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Research Paper

Multiple Myeloma Tumor Cells are Selectively Killed by Pharmacologically-dosed Ascorbic Acid

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ABSTRACT

High-dose chemotherapies to treat multiple myeloma (MM) can be life-threatening due to toxicities to normal cells and there is a need to target only tumor cells and/or lower standard drug dosage without losing efficacy. We show that pharmacologically-dosed ascorbic acid (PAA), in the presence of iron, leads to the formation of highly reactive oxygen species (ROS) resulting in cell death. PAA selectively kills $CD138^+$ MM tumor cells derived from MM and smoldering MM (SMM) but not from monoclonal gammopathy undetermined significance (MGUS) patients. PAA alone or in combination with melphalan inhibits tumor formation in MM xenograft mice. This study shows PAA efficacy on primary cancer cells and cell lines *in vitro* and *in vivo*.

1. Introduction

Multiple myeloma (MM) is a plasma cell neoplasm. Four active classes of drugs including glucocorticoids, DNA alkylators (melphalan), proteasome inhibitors (bortezomib and carfilzomib) and immunomodulatory agents (thalidomide, lenalidomide, and pomalidomide), combined with or without autologous stem cell transplantation (ASCT) have led to complete remissions (CRs) in the large majority of newly diagnosed patients with MM (Alexanian et al., 2014; Fu et al., 2013; Terpos et al., 2014; Wang et al., 2014; Sonneveld et al., 2013; Gay et al., 2013; Liu et al., 2013; Bergsagel, 2014). These treatments have greatly improved patient progression-free and overall survival. However, there are at least three major problems limiting the administration of these agents: 1. All these drugs target both tumor and non-tumor cells; 2. Increased hematologic toxicity has been identified by combining alkylators with either immunomodulatory drugs (IMIDs) (Bergsagel, 2014); and 3. High doses of the DNA alkalating agent, such as melphalan, have strong cytotoxicity on gut epithelial cells and hematopoietic stem cells (Shaw et al., 2014). One way to deal with non-selective toxicity of high dose melphalan is to combine it with another agent which very specifically targets tumor cells and therefore decreasing melphalan dosing without loss of efficacy.

In the 1970s, Cameron and Pauling reported that high doses of vitamin C increased survival of patients with cancer (Cameron and Pauling, 1976, 1978). Recently, reports have shown that pharmacologically dosed ascorbic acid (PAA) 50–100 g (Chen et al., 2008; Padayatty et al., 2004; Hoffer et al., 2008; Padayatty et al., 2006; Welsh et al., 2013), administered intravenously, has potent anti-cancer activity and its role as anti-cancer therapy is being studied at the University of Iowa and in other centers (Du et al., 2012; Ma et al., 2014). In the presence of catalytic metal ions like iron, PAA administered intravenously exerts pro-oxidant effects leading to the formation of highly reactive oxygen species (ROS), resulting in cell death (Yun et al., 2015; Ma et al., 2014; Du et al., 2012; Chen et al., 2007, 2005). In a previous study, we have reported that the labile iron pool (LIP) is significantly elevated in MM cells (Gu et al., 2015), suggesting that PAA treatment should target MM cells quite selectively. The higher LIP is the direct result of the low expression of the only known mammalian cellular iron exporter, Ferroportin 1 (Fpn1), in MM as demonstrated by our group (Gu et al., 2015). These findings led us to the hypothesis that PAA might specifically target MM cells with high iron content and may also act synergistically in combination with commonly used MM therapies.

Abbreviation: MM, multiple myeloma; ROS, reactive oxygen species; SMM, smoldering MM; IMIDs, immunomodulatory drugs; ASCT, autologous stem cell transplantation; CRs, complete remissions; PAA, pharmacologically dosed ascorbic acid; LIP, labile iron pool; GEP, gene expression profiling; BM, bone marrow; IVIS, *in vivo* imaging system; WT, wild-type; DFO, deferoxamine; AIF1, apoptosis inducible factor 1.

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2. Materials and Methods

2.1. Patients Samples

Peripheral-blood samples or bone marrow aspirates were obtained from patients with monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and multiple myeloma (MM). Written informed consent was obtained from all participants. The de-identified clinical specimens in this study were approved by the institutional review board at the University of Iowa (HawkIRB protocol 201302833).

2.2. Gene Expression

Gene expression profiling (GEP) has been described previously (Zhan et al., 2006; Shaughnessy et al., 2007). The GEP access number of normal plasma cell (NPC), MGUS, and primary myeloma samples is GSE2658.

2.3. Viability Assay

Pharmacological ascorbic acid (PAA) was kindly provided by Dr. Garry R. Buettner (University of Iowa). Dr. Buettner prepares PAA as previously described (Du et al., 2010). Briefly, L-ascorbic acid was from MACRON Fine Chemicals/Avantor Performance Materia (Center Valley, PA, USA). A stock solution of 1.0 M ascorbate in de-ionized water (pH adjusted to 7.0 with NaOH) was made under argon and stored in a volumetric flask with a tight-fitting stopper at 4 °C. Ascorbate concentration was checked at 265 nm, $\varepsilon = 14{,}500\ M^{-1}\ cm^{-1}$(Buettner, 1998). The solution can be kept for several weeks without significant loss of ascorbate due to the lack of oxygen. $CD138^+$ MM cells and $CD138^-$ non-MM cells were isolated from MGUS, SMM, and MM patient samples using anti-CD138 immunomagnetic beads (Miltenyl Biotec, Auburn, CA). Cells were cultured with or without PAA at the described concentration for 1 h. After incubation, the cells were washed and cultured up to 24 h. Cell counts and viable cell number were determined using Trypan Blue staining.

2.4. Xenograft Mouse Model

The animal study was performed according to the guidelines of the Institutional Animal Care and local veterinary office and ethics committee of the University of Iowa, USA under approved protocol (IACUC 5081482). NOD.Cγ-Rag1 mice 6–8 weeks old (Jackson Laboratory, Bar Harbor, Maine) were injected intravenously with ARP1 MM cells (1×10^6) expressing luciferase. After one-week injection of ARP1 cells, mice were treated with either PAA (4 mg/kg) injected intraperitoneal once a day, 5 days every week for 3 weeks. Melphalan (3 mg/kg) was injected intraperitoneal once a day, 2 days a week for 3 weeks (Sanchez et al., 2012). Carfilzomib (3 mg/kg) was injected by in vein once a day, 2 days every week for 3 weeks (Eda et al., 2014). Bortezomib (3 mg/kg) was injected intraperitoneal once a day, 2 days a week for 3 weeks. The mice were euthanized when a humane endpoint was reached.

2.5. In Vivo Imaging System

Xenogen IVIS-200, an *in vivo* imaging system (IVIS), was used to analyze tumor burden and was indicated by quantification of luciferase intensity of mice pre- and post-treatments.

2.6. Cell Culture

Human myeloma cell lines (ARP1, OCI-MY5 and their derivative cell lines) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FBS (Invitrogen), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified incubator at 37 °C and 5% CO_2/95% air. To increase cellular iron concentration, ferric nitrilotriacatate (Fe-NTA) was added in the cell culture media.

2.7. Western Blotting

Cells were harvested and lysed with lysis buffer: 150 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.4, 1% X-100 Triton. Cell lysates were subjected to SDS-PAGE, transferred onto a pure nitrocellulose membrane (BioRad) and blocked with 5% fat-free milk. Primary antibodies for immunoblotting included: anti-AIF1 (1:1000, Cell Signaling), anti-RIP (1:1000, Santa Cruz Biotechnology), anti-RIP3 (1:1000, Cell Signaling), anti-Caspase3 (1:1000, Cell Signaling), anti-Caspase 8 (1:1000, Cell Signaling), anti-Caspase 9 (1:1000, Cell Signaling), phosphorylated γ-H2AX (1:1000, Enzo Life Sciences), and β-actin (1:1000, Cell Signaling) as loading control. Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:10,000, Santa Cruz Biotechnology, cat#: sc-2005) or anti-rabbit secondary antibody (1: 10,000, AnaSpec Inc., cat#: AS-28177) for 1 h and chemi-luminescence signals were detected by HRP substrate (EMD Millipore). Pan-caspase inhibitor Q-VD-OPh (Sigma-Aldrich, MO) was at final concentration of 25 μM.

2.8. Statistical Analysis

GEP data were analyzed by one-way ANOVA test using log2 transformed Affymetrix Signals and presented by boxplot. The comparisons of tumor burden were analyzed either by student *t*-test (2 groups) or by one-way ANOVA test (>2 groups). Kaplan-Meier test was performed for survival with the use of SPSS 16.0 software (SPSS, Chicago, IL). Two-tailed *p* value at an alpha level of 0.05 was considered to indicate statistical significance. Graphs were generated using Prism 6 software.

2.9. Electron Microscopy

Electron microscopy was performed by the Central Microscopy Research Facility personnel at the University of Iowa. Images were captured on JEOL JEM 1230.

3. Results

3.1. Pharmacological Ascorbic Acid Selectively Kills Primary Myeloma Cells

We analyzed the response to PAA of both $CD138^+$ primary MM cells (high cytosolic iron) and $CD138^-$ non-MM bone marrow (BM) cells obtained from 13 patients. The 13 patients included 2 pre-cancer of monoclonal gammopathy of undetermined significance (MGUS), 2 smoldering MM (SMM) and 9 MM patients. Patient demographic, disease characteristics and therapy are listed in Supplementary Table 1 and Supplementary Fig. 1. The survival of $CD138^+$ cells in vitro was significantly decreased following PAA treatment in all 9 MM (Fig. 1A, grey bars, $p < 0.01$). In contrast, no significant change of cell viability was observed in $CD138^-$ BM cells from the same patients (Fig. 1A, black bars). The same effect of PAA was also observed in the SMM patients (Fig. 1B). However, almost no response to PAA was detected in $CD138^+$ cells from the 2 MGUS patients (Fig. 1C). We predicted that this would be the case because MGUS patients have much lower cytosolic iron compared to MM patients (Supplementary Fig. 2) as the consequence of lower expression of transferrin receptor 1 (TfR1), the cellular iron receptor-mediated importer (Supplementary Fig. 2A), and higher expression of Ferroportin 1 (Fpn1), the iron exporter (Supplementary Fig. 2B).

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