Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Cellular toxicity driven by high-dose vitamin C on normal and cancer stem cells

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ARTICLE INFO

Article history:
Received 6 February 2018
Accepted 8 February 2018
Available online xxx

Keywords:
Vitamin C
DNA damage
Stem cells
Oxidative stress

ABSTRACT

As a powerful antioxidant, vitamin C protects cells from oxidative damage by inhibiting production of free radicals. However, high levels of vitamin C shows cytotoxicity especially on cancerous cells through generating excessive ROS and blocking the energy homeostasis. Although the double-sided character of vitamin C has been extensively studied in many cell types, there is little research on the consequence of vitamin C treatment in stem cells. Here, we identified that high-dose vitamin C shows cellular toxicity on proliferating NSPCs. We also demonstrated that undifferentiated NSPCs are more sensitive to vitamin C-driven DNA damage than differentiated cells, due to higher expression of Glut genes. Finally, we showed that high-dose vitamin C selectively induces DNA damage on cancer stem cells rather than differentiated tumor cells, raising a possibility that vitamin C may be used to target cancer stem cells.

1. Introduction

Vitamin C is a crucial nutrient which cannot be synthesized in human body due to the deficiency of L-gulono-gamma-lactone oxidase, the enzyme catalyzing the terminal step in L-ascorbic acid biosynthesis [1]. Because vitamin C deficiency may result in the potentially fatal disease scurvy and human body has only limited storage capacity for the water-soluble vitamin C, a regular intake is essential to avoid hypovitaminosis C [2]. When vitamin C is administrated orally, the maximum vitamin C plasma concentration does not exceed 250 μM due to the limited absorptive capacity of the gastrointestinal tract [3]. With intravenous infusion of vitamin C, however, plasma ascorbate concentrations can be increased more than 100-fold: up to 30 mM [4].

Protective roles of vitamin C have been demonstrated in many previous experiments. As a powerful first-line antioxidant, vitamin C suppresses production of free radicals and attenuates oxidative damage [5,6]. Increasing evidence suggests that many chronic diseases such as diabetics, atherosclerosis, cardiovascular diseases, chronic inflammation, cancer, neurodegenerative diseases, and aging are highly related to oxidative stress [7,8], leading to study on the ROS scavenging activity of vitamin C [9,10]. Interestingly, however, high-dose vitamin C often shows cytotoxicity in some contexts. Although still controversial, high-dose vitamin C seems to be more cytotoxic to cancerous cells than normal cells [11]. Indeed, vitamin C selectively kills KRAS and BRAF driven colorectal cancer cells by inducing oxidative stress, suppressing glycolysis and the subsequent energy crisis [12]. In addition, vitamin C induces apoptosis in human breast cancers through the nuclear translocation of apoptosis-inducing factor (AIF) [13]. High-dose vitamin C inhibits the viability of MCF7 breast adenocarcinoma and HT29 colon cancer cells by blocking the energy flux in glycolysis and the TCA cycle, consequently leading to insufficient ATP production [14].

While the effect of vitamin C on cancer cells has been extensively studied, there is little research on the consequence of vitamin C treatment in other cell types including stem cells. Here, we identified that high-dose of vitamin C suppresses the sphere-forming ability of neural stem/progenitor cells (NSPCs) and induced expression of apoptotic genes through depletion of GSH and NAD+. Compared to differentiated cells, undifferentiated NSPCs are more sensitive to high-dose vitamin C probably due to enhanced expression of Glut1 and Glut3. Similarly, undifferentiated cancer stem cells, the neoplastic counterparts of NSPCs, showed higher DNA damage signals upon high-dose vitamin C treatment

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https://doi.org/10.1016/j.bbrc.2018.02.083
0006-291X/

than differentiated cells did, raising the possibility that high-dose vitamin C may be used to block tumor recurrence through eradicating cancer stem cells.

2. Materials and methods

2.1. Neural stem cell culture and differentiation

Primary NSPCs were isolated from the brain of mouse embryos and maintained in NSPC culture medium supplemented with 20 ng/ml EGF and bFGF. For differentiation, NSPCs were dissociated into single cells using TrypLE (Life Technologies), and plated on polyornithine and fibronectin-coated plates in NSPC culture medium including 1% FBS (Life Technologies) for 4 days.

2.2. Quantitative Real-time RT-PCR

Total RNA was isolated from primary NSPCs using RNA purification kit (Thermo Scientific). 200 ng of total RNA was treated with RNase-free DNase (Sigma-Aldrich) for 15 mins. After inactivation of DNase with EDTA and heating, RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Quantitative RT-PCR was performed on cDNA samples using the Power SYBR Green Master mix and was performed the qPCR on the Mic qPCR Cycler (bio molecular systems). The relative mRNA level was presented as values of 2^{-}[Ct(β-actin) − Ct(gene of interest)]. The sequences of the forward and reverse primers are as follows: Bax, 5′-TGTTTGCTGATGGCAACTTC-3′ and 5′-GATCAGCTCGGGCACTTTAG-3'; Gabarapl1, 5′-GGACCACCCCTTC-GAGTATC-3′ and 5′-CCTCTTATCCAGATCAGGGACC-3'; Cited2, 5′-TGTCCCTCTATGTGCTGCTG-3′ and 5′-ATGGTCTGCCATTTCCAGTC-3'; Myc, 5′-TGAAGGCTGGATTTCCTTTG-3′ and 5′-TTCTCTTCCTCGTCGCAGAT-3'; Glut1, 5′-CAGTTCGGCTATAA-CACTGGTG-3′ and 5′-GCCCCCGACAGAGAAGATG-3'; Glut3, 5′-ATGGGGACAACGAAGGTGAC-3′ and 5′-GTCTCAGGTGCATTGAT-GACTC-3'; Svct2, 5′-TGGACGGCATACAAGTTCCAG-3′ and 5′-GAAGA-CATCAGTCACCGTGAAG-3'; β-actin, 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3'.

2.3. Protein preparation and immunoblot analysis

For immunoblotting, cells were disrupted directly with laemmli buffer, followed by sonication and heat-denaturation. Immunoblot analyses were performed with anti-γH2AX (cusabio), anti-Cleaved caspase3 (thermo fisher scientific), anti-Oct4 (Bioworld), anti-Nestin (Novus Biologicals), anti-GFAP (abcam), anti-DCX (Neuromics), anti-β actin (sigma aldrich), anti-total AMPK, and anti-phospho AMPK (Cell Signaling). The signals were detected with Clarity™ Western ECL Substrate (bio-rad).

2.4. GSH/GSSG-Glo assay

Primary NSPCs were maintained with N2 culture media supplemented with 20 ng/ml EGF and bFGF. At 24 hrs after DW or 5 mM ascorbic acid treatment, growth media was removed and cells were washed with PBS. Total glutathione and GSSG were assayed in triplicate with GSH/GSSG Glo kit (Promega), following manufacturers' instructions. Ratio GSH/GSSG was calculated as follow: [luminescence of total glutathione − luminescence of GSSG]/[luminescence of GSSG/2].

2.5. NAD + measurement

Primary NSPCs were maintained with N2 culture media supplemented with 20 ng/ml EGF and bFGF. At 24 hrs after DW or 5 mM ascorbic acid treatment, growth media was removed and cells were washed with PBS. NAD+ and NADH were assayed in triplicate with NAD/NADH Glo kit (Promega), following manufacturers' instructions.

2.6. Statistical analysis

The unpaired two-tailed Student's t-test was used for experiments comparing two sets of data unless noted. All results are expressed as mean ± s.e.m. Differences were considered significant when $^{*}P<0.05$ and $^{**}P<0.01$.

3. Results

3.1. High-dose vitamin C shows cellular toxicity on NSPCs

To test the effect of high-dose vitamin C on proliferating NSPCs, cell morphology was monitored after treatment of vehicle or vitamin C. When NSPCs were maintained with serum-free culture media supplemented with 20 ng/ml EGF and bFGF (proliferating condition), cells were floating and form spheres. Upon Low-dose (400 μM) ascorbic acid treatment, sphere-forming ability of NSPCs was unaffected. However, when cells were challenged with 2 mM ascorbic acid, cells became loosely connected and started to adhere to the plate. At a higher concentration of vitamin C (5 mM), the number and size of NSPC spheres were dramatically reduced (Fig. 1A). To check the cytotoxic effect mediated by high-dose vitamin C on NSPCs, we examined the phosphorylation of γH2AX, a marker of DNA damage, and the active form of caspase 3, a marker of apoptosis through immunoblotting. Clearly, high-dose vitamin C induced DNA damage, leading to the death of NSPCs (Fig. 1B). To confirm the apoptotic event driven by high-dose vitamin C, we evaluated mRNA expression of pro-apoptotic genes. Bax has been known to counter Bcl2 and promotes apoptosis. Bax deficiency caused lymphoid hyperplasia and excessive expansion of granulosa cells [15]. The mRNA expression of Bax was dramatically elevated by high-dose vitamin C. Another gene tested was Cited2. Down-regulation of Cited2 is frequently observed in various types of leukemia, and Cited2 induces p53 acetylation which leads to the apoptosis [16]. The mRNA level of Cited2 was also elevated by high-dose vitamin C. We also analyzed the expression of Gabarapl1. Recent report demonstrated that overexpression of Gabarapl1 inhibited cell growth of hepatocellular carcinoma and low level of Gabarapl1 is associated with a poor outcome for liver cancer patients [17]. Not surprisingly, the mRNA expression of Gabarapl1 was also increased under high-dose vitamin C treatment (Fig. 1C). Together, high-dose vitamin C induced DNA damage and apoptosis of proliferating NSPCs, indicating that the effect was cytotoxic rather than cytostatic.

Recently, it was identified that vitamin C selectively disrupted the growth of *KRAS* and *BRAF* mutant colorectal cancers [12]. Excessive vitamin C uptake causes the depletion of GSH inside cells. Because GSH is a crucial intracellular and extracellular antioxidant [18,19], the reduction of GSH increased cellular reactive oxygen species (ROS), leading to cell death [18]. To check whether high-dose vitamin C has similar effect on proliferating NSPCs, we analyzed the relative ratio of reduced to oxidized glutathione (GSH/GSSG) in vitamin C treated cells. Interestingly, the relative GSH/GSSG value was significantly decreased upon 5 mM vitamin C treatment (Fig. 1D). In culture media, extracellular vitamin C is oxidized to dehydroascorbate (DHA) and then DHA is imported into cells through glucose transporters including Glut1 and Glut3 [20,21]. Intracellular DHA is nonenzymatically reduced to vitamin C by GSH. Therefore, downregulation of GSH/GSSG by high-dose vitamin C treatment is probably due to the expense of

Please cite this article in press as: T.-J. Kim, et al., Cellular toxicity driven by high-dose vitamin C on normal and cancer stem cells, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.02.083