Toxicology in Vitro 27 (2013) 1542–1549

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

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

# Oxidative stress mediates apoptotic effects of ascorbate and dehydroascorbate in human Myelodysplasia cells *in vitro*

Ana Cristina Gonçalves [a,b,c], Vera Alves [d], Teresa Silva [e], Cristina Carvalho [c,f], Catarina Resende de Oliveira [c,g], Ana Bela Sarmento-Ribeiro [a,b,c,*]

[a] Applied Molecular Biology and Hematology University Clinic, Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal
[b] Centre of Investigation in Environment, Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal
[c] Centre for Neuroscience and Cell Biology (CNC), University of Coimbra, 3004-504 Coimbra, Portugal
[d] Immunology Institute, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal
[e] Hematopathology, Institute of Pathological Anatomy, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[f] Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, 3004-504 Coimbra, Portugal
[g] Biochemistry Institute, Faculty of Medicine, University of Coimbra, 3000-548 Coimbra, Portugal

ARTICLE INFO

*Article history:*
Received 5 July 2012
Accepted 19 March 2013
Available online 27 March 2013

*Keywords:*
Myelodysplastic Syndrome
Ascorbate
Dehydroascorbate
Oxidative stress
Apoptosis
Anti-cancer therapy

ABSTRACT

The Myelodysplastic Syndromes are stem cell heterogeneous disorders characterized by peripheral cytopenias and hypercellular bone marrow, which can evolute to acute leukaemia. Vitamin C can act as an antioxidant, ascorbic acid (AA) donates two electrons and becomes oxidized to dehydroascorbic acid (DHA). Under physiological conditions, vitamin C predominantly exists in its reduced (AA) form but also exists in trace quantities in the oxidized form (DHA). This study evaluates the therapeutic potential of vitamin C in Myelodysplastic Syndromes (MDSs).

F36P cells (MDS cell line) were treated with ascorbate and dehydroascorbate alone and in combination with cytarabine. Cell proliferation and viability were assessed by trypan blue assay and cell death was evaluated by optical microscopy and flow cytometry. The role of reactive oxygen species, mitochondrial membrane potential, BAX, BCL-2 and cytochrome C were also assessed.

Vitamin C decreases cell proliferation and viability in a concentration, time and administration dependent-manner inducing cell death by apoptosis, which was shown to be associated to an increased in superoxide production, mitochondrial membrane depolarization. These compounds modulate BCL-2, BAX and cytochrome C release.

These results suggest that vitamin C induces cell death trough apoptosis in F36P cells and may be a new therapeutic approach in Myelodysplasia.



## 1. Introduction

Naturally occurring dietary agents, known to produce chemopreventive effects in experimental cancer models, have been shown to target signalling intermediates in apoptotic pathways, especially because diet and nutrition are key factors in cancer risk modulation. These dietary agents display numerous functions on genetic transcription modulation, being involved in activation or inhibition of specific genes and in induction of cell death (Aggarwald and Shishodia, 2006; Martin, 2006). In recent years, because of their low systemic toxicity, vitamins have been evaluated for their anti-tumour activities and have gained importance because of their prophylactic and therapeutic potential role in several diseases. Antioxidants, such as vitamin C, show protective effects and, on the other hand, they can develop pro-oxidant properties, dependably on their concentration and cell type (Ratnam et al., 2006).

Vitamin C is a major water-soluble vitamin that exhibits various biological functions, such as antioxidant properties, being implicated in the regeneration of α-tocopherol. Humans cannot synthesize vitamin C *de novo* and thus have to acquire most body storage of vitamin C through fruits and vegetables or vitamin supplements. Under physiological conditions, vitamin C predominantly exists in its reduced form but also exists in trace quantities in the oxidized form. Vitamin C is mainly transported in the dehydroascorbic acid (DHA) form and can be regenerated to ascorbic acid (AA) either enzymatically or non-enzymatically. Besides the antioxidant proprieties, vitamin C displays also a pro-oxidant function by inducing an increase in reactive oxygen species (ROS) production (McEligot et al., 2005; Verrax and Calderon, 2008). The antioxidant activity of vitamin C resides primarily in its ability to donate electrons and

* Corresponding author. Address: Applied Molecular Biology, Faculty of Medicine, University of Coimbra, Azinhaga de Sta Comba, Celas, 3000-548 Coimbra, Portugal. Tel.: +351 239480217; fax: +351 239480048.
*E-mail address:* absarmento@fmed.uc.pt (A.B. Sarmento-Ribeiro).

0887-2333/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2013.03.009

therefore by acting as a reductive agent. In fact, AA donates two electrons and becomes oxidized to DHA. However, in a concentration dependent manner, vitamin C can produce hydrogen peroxide at cytotoxic levels, which can generate hydroxyl radical in the presence of divalent cations such as iron and cooper (González et al., 2005; Bhat et al., 2006).

Several studies have shown that vitamin C alone or combined with other antioxidants can potentiate the efficacy of several chemotherapeutic drugs, such 5-fluorouracil, doxorubicine, vincristin, adriamycin or gencitabin, either *in vitro* or *in vivo*. However, other authors report that vitamin C may have a negative effect decreasing the activity of new anti-tumoral drugs, such as bortezomib (Zou et al., 2006) or Trail Ligand (Perez-Cruz et al., 2007).

Reactive oxygen species (ROS) are formed constantly as a consequence of metabolic and other biochemical reactions mainly in mitochondria as well as induced by external factors such as drugs exposure. Many cancer cells show an increase in ROS production as a result of oxidative metabolism. Since cells can only tolerate certain ROS levels, the increase in oxidative stress can lead to oxidative damage of lipids, nucleic acids and proteins (McEligot et al., 2005; Valko et al., 2007).

On the other hand, cancer cells readily take up vitamin C *in vitro* and certain human tumour cells have higher vitamin C levels compared with adjacent normal cells. The higher intracellular concentration of vitamin C may have effects on tumour growth and in the tumour's ability to respond to chemotherapy and radiation therapy (Agus et al., 1999).

The Myelodysplastic Syndromes (MDSs) are a heterogeneous group of stem cell disorders characterized by ineffective haematopoiesis, cytopenia and a higher potential of evolution to myeloid leukaemia. MDS may arise both *de novo* and as a consequence of chemo- or radiotherapy. The natural history of these diseases ranges from a chronic course to a rapid course towards leukeamic progression, where approximately 30% of MDS cases transform in acute myeloid leukaemia (AML) (Hirai, 2003; Nimer, 2008; Mufti et al., 2008). Different treatment options are available for MDS patients ranging from supportive care, which helps relieve symptoms, to aggressive treatment that may slow or prevent progression of the disease. Cytarabine is a drug effective in MDS, producing complete response rates of 15–20% when used in a low-dose. However, virtually all patients treated with low-dose cytarabine relapse (Nimer, 2008).

The pathogenesis of MDS is complex, since hematopoietic cells and the hematopoietic bone marrow microenvironment are both involved in disease establishment and progression. The presence of oxidative stress markers in MDS patients indicates a potential role for pro-oxidant pathways in the maintenance/progression of these disorders (Farquhar and Bowen, 2003). Moreover, the treatment with the antioxidant aminothiol amifostine suggests that oxidative stress could be a new strategy in MDS treatment (Farquhar and Bowen, 2003).

In this study, we pretend to evaluate the therapeutic potential of vitamin C in MDS, as in monotherapy and/or as adjuvant to conventional anti-carcinogenic therapies.

## 2. Materials and methods

### 2.1. Materials

Roosevelt Park Memorial Institute Medium 1640 medium (RPMI 1640, Gibco, Invitrogen, Barcelona, Spain), foetal bovine serum (FBS, Gibco, Invitrogen, Barcelona, Spain), penicillin/streptomycin (Gibco, Invitrogen, Barcelona, Spain), recombinant interleucin-3 (rh-Il-3, GIBCO, Invitrogen, Barcelona, Spain), phosphate buffered saline (PBS, Sigma, Sintra, Portugal), ascorbic acid (AA, Sigma, Sintra, Portugal), dehydroascorbic acid (DHA, Sigma, Sintra, Portugal), cytarabine (Ara-C, Sigma, Sintra, Portugal), annexin V-FITC (AV) and propidium iodide (PI, Immunotech kit, Beckman Coulter, Inc., Marseille, France), 2′,7′-dichlorodihydrofluorescin diacetate ($H_2$DCF-DA, Molecular Probes, Invitrogen, Barcelona, Spain), hydroetidine (HE, Sigma, Sintra, Portugal), JC-1 (Molecular Probes, Invitrogen, Barcelona, Spain), mercury orange (MO, Sigma, Sintra, Portugal), hydrogen peroxide (Sigma, Sintra, Portugal), culture flasks and well-plates (Sarstedt, Rio Tinto, Portugal).

### 2.2. Cell culture

The F36P cells, a Myelodysplastic Syndrome cell line established from a patient with refractory anaemia with excess of blast in transformation (RAEB-t), was purchased from European Collection of Cell Cultures (ECACC, UK). Cell line was routinely grown in RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 25 mM, penicillin 100U/mL and streptomycin 100 μg/mL) supplemented with 10 ng/mL recombinant interleucin-3 (rh-Il-3) and 10% heat-inactivated foetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% $CO_2$. All experiments were performed between 10 and 25 passage numbers and F36P cells were seeded at a density of $0.75 \times 10^6$ cells/mL. Ascorbic acid (AA) and dehydroascorbic acid (DHA) was always freshly prepared by dissolving these compounds in water at a final concentration of 100 mM, immediately prior to the experiments. After preparation AA and DHA were sterilised by passing through a 0.20 μm membrane. Cells were incubated in the absence and in the presence of increasing AA and DHA concentrations, ranging from 50 μM to 5 mM, and 50 nM cytarabine (Ara-C).

### 2.3. Cell viability and proliferation assays

Cell viability and proliferation were assessed by the trypan blue exclusion test. Briefly, viable cells were identified by their ability to exclude dye, whereas dye stained nonviable cells. At each 24 h, exposure and not exposure (control) cells were harvest and the number of stained (nonviable) and unstained (viable) cells were counted using a haemocytometer (Neubauer chamber). The viability was calculated as percentage of viable cells and cell proliferation was determinate by the number of viable cells (density).

### 2.4. Cell death evaluation

F36P cell death was evaluated under the conditions describe above by optical microscopy through morphological assessment of May–Grünwald–Giemsa stained slides and by flow cytometry using the Annexin V and Propidium Iodide double staining. For morphological assessment, cells were transferred to slides fixed, stained and evaluated under light microscopy, using a Nikon Eclipse 80i equipped with a Nikon Digital Camera DXm 1200F. For flow cytometry analysis, F36P cells were stained simultaneously with Annexin V (AV), labelled with the fluorescent probe fluorescein isothyocianate (FITC) and with PI. This assay discriminates among intact cells (AV−/PI−), early apoptotic cells (AV+/PI−) and late apoptotic or necrotic cells (AV+/PI+). After drug treatments in the above conditions, cells were co-stained with AV-FITC and PI using the manufacturer's recommended protocol (Immunotech Kit). Briefly, cells were washed with ice-cold PBS (centrifuged at 500*g* for 5 min), resuspended in 100 μL of binding buffer and incubated with 1 μL of AV-FITC solution and 5 μL of PI solution for 10 min on ice in the dark. After incubation time, cells were diluted in 400 μL of ice-cold binding buffer, and analyzed by flow cytometry (Dourado et al., 2007). Results are expressed in % ± SD of at least three independent experiments. Flow cytometry analysis was performed using a six-parameter, four-colour FACSCalibur™