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Negative modulation of mitochondrial oxidative phosphorylation by epigallocatechin-3 gallate leads to growth arrest and apoptosis in human malignant pleural mesothelioma cells

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ABSTRACT

Increasing evidence reveals a large dependency of epithelial cancer cells on oxidative phosphorylation (OXPHOS) for energy production. In this study we tested the potential of epigallocatechin-3-gallate (EGCG), a natural polyphenol known to target mitochondria, in inducing OXPHOS impairment and cell energy deficit in human epitheliod (REN cells) and biphasic (MSTO-211H cells) malignant pleural mesothelioma (MMe), a rare but highly aggressive tumor with high unmet need for treatment. Due to EGCG instability that causes H_2O_2 formation in culture medium, the drug was added to MMe cells in the presence of exogenous superoxide dismutase and catalase, already proved to stabilize the EGCG molecule and prevent EGCG-dependent reactive oxygen species formation. We show that under these experimental conditions, EGCG causes the selective arrest of MMe cell growth with respect to normal mesothelial cells and the induction of mitochondria-mediated apoptosis, as revealed by early mitochondrial ultrastructure modification, swelling and cytochrome *c* release. We disclose a novel mechanism by which EGCG induces apoptosis through the impairment of mitochondrial respiratory chain complexes, particularly of complex I, II and ATP synthase. This induces a strong reduction in ATP production by OXPHOS, that is not adequately counterbalanced by glycolytic shift, resulting in cell energy deficit, cell cycle arrest and apoptosis. The EGCG-dependent negative modulation of mitochondrial energy metabolism, selective for cancer cells, gives an important input for the development of novel pharmacological strategies for MMe.

1. Introduction

Mitochondrial bioenergetics and dynamics and their proper regulation are crucial for tumor cell growth. Under normal conditions, cells rely on mitochondrial oxidative phosphorylation (OXPHOS) to provide energy for cellular activities. Cancer cells are generally characterized by a strong enhancement of aerobic glycolysis, the so-called Warburg effect, often associated to the decrease in mitochondrial respiration and OXPHOS [1]. However, it is becoming increasingly evident that enhanced OXPHOS occurs in a variety of tumors and consequently mitochondria could be a promising target for the development of efficient anticancer therapies [2–4].

Consistently, it has been recently reported that mitochondria-targeted molecules, able to disrupt mitochondrial architecture and induce swelling, are effective to reduce the growth of epithelial tumor cells, such as human breast cancers [5] and malignant mesothelioma (MMe) [6]. MMe is a rare, but with an increasing incidence, insidious and highly aggressive cancer with poor prognosis [7]; due to its high drug resistance, despite the reported improvements in the clinical management, the unmet need for malignant mesothelioma treatment is high. Thus, in the light of current failures in MMe therapies, attempts to induce cell energy deficit can be an attractive strategy to reduce MMe tumor growth.

In this study we aimed at determining whether epigallocatechin-3-gallate (EGCG), known to be a mitochondrial-targeted drug [8,9], is able to induce mitochondrial energy deficit and a selective growth arrest of MMe cells. EGCG, a natural polyphenol component of green tea, has been extensively studied for its anticarcinogenic effect in a wide variety of cancer cells (for Refs. see [10]) and it has been shown

Abbreviations: CAT, catalase; cyt *c*, cytochrome *c*; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; EGCG, epigallocatechin-3-gallate; GLU, glutamate; MAL, malate; MMe, malignant mesothelioma; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SOD, superoxide dismutase; STAT3, phospho-signal transducer and activator of transcription 3; SUCC, succinate; XTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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to interact with a large set of protein targets [11] modulating a variety of cell signaling pathways (for Refs. see [12]). Although EGCG targets mitochondria in tumor cells [9], little is known about the effect of EGCG on mitochondrial function in cancer [13,14].

Another open question is the proper use of EGCG for *in vitro* studies necessary to characterize its mechanism of action. It has been shown that EGCG induces apoptosis in a variety of cultured cancer cell types, including MMe cells, through its pro-oxidant activity [15–18]. However, there is evidence that some of the cytotoxic effects of this compound may be related to its instability under culture conditions, since, as a result of its auto-oxidation, oxidative products and reactive oxygen species (ROS) are formed in the extracellular phase. The addition of superoxide dismutase (SOD) and catalase (CAT) to the culture medium has been shown to stabilize EGCG and to increase its half-life to more than 24 h [19–21].

In this study, we emphasize the need to stabilize EGCG in order to obtain a selective inhibitory action on human MMe-derived REN cells. Our results disclose for the first time a novel mechanism by which EGCG, through the early impairment of mitochondrial respiratory chain (MRC) complexes and ATP synthase, induces a strong cell energy deficit followed by cell cycle arrest in G2/M phase and mitochondria-mediated apoptosis in MMe cells.

2. Materials and methods

2.1. Cell cultures and materials

The epithelioid MMe derived REN cell line, used as the principal experimental model in this investigation, is a tumourigenic, p53-mutant, epithelial subtype [22]. It was characterized and kindly provided by Dr. Albelda S.M. (University of Pennsylvania, Philadelphia; PA) and characterized [23]. MSTO-211H, derived from biphasic mesothelioma, and mesothelial MET5A cell lines were obtained from Istituto Scientifico Tumori (IST) Cell-bank, Genoa, Italy. Cells were cultured at 37 °C in humidified 5% CO_2/95% air in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) (GIBCO/BRL). Cells were subjected to a 1:3 split every 3 days.

All reagents used were from Sigma-Aldrich unless otherwise specified.

2.2. EGCG treatment

EGCG – extracted from green tea leaves with a purity >95% (Sigma-Aldrich) – was freshly prepared for each experiment at 20 mM concentration in PBS. Cells were seeded into 10-cm Petri dishes, 6-well or 96-well plates, according to the experiment, and cultured until they have reached 60–70% confluence (about 24 h). For dose-dependent experiments, cells were treated with EGCG (from 20 to 200 μM) added to the fresh culture medium for 24 h in the absence or presence of SOD (5 units/ml) and CAT (30 units/ml) [20]. For time-dependent experiments, the medium containing 100 μM EGCG plus SOD and CAT was replaced after 24 h of incubation.

2.3. Proliferation assays

Cell proliferation assay was performed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (XTT, Cell Proliferation Kit II, Roche). 6000 cells per well were seeded into a 96-well plate and incubated over-night at 37 °C in a CO_2 incubator to allow adherence. To assay cell proliferation, 50 μl of XTT labeling mixture were added to each well and the plate incubated at 37 °C for 2–4 h following the manufacturer's instructions. Formazan dye accumulation produced by metabolically active cells was monitored by reading absorbance at 490 nm, with a reference wavelength at 655 nm, by using the microplate reader mod. 680 (Bio-Rad).

For cell count experiments, cells were seeded at a density of 1×10^6 cells in 10 cm culture plates and incubated over-night at 37 °C. Cells were then trypsinized, stained with Trypan blue and counted in a Bürker haemocytometer within 5 min after staining.

2.4. ROS detection

The pro-oxidant dose-dependent response of EGCG was checked by measuring H_2O_2 concentration in the culture medium after 1 h of incubation with different EGCG concentrations either in the absence or presence of SOD (5 units/ml) plus CAT (30 units/ml), by using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes).

Quantitative analysis of intracellular ROS was performed by means of an LS50 Perkin Elmer spectrofluorimeter, using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), a non-fluorescent dye which is hydrolyzed inside the cells and reacts with multiple types of ROS, mainly H_2O_2, to give the fluorescent product, dichlorofluorescein (DCF) [24]. Cultured cells were incubated with 5 μM DCFH-DA for 30 min under growth conditions, washed and suspended in PBS. Fluorescence emission was recorded at the excitation–emission wavelengths of 488 and 520 nm, respectively and normalized to the protein content to determine the relative ROS production. Protein concentration was quantified by using a modified Bradford assay (Bio-Rad protein assay) according to the manufacturer's instructions.

ROS were visualized in live REN cells by using laser scanning confocal microscopy imaging. Cells were cultured at low density on fibronectin-coated 35-mm glass-bottom dishes and treated with 100 μM EGCG in the presence or absence of SOD and CAT. After 24 h, cells were incubated for 20 min at 37 °C with both 3 μM DCFH-DA and 3 μM MitoSOX™ (Molecular Probes), a selective mitochondria-targeted probe, specific for superoxide anion [25]. After washing with PBS, stained cells were examined under a Leica TCS SP5 II microscope (images collected using a 60× objective). The green fluorescence of oxidized DCF was analyzed by exciting the sample with a Diode 405 laser (λex 488 nm); the red fluorescence of MitoSOX was analyzed by exciting the sample with a HeNe laser 543 (λex 543 nm).

2.5. Cell cycle analysis

Cell cycle analysis was carried out by seeding 5×10^5 cells per well in 96-well cell culture plates in the presence or in the absence of 100 μM EGCG plus SOD (5 U/ml) and CAT (30 U/ml) for 1, 3, 6, 18 and 24 h at 37 °C in a 5% CO_2 atmosphere.

After incubation, adherent cells were detached with trypsin (0.5% trypsin/0.1% EDTA in PBS), harvested in complete RPMI and centrifuged at 500 ×*g* for 10 min. Pellets were washed with PBS and fixed with ice-cold 75% ethanol overnight at 4 °C, treated with 100 μg/ml RNase A, and subsequently stained with 25 μg/ml propidium iodide. Samples were analyzed by using a flow cytometer FACS (Becton Dickinson) and ModFit software (Verity Software House).

2.6. Apoptosis assessment

Cells were plated in 96-well culture plates at 6000 cells per well and incubated over-night at 37 °C in a CO_2 incubator to allow adherence. After 24 h, cells were exposed to EGCG in a culture medium containing SOD and CAT for an additional 48 h. Apoptosis was measured with the Cell Death Detection ELISA (Roche), which was performed according to the manufacturer's protocol. The amount of histone-associated-DNA-fragments (mono- and oligonucleosomes) was assayed in the cytoplasmic cell fraction by measuring absorbance at 405 nm, with a reference wavelength at 655 nm, using the microplate reader mod. 680 (Bio-Rad).

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