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Ascorbyl stearate and ionizing radiation potentiate apoptosis through intracellular thiols and oxidative stress in murine T lymphoma cells

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ABSTRACT

Ascorbyl stearate (Asc-s) is a derivative of ascorbic acid with better anti-tumour efficacy compared to its parent compound ascorbic acid. In this study, we have examined radio-sensitizing effect of Asc-s in murine T cell lymphoma (EL4) cells at 4 Gy. Asc-s and radiation treatment reduced cell proliferation, induced apoptosis in a dose dependent manner by arresting the cells at S/G2-M phase of cell cycle. It also decreased the frequency of cancer stem cells per se, with significantly higher decrease in combination with radiation treatment./Further, Asc-s and radiation treatment increased the level of reactive oxygen species (ROS), drop in mitochondrial membrane potential (MMP) and increased caspase-3 activity resulting in apoptosis of EL4 cells. Further it also significantly decreased GSH/GSSG ratio due to binding of Asc-s with thiols. The increase in oxidative stress induced by Asc-s and radiation treatment was abrogated by thiol antioxidants in EL4 cells. Interestingly, this redox modulation triggered significant increase in protein glutathionylation in a time dependent manner. Asc-s treatment resulted in glutathionylation of IKK, p50-NF-kB and mutated p53, thereby inhibiting cancer progression during oxidative stress. Asc-s quenches GSH ensuing Asc-s + GSH adduct thereby further modulating GSH/GSSG ratio as evident from HPLC and docking studies. The anti-tumour effect of Asc-s along with radiation was studied by injecting EL4 cells in synegenicC57/BL6 male mice. Intraperitoneal injection of Asc-s followed by radiation exposure at 4 Gy to the tumour bearing mice resulted in radio-sensitization which is evident from significant regression of tumour as evident from tumour burden index. The survival study supports the data that Asc-s pre-treatment enhances radio-sensitization in murine lymphoma. Our data, suggest that Asc-s and ionizing radiation induced cell cycle arrest and apoptosis by perturbing redox balance through irreversible complexes of thiols with Asc-s, disturbed mitochondrial membrane permeability and activation of caspase-3 in EL4 cells.

1. Introduction

Radiation therapy is an integral part of treatment of different types of solid cancers. Ionizing radiation precipitates direct and indirect damage to the cells. Reactive oxygen species (ROS) generated during radiation treatment is the paramount negotiator of radiation induced catastrophe to biological systems. ROS generate oxidative stress and perturbs intracellular redox balance in cell [1]. Owing to its attribute of high reactivity, electrophilicity and short lifespan, ROS react with critical biomolecules in cell such as lipids, proteins and DNA, damage them and drive the cells to undergo apoptosis [2,3]. High oxidative stress environment prevail in metabolically active cancer cells [4,5]. However, development of radioresistance in cancer cells implies mechanism devised by its intracellular antioxidant system to tackle the oxidative stress and maintain low steady level. It is well established that intrinsic radio-resistance of lymphoma cells vis-à-vis normal lymphocytes accounts to lower basal, inducible ROS levels. It is well established that GSH levels and antioxidant enzymes were higher in lymphoma cells as compared to normal lymphocytes [3]. Radiation therapy has drawback of toxicity and resistance in cancer cells. A number of natural phytochemicals, such as curcumin, demethoxycurcumin, quercetin, genistein etc., are shown to possess radio-sensitizating potential in cancer cells [6,7].

Ascorbyl stearate (Asc-s), a fatty acid ester derivative of ascorbic acid is a potent anticancer compound. Asc-s is found to be effective at relatively low doses due to improved bio-availability over its parent compound ascorbic acid. Our earlier reports demonstrated that Asc-s treatment inhibited cancer cell growth by interfering with cell-cycle progression, clonogenicity and induced apoptosis by modulating signal transduction pathways of insulin-like growth factor 1 receptor (IGFIR)/p53/p21/cyclin and interfering with NF-κB expression, responsible for cancer cell survival [8–11].

Cellular redox status plays an important role in the biological effector functions of lymphocytes and leukocytes [12,13]. Since oxidative

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stress has been shown to modulate signalling pathways through modulation of thiol groups present on proteins and glutathionylation of many proteins [14,15], we hypothesized that the apoptotic effects of Asc-s may be due to its ability to perturb the redox balance in EL4 cells and depleting GSH reserves leading to modulation of mitochondrial membrane potential and activation of caspase-3. To understand the modulation of intracellular redox, we have tested the effect different antioxidants (thiol/non-thiol) on cell death induced by Asc-s in combination with exposure to ionizing radiation. We also studied the effect of Asc-s on intracellular GSH reserves and glutathionylation which may inactivate the function of tumour promoter proteins. Further, the antitumour effect of Asc-s in combination with ionizing radiation was tested on EL4 cells in synegenicC57/BL6 mice.

2. Materials and methods

2.1. Chemicals

Ascorbyl stearate (Asc-s) was purchased from Tokyo Chemical Industry (TCI), Japan. RPMI 1640 medium, N-acetylcysteine (NAC), propidium iodide (PI), Hoechst-33258, dithiothreitol (DTT), glutathione (GSH), N-acetyl cysteine (NAC), nonidet P-40, propidium iodide (PI), dimethyl sulfoxide (DMSO), PEG-catalase (CAT), superoxide dismutase (SOD), Caspase 3 Assay Kit and Immunoprecipitation Kit (Protein G) were purchased from Sigma Chemical Co. (USA). Fetal calf serum (FCS) was obtained from GIBCO BRL (MD, USA). Homogeneous caspase assay kit was purchased from Roche Applied Science (Germany). Trolox (TRO) was from Calbiochem (USA). Carboxy fluorescein diacetate succinimidyl ester (CFSE), 5-(and-6)-carboxy-2,7-dichloro fluorescein diacetate (DCF-DA) and 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbo cyanine iodide (JC-1) was procured from Molecular Probes, Invitrogen. Anti-GSH, anti-p53, anti-NFkB, anti-HRP IgG and anti-IKK antibodies were procured from Cell Signalling Technologies (USA). All other chemicals used in this study were obtained from reputed manufacturers and were of analytical grade. Methotrexate (Alkem, India) was used as positive control drug.

2.2. Ascorbyl stearate (Asc-s) preparation

Asc-s (1.0 mM) was prepared in RPMI 1640 medium by dissolving Asc-s in DMSO and the pH was adjusted to 7.0 with 0.1 mM sodium hydroxide in sterilised Milli Q (MQ) water.

2.3. Cell culture

Murine T cell lymphoma (EL4) cells were procured from Health Protection Agency Culture Collections, UK. Mouse lymphoma EL4 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere in a CO_2 incubator.

2.4. Animal maintenance

C57/BL6 male mice, weighing approximately 20–25 g, of six to eight week old inbred reared in the animal house of Bhabha Atomic Research Centre, Trombay were used for *in vivo* experiments. Mice were housed at constant temperature (23 °C) with a 12/12 h light/dark cycle. Mice were given *ad libitum* of chow and water. The animal experiments were carried out as per the guidelines of Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India. Project No. BAEC/14/16 and Date of approval April, 2016.

2.5. Exposure to ionizing radiation to Mouse lymphoma EL4 cells

Blood Irradiator (BRIT, Mumbai) ^{60}Co was used as source of γ-irradiation for treating Mouse lymphoma EL4 cells and mice. Lymphoma cells (5×10^5) suspended in RPMI medium without fetal calf serum was exposed to 4 Gy ionizing radiation for 15 min and the cells were cultured with different concentrations of Asc-s (0–200 μM) for 48 h in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Unirradiated cells served as control. C57/BL6 male mice were exposed to a dose of 4 Gy at a dose rate of 1.0 Gy/min.

2.6. Cell cycle analysis

EL4 cells were exposed to γ-irradiated and incubated with different doses of Asc-s (0–200 μM) and Methotrexate (MTX) (1–10 μM) for 48 h at 37 °C in RPMI 1640 medium with 10% FCS in CO_2 incubator. The cells were washed with PBS and subjected to Nicoletti assay to identify apoptosis by analysing cells with a DNA content less than 2n ("sub-G_1 cells") and document cell cycle phases by Flow cytometry [16]. Cells harvested were stained by addition of 1 ml of staining solution containing 0.5 mg/ml PI, 0.1% sodium citrate, and 0.1% Triton X-100 overnight at 4 °C (*23*) incubated in buffer (PBS + 0.1% Triton-X + 0.1% sodium citrate, pH 7.4, supplemented with 50 μg/mL PI) overnight at 4 °C. Cell analysis was performed using Flow cytometer (Partec, Germany). The PI fluorescence signal at FL3-PI versus histogram was used to ascertain sub-G_1 and cell cycle distribution by Flowjo software.

2.7. Enumeration of side population cells

Exponentially growing EL4 cells in 96-well plate were treated with Asc-s and Methotrexate (MTX) (1–10 μM) followed by exposure to radiation (4 Gy). Forty eight hours after incubation, cells were spun down, washed and resuspended in DMEM devoid of phenol red. Hoechst 33342 stain (10 mg/ml) was added to the cells, incubated at 37 °C for 1.5 h. Cells were spun down, and acquired using high content screening instrument (Acumen Celista) from TTP Labtech, UK. The live cells showing low Hoechst fluorescence in red channel (620 nm) and blue channel (450 nm) were gated and plotted as side population cells [7].

2.8. Effect of Asc-s on EL4 cell proliferation

The effect of Asc-s and Methotrexate (MTX) (1–10 μM) on EL4 cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylltetrazolium bromide (MTT) assay [8]. In brief, 2×10^4 EL4 cells were seeded in 96 well plates in 0.15 ml RPMI. EL4 cells were treated with Asc-s at concentration of 0–200 μM and MTX at 1–10 μM with and without 4 Gy radiation treatment as two experiment sets. After 48 h, cell proliferation and viability was determined by MTT assay and absorbance was read at 570 nm using microplate reader. The decrease in absorbance is commensurable with loss of cell viability.

2.9. Measurement of ROS in EL4 cells

Intracellular ROS in Mouse lymphoma EL4 cells were measured by spectrofluorimetric method of Checker et al. [17]. To detect intracellular ROS, EL4 cells were incubated with 20 μM oxidation-sensitive dihydrodichlorofluoresceindiacetate (H_2DCF-DA) for 25 min at 37 °C before exposure to 4 Gy ionizing radiation (30–120 Min) and treatment with different concentrations of Asc-s (0–200 μM). An increase in fluorescence due to oxidation of H_2DCF to DCF was measured at 485 nm excitation and 535 nm emission in a spectrofluorimeter (Biotek Synergy Hybrid multimode plate reader, US).