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Combination of ascorbate/epigallocatechin-3-gallate/gemcitabine synergistically induces cell cycle deregulation and apoptosis in mesothelioma cells

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ABSTRACT

Malignant mesothelioma (MMe) is a poor-prognosis tumor in need of innovative therapies. In a previous *in vivo* study, we showed synergistic anti-MMe properties of the ascorbate/epigallocatechin-3-gallate/gemcitabine combination. We have now focused on the mechanism of action, showing the induction of apoptosis and cell cycle arrest through measurements of caspase 3, intracellular Ca^{2+}, annexin V, and DNA content. StellArray™ PCR technology and Western immunoblotting revealed DAPK2-dependent apoptosis, upregulation of cell cycle promoters, downregulation of cell cycle checkpoints and repression of NFκB expression. The complex of data indicates that the mixture is synergistic in inducing cell cycle deregulation and non-inflammatory apoptosis, suggesting its possible use in MMe treatment.

Introduction

Malignant mesothelioma (MMe) is an incurable tumor, showing resistance to chemo- and radio-therapy, with a median survival of 8 to 18 months (Martino and Pass, 2004). Approximately 10,000 to 15,000 cases are diagnosed annually worldwide. The incidence of the disease is rising because its etiology is associated with prior exposure to asbestos, which has been worldwide used as construction material. In the majority of patients, responses to chemotherapeutic approaches are not effective and remain largely unsuccessful due to the development of chemoresistance (Kindler and van Meerbeeck, 2002; van Meerbeeck et al., 2005). However, because of hitherto low response rates, several trials are being made with new targeted agents, in the effort of improving treatment outcome. Growing evidence suggests that combining chemopreventive agents with chemotherapy or radiotherapy may reduce adverse effects and lead to enhanced antitumor activity through synergistic actions. A standard treatment consists in the association of cisplatin with the antifolate drugs pemetrexed or ralitrexed (Neoptolemos et al., 2003; Sarkar and Li, 2007).

We have put forward the idea of combining active nutrients and pharmaceutical drugs in the treatment of MMe. Antitumor nutrients are generally better tolerated by the organism than drugs, and they can therefore help in increasing the efficiency of drugs without using higher doses, if the mixture is synergistic. Ascorbate is highly tolerated by the human body, while it has been reported to be useful in the treatment and prevention of cancer (Ohno et al., 2009). We have found that MMe cells are more susceptible to ascorbate than normal mesothelium because of their higher rate of superoxide production (Ranzato et al., 2011).

Following these outcomes, we have made an *in vitro* survey aimed at finding out synergistic partners of ascorbate, chosen among drugs used in cancer therapy and active nutrients with antitumor properties. These tests have revealed a synergistic cytotoxicity of ascorbate in combination with gemcitabine or the green tea polyphenol epigallocatechin-3-gallate (EGCG) (Martinotti et al., 2011). Thereafter, a triple mixture of these compounds, named AND (active nutrients/drug), has been found to act synergistically *in vitro* on various MMe cell lines, and moreover to arrest the growth and invasiveness of mouse MMe xenografts (Volta et al., 2013). Gemcitabine (2′,2′-difluorodeoxycytidine) is widely used in the clinical management of MMe patients (Kindler and van Meerbeeck, 2002), while EGCG has raised interest in cancer research because of its ability to induce apoptosis without affecting normal cells (Gupta et al., 2004; Ranzato et al., 2012).

However, before proposing this mixture as an alternative clinical treatment for MMe cancer, we wanted to achieve more in-depth information about its mechanism of action. The induction of apoptosis is a main requisite for chemotherapeutic agents. Therefore, in the present study we investigated whether the induction of cell cycle arrest and apoptosis is mechanistically linked to the synergistic action of AND. The tumorigenic REN cell line was chosen as an *in vitro* MMe model

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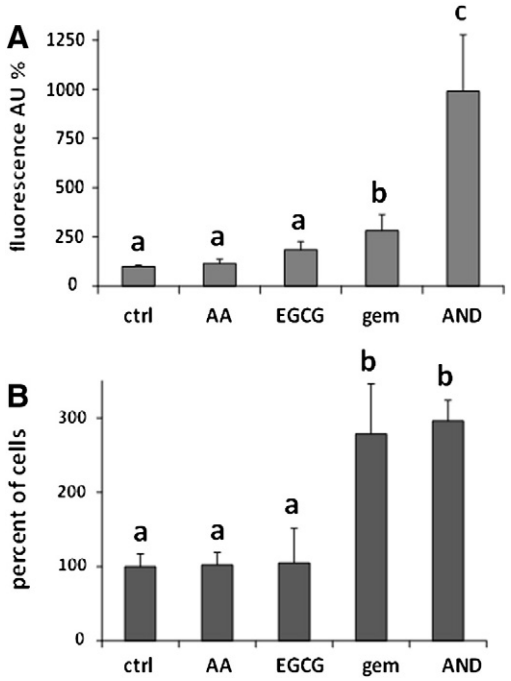

Fig. 1. A. Caspase 3 activity, measured in REN cells by the Cayman Caspase-3 Fluorescence kit, after exposure for 24 h, to 228 μM ascorbate (AA), 20 μM EGCG (epigallocatechin-3-gallate), or 9 μM gemcitabine (gem), or alternatively, after exposure to triple ascorbate/EGCG/gemcitabine combination (AND), with individual concentrations equivalent to one third of the above doses. Data are means ± SD, derived from 8 independent treatments, expressed as fluorescence arbitrary units (see Materials and methods section). Different letters on bars indicate statistical significance. Values with different letters are statistically different according to the Tukey's test ($p < 0.01$). B. Cells were treated as above, stained with annexin V-FITC/propidium iodide for apoptosis assay and analyzed by flow cytometry (see Materials and methods section). Data are means ± SD ($n = 6$) of the number of cells in early or late apoptosis. The value of control has been set to 100%. Statistics as in A.

(Bertino et al., 2008). In addition, we aimed at detecting apoptosis- and cell cycle-related molecular targets of AND by subjecting the mRNA of MMe cells to compatible PCR arrays.

Materials and methods

Reagents and solutions

(−)-Epigallocatechin-3-gallate (EGCG) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA); gemcitabine (2′-deoxy-′, 2′-difluorocytidine monohydrochloride) was from Ely Lilly Italia SpA (Sesto Fiorentino, Italy). All other reagents were from Sigma (St. Louis, MO, USA), unless otherwise specified.

Cell culture

REN cells are a p53-mutant deriving from MMe epithelial type (Ranzato et al., 2009; Smythe et al., 1994). These cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% antibiotic mixture (Gibco, Invitrogen Life Technologies, S. Giuliano Milanese, Italy), and maintained at 37 °C in humidified atmosphere with 5% CO_2.

Caspase-3 assay

Caspase-3 activity was determined in cell lysates by a fluorimetric assay kit (Caspase-3 Fluorescence kit, Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. 96-Well plate fluorescence was read in the Infinite 200 Pro microplate reader (Tecan, Wien) by using 485-nm excitation and 535-nm emission filters.

Flow cytometric analyses

Cell cycle analysis was performed by propidium iodide staining for DNA content and flow cytometric analysis. Briefly, cells ($2 \cdot 10^6$) were collected by centrifugation at 300 *g* for 10 min, washed with 10 mM sodium phosphate buffer, pH 7.5, containing 0.14 M NaCl (PBS), fixed in 1 mL of 70% ethanol in PBS, and stored at −20 °C overnight. Cell suspensions were washed three times with PBS and treated for 30 min at room temperature with PBS containing 0.1 mg/mL RNAse, and with 40 μg/mL propidium iodide (PI). Stained cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

For annexin V/PI assay, cells were stained with annexin V-FITC and PI, and evaluated for apoptosis by flow cytometry, according to the manufacturer's protocol (Bender System, Wien, Austria). Cells were washed twice with PBS and stained with 5 μL of annexin V-FITC in 195 μL Binding buffer for 10 min at room temperature. Cells were then washed with PBS and resuspended in 190 μL Binding buffer and stained with 10 μL of PI. Apoptotic cells were determined using the FACSCalibur. Both early apoptotic (annexin V-positive, PI-negative) and late apoptotic (annexin V-positive and PI-positive) cells were included in cell death determinations.

Measurements of free cytosolic Ca^{2+}

Variations of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were evaluated as previously described (Ranzato et al., 2011). Briefly, cells were plated on glass-base dishes (Iwaki Glass Inc., Tokyo, Japan), loaded with the fluorescent Ca^{2+} probe fluo 3-AM (20 μM) in loading buffer, washed, and examined by confocal time-lapse analysis with a Zeiss LSM 510 confocal system (Carl Zeiss) (exc 488 nm, em 505–550 nm). Cells were observed through a 20× Plan-Neofluar Zeiss objective (0.5 NA).

Western blotting

Western blotting was carried out as previously described (Ranzato et al., 2011). Amounts of 100 μg protein from cell lysates were subjected to SDS-PAGE (12% gel), blotted to nitrocellulose membrane, probed with primary monoclonal antibody against human DAPK2 (cat. N. D3069, Sigma Aldrich), dilution 1:500, incubated with horseradish peroxidase-conjugated secondary antibody (Bethyl Laboratories, Montgomery, TX, USA; dilution 1:1000), developed by an ECL kit (Millipore, Billerica, MA), acquired by the ChemiDoc XRS equipment (Bio-Rad Laboratories, Hercules, CA), and digitized with the Quantity One Imaging system (Bio-Rad). Equal loading was confirmed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The degree of NFκB activation was assessed by the NF-kappaB Activation Assay Kit (FIVEphoton Biochemicals, San Diego, CA, USA) according to the manufacturer's instructions. The presence of NF-κB-p65 within the cytoplasmic and nuclear fractions of REN cells was detected via Western blotting by using an anti-p-65 antibody (1:400), followed by hybridization to goat anti-rabbit IgG-HRP secondary antibody (1:3000). Peptide bands were acquired and digitized as above.

RNA isolation, cDNA synthesis and PCR array analysis

NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) was used to purify total RNA. Complementary DNA was synthesized from RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Penzberg, Germany). Finally, cDNA was mixed with the Sybr green master mix (FastStart SYBR Green Master, Roche Diagnostics), and loaded onto the Human Apoptosis 96 StellArray™, or onto the Human Cell Cycle Tox and Cancer 96 StellArray™ kits (Lonza, Rockland, ME, USA), on a Bio-Rad CFX384™ real-time PCR detection system. A list of the 96 genes included in each array is available online at https://array.bhbio.com/BHB/GUI/SP/PlateDetails.aspx. Data from three independent