

Ascorbate (also known as vitamin C), which is generally regarded as an antioxidant, has been known as an essential micronutrient and as an unorthodox therapy for cancer treatment. Initial ascorbate treatment of cancer patients conducted by Pauling and Cameron [1] showed conflicting results with regard to efficacy [2,3]. Despite these uncertainties, ascorbate recently has been reevaluated as a potential cancer treatment. For effective cytotoxicity, the ascorbate concentration must reach a pharmacological level that varies depending on the cancer cell line. Emerging evidence indicates that high concentrations of ascorbate can be easily achieved by intravenous or intraperitoneal injection but not by oral dosing [3,4]. Furthermore, pharmacological concentrations of ascorbate selectively kill cancer cells but not normal cells, a characteristic of an ideal cancer drug [5].

It is still questionable, however, to apply ascorbate for clinical uses, because it has been reported that ascorbate has quite different anticancer effects on various cancer cell lines [4,6–8]. These data demonstrate that the concentration of ascorbate that can kill one cancer cell line does not have same effect on others. This relatively different cytotoxicity of ascorbate may have caused inconsistent therapeutic outcomes. Although a major cell death pattern can be shown, it has been reported that ascorbate-mediated cell death shows a mixed pattern between apoptosis, necrosis, and cell cycle arrest [9–11]. Because ascorbate cytotoxicity differs among cancer cell lines, the cell death mechanism can be also different among cell lines. This can make it difficult for researchers or clinics to analyze or anticipate results.

According to many studies, ascorbate at pharmacological concentrations can act as a DNA-damaging stress and pro-oxidant on cancer cells [9,12,13]. Based on these studies, we propose that a factor(s) that regulates DNA-damaging stress and oxidative stress might be extensively involved in ascorbate cytotoxicity. Because it was shown that ascorbate exerts its cytotoxicity via hydrogen peroxide (H_2O_2) generation [9], an element that can modify the balance between oxidants

Abbreviations: NAC, *N*-acetyl-l-cysteine; CHX, cycloheximide; DCFDA, 2′, 7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; ip, intraperitoneal; PBS, isotonic phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription–polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; wt, wild type

* Corresponding author. Fax: +82 2 2077 7257.
E-mail address: mslee@sookmyung.ac.kr (M.-S. Lee).

0891-5849/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.07.079

and antioxidants in cancer cells may be very helpful in explaining the differences in ascorbate cytotoxicity. So we focused on p53, which is a major tumor suppressor and transcription factor that responds to various DNA-damaging stresses and orchestrates the transcription of genes involved in cell cycle arrest, senescence, and apoptosis [14]. It has been shown that cellular oxidative stress can be related to one of several p53-related functions that are oxidative stress-generating phenomena [15]. However, it is still debated as to what mechanisms are involved in influencing p53 to promote intracellular oxidative stress or vice versa.

An ideal anticancer agent should be toxic to malignant cells but only minimally toxic to normal cells. In this regard, ascorbate might be a good cancer therapeutic agent. However, more basic work should be done before using it as a common anticancer drug, because the mechanism of action is not fully understood. Therefore, our study has focused on the conditions providing for a more consistent therapeutic effect of ascorbate. We have examined the role of p53 as a sensitizer for ascorbate cytotoxicity and hypothesize that p53 renders cells more sensitive to ascorbate-induced oxidative stress. We found that the presence of p53 can influence ascorbate cytotoxicity, and reactivation of p53 in cancer cells enhances anticancer activity of ascorbate by increasing reactive oxygen species (ROS) generation via the p53 transcriptional network. Furthermore, ascorbate-mediated p53 activation was achieved by ubiquitination of MDM2 and activation by p38MAPK. Finally, we identified that ascorbate had similar preferential cytotoxicity on wild-type p53-bearing tumors over p53-deficient tumors *in vivo*, which result was similar to the *in vitro* p53 effect on ascorbate cytotoxicity.

Materials and methods

Cell lines

Human cancer cells HCT116$^{+/+}$, MCF7, HeLa, HCT116$^{-/-}$, and SKOV3 were grown in Dulbecco's modified Eagle's medium (WelGene, Seoul, South Korea), and A549 and H1299 cells were maintained in RPMI 1640 medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2 mM glutamine. All media were supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 U/ml streptomycin. Cultures were grown at 37 °C in a 5% CO_2 atmosphere. HCT116$^{+/+}$ and HCT116$^{-/-}$ cells were a kind gift from Dr. Vogelstein.

Measurement of ascorbate cytotoxicity

The MTT assay (Sigma–Aldrich, St. Louis, MO, USA) was used to determine cell viability [16]. Cells in 96-well plates were treated with ascorbate (0.5–10 mM) for 8–24 h or with H_2O_2 (10–200 μM) for 5 h. After treatment with each drug for the specified times, cultures were incubated with MTT for 4 h and results were measured by an enzyme-linked immunosorbent assay (ELISA) plate reader. The EC_{50} value is defined as the concentration that reduced survival by 50%.

Modification of p53 expression and cytotoxicity of ascorbate-induced ROS

p53 expression in several p53-null or p53-wild-type (wt) cell lines was varied by p53 expression vectors or short hairpin RNA (shRNA). pcDNA3.1/p53 expression plasmid or pLKO/p53 shRNA plasmid was transfected onto 90% confluent cultures in 60-mm^3 dishes using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Expression levels of p53 protein were measured by immunoblot analysis. To examine the cytotoxic effect of oxygen radicals induced by ascorbate, cells were pretreated for 2 h with 100 μM ROS scavenging agent, *N*-acetyl-L-cysteine (NAC), and then 2 mM ascorbate was added. Cell viability was subsequently measured by MTT assay.

Measurement of endogenous ROS levels

To examine the effects of p53 on ROS generation, the p53 expression levels were altered in various cancer cell lines with the same genetic background. Twenty-four hours after modification of p53 expression, the cells were incubated in 96-well plates with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA) for 30 min, followed by three washes with phosphate-buffered saline (PBS). Fluorescence was then measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using an ELISA plate reader [17].

Measurement of ascorbate-mediated H_2O_2

To examine the alteration in intracellular ROS level caused by ascorbate and modified p53 level, cells were seeded at a density of 1×10^6 cells per 60-mm^3 dish and transiently transfected. At 24 h posttransfection, cells were reseeded onto 24-well plates at a density of 5×10^4 cells/well and cultured for 24 h. The cells were incubated with 2 mM vitamin C for 6 h and then incubated with 100 μM DCFDA for 30 min at 37 °C. After two rinses with PBS, 0.5 ml of PBS was added to each well and imaged using a confocal microscope (Model FV300; Olympus).

Rt-PCR

Total RNA was isolated from HCT116$^{+/+}$ and HCT116$^{-/-}$ cells using an RNA isolation kit (Qiagen) and then reverse transcribed with ImProm-II RT (Promega), according to the manufacturer's instructions. The cDNA was amplified by PCR using the AccuPower PCR premix (Bioneer) with the following primers: *p53*, 5′–AGGGTTAGTCCACAATCAGC-3′ and 5′–GGTAGGTGCAAATGCC-3′; *MDM2*, 5′–CAGCAGGAATCATCGGACTCA-3′ and 5′–CCTTATTACACACAGAGCCAGGC3′; *NQO1*, 5′–AGGCTGGTTTGAGCGAGTGTTC-3′ and 5′–TTTGAATTCGGGCGTCTGCTG-3′; *POX*, 5′–GCCATTAAGCTCACAGCACTGGG-3′ and 5′–CTGATGGCCGGCTGGAAGTA-3′; *PUMA*, 5′–GACGACCTCAACGCACAGTA-3′ and 5′–CCAGGGTGTCAGGAGGTG-3′; *NOXA*, 5′–ATGCCTGGGAAGAAGGCGCGC-3′ and 5′–TCAGGTTCCTGAGCAGAAGAG-3′; *BAX*, 5′–CCAGCTCTGAGCAGATCATG-3′ and 5′–GGAGTCTGTGTCCACG-3′; *MNSOD*, 5′–GCGGCCTACGTGAACAACCTGAACG-3′ and 5′–TCAATCCCCAGCAGTGGAATAAGGC-3′; *CATALASE*, 5′–TGCTGAATGAGGAACAGAGG-3′ and 5′–GTGTGAATCGCATTCTTAGG-3′; *GPX1*, 5′–TTCCCGTGCAACCAGTTG-3′ and 5′–TTCACCTCGCACTTCTCGAA-3′; *PIG12*, 5′–AGGCTGCGGAAGAAGGCC-3′ and 5′–AAAGGAAGGGGTAGATGGTCTC-3′; *ALDH4*, 5′–CCTGAAGCCTATTGCAGACC-3′ and 5′–TGAAGTTGATGCCACAGAGG-3′. The amount of amplified DNA in each stained band after separation by electrophoresis was determined by densitometry, and the expression of each mRNA was normalized to that of β-actin.

Immunoblot analysis and immunoprecipitation (IP)

Cells were lysed in NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40) containing protease inhibitor, and IP was performed by lysing cells, followed by preclearing with protein A/G–Sepharose (Santa Cruz Biotechnology). Precleared lysates were incubated with antibody for 16 h at 4 °C with continuous agitation, and then protein A/G–Sepharose was added. After 4 h of incubation, the lysate–antibody–agarose A/G bead complex was collected by centrifugation at 10,000 *g* for 5 min and washed five times with lysis buffer. Proteins were eluted from beads by boiling for 5 min in sodium dodecyl sulfate (SDS)–polyacrylamide gel