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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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 epithelial (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 H2O2 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.

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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 a strong enhancement of aerobic glycolysis, the so-called Warburg effect, which 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.
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 MM-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 METSA cell lines were obtained from Istituto Scientifico Tumori (IST) Cell-bank, Genoa, Italy. Cells were cultured at 37 °C in humidified 5% CO2/95% 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-dimethyl-thiazol-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 CO2 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 × 10⁶ 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 H2O2 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’-dichlorodihydrofluorescein diacetate (DCFH-DA), a non-fluorescent dye which is hydrolyzed inside the cells and reacts with multiple types of ROS, mainly H2O2, 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 wavelength 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 × 10⁵ 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% CO2 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 CO2 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).
2.7. Immunoblot analysis

Cells were lysed with 0.1% Triton X-100 in PBS in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates (0.05 mg protein) were resolved by a 10%–12% SDS-polyacrylamide gel (depending on the molecular weight) and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with TBS-T (50 mM Tris, 150 mM NaCl, 0.02% Tween 20, pH 7.5) containing 5% non-fat milk and probed with primary antibodies overnight at 4 °C. Immunoblot analysis was performed, essentially as described in [26], using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence Western blotting reagents (Amersham, Pharmacia Biotech). Membranes were also probed with anti-β-actin antibody as internal loading control and densitometry values of immunoreactive bands for each sample were normalized versus the corresponding densitometry value of β-actin.

2.8. Preparation of mitochondrial and cytoplasmic protein fractions

Cells were trypsinized, washed with ice-cold PBS and centrifuged at 800 × g for 5 min. Cell pellets were lysed in Buffer A consisting of 20 mM HEPES-Tris (pH 7.6), 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 250 mM sucrose, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM sodium orthovanadate. Samples were then homogenized with a Dounce homogenizer (20 strokes for 30 min on ice) and centrifuged twice at 800 × g for 5 min to remove nuclei and unlysed cells. The final supernatant was centrifuged at 13,000 × g for 15 min to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant).

Supernatants were then centrifuged at 10,000 × g for 90 min, to obtain a clean cytosolic fraction, while the mitochondrial fraction was solubilised in Buffer B (1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 10 mM benzamidine, 1 mM PMSF and 0.2 mM sodium orthovanadate) for 30 min on ice.

Mitochondrial and cytosolic fractions were centrifuged at 13,000 × g for 10 min and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad). Both sources. Then ADP (0.5 mM) was added and the reduction of NADP+, plus rotenone (3 μM) and succinate (3 μM) plus rotenone (3 μM) and succinate (3 μM) was measured enzymatically using NADPH and diaphorase coupled enzymes to ensure the presence of either glutamate plus malate (GLU/MAL, 5 mM each) or succinate (SUC, 5 mM) plus rotenone (3 μM) was measured enzymatically using NADPH and diaphorase coupled enzymes to ensure the presence of either glutamate plus malate (GLU/MAL, 5 mM each) or succinate (SUC, 5 mM) plus rotenone (3 μM).

2.10. Measurement of MRC complex activities

Measurements of MRC complex activities were carried out in mitochondrial membrane-enriched fractions from cultured cells. Aliquots of trypsinized cells were washed with ice-cold PBS, frozen in liquid nitrogen and kept at −80 °C until use. Isolation of mitochondrial membrane-enriched fractions was carried out as described in [28] and measurement of MRC complex activities performed essentially as in [29], by three assays which rely on the sequential addition of reagents to measure the activities of: i) NADH:ubiquinone oxidoreductase (complex I) followed by ATP synthase (complex V), ii) succinate:ubiquinone oxidoreductase (complex II) and iii) cytochrome c oxidase (complex IV) followed by cytochrome c oxidoreductase (complex III).

2.11. Measurement of mitochondrial ATP production

Cells were detached from a 10-cm plate by trypsin treatment, washed with PBS and suspended in ice-cold sucrose medium (0.25 M sucrose, 10 mM Tris-HCl pH 7.2, 1 mM EGTA). To measure the mitochondrial ATP production, cells (1 mg of protein) were incubated at 37 °C in 2 ml of the respiration medium (210 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 5 mM KH2PO4/K2HPO4 pH 7.4, 3 mM MgCl2 and 5 mg/ml BSA) and permeabilized with digitonin (0.01% w/v for 5 min). Then the ATP detecting system (2.5 mM glucose, 2 U hexokinase, 1 U glucose 6-phosphate dehydrogenase, 0.25 mM NADP+) was added in the presence of 10 μM diadenosine pentaphosphate (Ap5A), used to inhibit adenylyl kinase; the measurement was made in the presence of either glutamate plus malate (GLU/MAL, 5 mM each) or succinate (SUC, 5 mM) plus rotenone (3 μM), used as respiratory energy sources. Then ADP (0.5 mM) was added and the reduction of NADP+, which reveals ATP formation, was monitored as an increase in absorbance at 340 nm. Care was taken to use enough hexokinase/glucose 6-phosphate dehydrogenase coupled enzymes to ensure a not-limiting ADP-regenerating system for the measurement of ATP production.

2.12. Measurement of cellular ATP content

Cells were detached from a 10-cm plate by trypsin treatment, washed with PBS and cellular ATP content was assayed in cell extracts obtained by the boiling water procedure as described in [30] by enzymatic procedure as described in [31].

2.13. Measurement of extracellular L-lactate levels

REN cells were plated in 5-cm dishes, grown until they reached sub-confluence and incubated with 100 μM EGCG plus SOD and CAT for 1, 3, 6, and 24 h. The extracellular L-lactate levels were measured in the culture medium by using the L-lactate dehydrogenase method, which gives a reliable estimate of L-lactate production inside the cells [32].

3. Results

3.1. EGCG, stabilized by SOD and CAT, selectively inhibits MMe REN cell growth and impairs mitochondrial bioenergetics in a dose-dependent manner

In order to ascertain the capability of EGCG to selectively inhibit MMe cell growth, we first examined the dose-response effect of EGCG treatment on MMe REN cell proliferation in comparison to that produced on normal mesothelial MET5A cell proliferation, both in the absence or presence of SOD and CAT used to prevent EGCG auto-oxidation [18–21]. As shown in Fig. 1A, 24 h-treatment with EGCG selectively inhibited the growth of the MMe REN cells in a dose-dependent manner when incubated in the presence of SOD/CAT, with an estimated IC50 of 100 μM, as measured by XTT assay; conversely, the viability of the normal mesothelial MET5A cells was not affected by EGCG in the presence of SOD/CAT. On the contrary, EGCG proved to inhibit cell growth of both REN and MET5A cells when added in the absence of SOD/CAT (Fig. 1A).

Importantly, in the absence of SOD/CAT, EGCG induced a dose-dependent cell death in both REN and MET5A cells (Fig. 1B). On the contrary in the presence of SOD/CAT, stabilized EGCG was still able to induce cell apoptosis in MMe REN cells at 100 μM and 200 μM, although to a less extent than in the absence of SOD/CAT, but completely lost its pro-apoptotic activity in normal MET5A cells (Fig. 1B).
Consistently, the phase-contrast microscopy analysis of REN cells treated with 100 or 200 μM EGCG in the absence or presence of SOD/CAT showed a reduction in cell density, several cellular debris and signs of cellular suffrage and in addition, EGCG-treated REN in the absence of SOD/CAT showed several cytoplasmic vacuoles (Fig. 1C). Similar changes occurred in normal MET5A cells treated with EGCG alone, whereas in the presence of SOD/CAT, even up to 200 μM EGCG, cells were well spread and with a flattened morphology, as in non-treated controls.

Since the observed cytotoxic effect of EGCG in the absence of SOD/CAT could depend on ROS production due to its auto-oxidation [17–21], H$_2$O$_2$ concentration was measured in the culture medium of both EGCG-treated REN and MET5A cells as a function of EGCG concentration (Fig. 1D). An increase in extracellular H$_2$O$_2$ concentration was already detectable in control cells, and dramatically increased in EGCG-treated REN cells (Fig. 1D). The presence of SOD/CAT completely prevented the cytoplasmic ROS accumulation, while the EGCG-triggered overproduction of superoxide anion by mitochondria remained still substantial.

Since it is well established that the impairment of the mitochondrial respiratory chain complexes, in particular complexes I and III, induces superoxide anion formation [33], we hypothesized that the overproduction of mitochondrial ROS in REN cells treated with EGCG stabilized by SOD/CAT (see Fig. 1E) was a consequence of an EGCG-mediated mitochondrial dysfunction. The functional analysis of the mitochondrial respiratory...
chain (MRC) complexes revealed a generalized dose-dependent decline in complex activities 24 h after the treatment of REN cells with EGCG stabilized by SOD/CAT (Fig. 2A). Moreover, a remarkable decline in ATP production via mitochondrial OXPHOS was observed upon the addition of the respiratory substrates GLU/MAL or SUCC (Fig. 2B). In addition, the cellular ATP level was decreased in a dose-dependent manner by the stabilized EGCG, becoming 50% lower than that of the untreated cells, with 100 μM EGCG (Fig. 2C). It is interesting to note that no change in both MRC complex activities and ATP cellular level was observed in normal MTS5A cells incubated with 100 μM EGCG stabilized with SOD/CAT (not shown).

Altogether these data demonstrate that a 24 h-treatment of REN cells with stabilized 100 μM EGCG, induces a significant impairment of the MRC function and a decline in the ATP production, selectively in MMe REN cells.

All the further analysis of EGCG-dependent effects on MMe cells were carried out incubating REN cell with 100 μM EGCG in the presence of SOD and CAT.

3.2. EGCG induces cell cycle arrest in G2/M phase and mitochondria-mediated apoptosis in MMe REN cells

To further investigate EGCG-dependent growth inhibition in MMe REN cells, cell cycle distribution analysis was performed during the 24-hour period of EGCG treatment (Fig. 3A). In an early phase (1–6 h) of the treatment no significant changes were observed in cell cycle distribution with respect to untreated control cells. After 18/24 h, instead, the REN surviving population in G2/M phase increased about 3-fold as compared to untreated cells whose G2/M phase population remained unchanged during all the time period investigated (not shown). The arrest in G2/M was associated with a concomitant decrease in the percentage of cells in G0/G1 phase (Fig. 3A).

In parallel, we also evaluated the cell number and apoptosis in EGCG treated REN cells. After prolonged exposure to EGCG (48 h), a 2-fold decrease in cell number (Fig. 3B) and an increase in cell apoptosis (Fig. 3C) occurred with respect to untreated cells.

Since mitochondria are known to play a central role in eliciting apoptosis in response to many triggers, including EGCG [13,34,35] and being the mitochondrial release of cytochrome c (cyt c) an early step in the time course of the events leading to mitochondria-mediated apoptosis [36], we analyzed the distribution of cyt c between mitochondrial and cytosolic fractions during the 24 hour period of EGCG-treatment in REN cells. The release of cyt c from mitochondria to the cytosol started 6 h after EGCG addition. Cytosolic cyt c level reached about 3-fold with respect to controls 24 h after the treatment, as shown by immunoblotting analysis (Fig. 3D and E).

Ultrastructural observations under transmission electron microscope have been performed to analyze the early and late effects of EGCG treatment on subcellular cytoarchitecture and mitochondria. Control REN cells (treated with SOD/CAT from 1 to 48 h) showed an electron dense cytoplasm rich in ribosomes with several mitochondria, one or two Golgi apparatus and rare endoplasmic reticulum cisternae (Fig. 4A, B). Most of mitochondria showed a dense expanded matrix and medium/small intracristae compartments, which extend parallel to the organelle main axis (Fig. 4 A, B). No ultrastructural differences with respect to controls were observed in REN cells treated with the stabilized EGCG for 1 h (data not shown). Differently, 6 h after treatment, an accurate analysis of mitochondrial morphology revealed an initial
3.3. EGCG early affects activity and protein levels of MRC complexes inducing 6 h after EGCG treatment (see Fig. 4), when neither changes in REN data shown in Fig. 3C. and mitochondrial protein markers, respectively, were also analyzed. The levels of cyt detects DNA fragments within immunocaptured nucleosomes. Graph is representative of three independent experiments. (B) Representative immunoblotting and (E) densitometric experiments. (B) Cell survival at different times. Graph is representative of six independent experiments. (C) Cell apoptosis measured with the Cell Death Detection ELISA (Roche), which time periods. (A) DNA histograms indicate cell cycle kinetics. The percentage of cells in G1/G0, S and G2/M phases is indicated. Data shown are representative of three independent ex-

The early ultrastructural modification of mitochondria occurring at 6 h after EGCG treatment (see Fig. 4), when neither changes in REN cell growth nor cell apoptosis were observed (see Fig. 3 A–C), prompted us to investigate whether EGCG caused early specific alterations in the mitochondrial functions. The measurement of MRC complex activities in mitochondrial membrane-enriched fractions from EGCG-treated REN cells during the 24 hour period of treatment showed that a significant reduction in MCR activity already occurred 3 h after the treatment for complex I, II, IV and ATP synthase (complex V). The activity of all complexes was reduced of about 50% 18 h after the treatment (Fig. 5A).

To verify whether the EGCG inhibitory effect on the activity of MRC complexes was associated with changes in their content, the levels of some OXPHOS complex subunits were analyzed (Fig. 5B). In line with the functional data, a shared pattern of decrease in the levels of subunits of all OXPHOS complexes was observed in EGCG-treated REN cells starting from 3 h after EGCG incubation and involving particularly the subunits of complexes I, II and V (Fig. 5C), as well as complex IV, as shown in a representative immunoblot (Fig. 5B), and in the statistical analysis of densitometry values (Fig. 5C).

To give some insight into the molecular basis of this early MRC complexes deficit in EGCG-treated REN cells, we investigated the protein levels of the phospho-signal transducer and activator of transcription 3 (STAT3) that is known to regulate MRC complex expression and activity [37] and has been recently reported to be down-regulated by EGCG in human pancreatic cancer cells [38]. Interestingly, as shown by the
immunoblot analysis (Fig. 5D and E), a 50% decrease in STAT3 protein level was found starting from 3 h after treatment with EGCG with respect to untreated REN cells.

To assess whether the alterations in MRC complex activities and protein level have consequences on energy status, the cellular ATP level was measured in EGCG-treated REN cells. Interestingly, a marked elevation of cellular ATP amount was found 1 h after EGCG treatment and was maintained up to 3 h. The ATP amount then gradually declined, dropping under the level of controls at 24 h (Fig. 6A). This drug-induced ATP surge is not unusual in cells en route to apoptosis and could derive from both OXPHOS and anaerobic glycolysis [39,40]. To check this, we measured both the mitochondrial ATP production and the extracellular release of L-lactate, the end-product of glycolysis, as a function of EGCG incubation time. We found that the rate of mitochondrial ATP production induced by complexes I and II substrates (MAL/GLU and SUCC, respectively) increased after 1 h of EGCG incubation and progressively decreased thereafter, declining under control values at 3 h of incubation (Fig. 6B). In parallel, we found that levels of L-lactate strongly increased after 3 h of EGCG treatment (insert of Fig. 6C) remaining slightly higher with respect to untreated cells at all the following incubation times (Fig. 6C).

**3.4. EGCG inhibits mitochondrial metabolism inducing cell energy deficit and apoptosis in biphasic MMe MSTO-211H cells**

In order to confirm the efficacy of EGCG on MMe cell proliferation and the molecular mechanism by which it acts in these cells, we checked whether and how 100 μM EGCG stabilized with SOD/CAT induces cell death in MSTO-211H, a biphasic malignant cell line more aggressive than the other MMes [41].

Similarly to REN cells, the exposure of MSTO-211H to EGCG up to 48 h inhibited cell growth (Fig. 7A) and determined an increase in cell apoptosis as compared to untreated cells of 1.6- and 3-folds 24 and 48 h after EGCG treatment, respectively (Fig. 7B). Likewise, analysis of mitochondrial energy metabolism of EGCG-treated MSTO-211H cells, showed that both the mitochondrial ATP production induced by complexes I and II substrates (Fig. 7C) and the cell ATP content (Fig. 7D), increased 1 h after EGCG treatment and then progressively reduced up to 24 h. We show that in MSTO-211H, as in the case of REN cells, EGCG acts via an early impairment of the MRC complexes, being their activity, in particular that of complex I, II and ATP synthase, already decreased 3 h after the treatment and reaching a 50% inhibition 24 h after treatment (Fig. 7E).
4. Discussion

EGCG is a very multifunctional molecule known for its beneficial effects in a broad range of diseases including neurodegenerative and cardiovascular diseases [42–45], as well as cancer (for Refs. see [10,46]). In pathological conditions in which a deficit in OXPHOS is present, as in the case of Down syndrome, EGCG exerts a cytoprotective effect at low concentrations (10–20 μM), activating the mitochondrial energy metabolism [43]. Conversely, in several cancer types EGCG, used at higher concentrations, becomes cytotoxic and exerts an inhibitory action on cell proliferation [10,46,47]; however, studies on the effect of EGCG on mitochondrial energy metabolism in cancer cells are lacking. Here we report a molecular and functional study which shows for the first time that EGCG, in vitro stabilized with SOD/CAT, selectively induces apoptosis in...
MMe cells, by decreasing MRC complex protein level and activity, impairing mitochondrial energy metabolism and leading to cellular ATP deficit and cell cycle arrest.

It has been recently shown that EGCG can undergo auto-oxidation causing a dose-dependent ROS production leading to cancer cell apoptosis [15–21]. In the present study, we show that EGCG added to cell culture medium induces a dose-dependent H$_2$O$_2$ formation and decreases the proliferation of both MMe REN and normal mesothelial MET5A cells to indicate that H$_2$O$_2$ generated from EGCG auto-oxidation might be responsible for its non-selective cytotoxicity. EGCG stabilized by SOD and CAT [20,21] which prevent H$_2$O$_2$ formation in culture medium, induces a selective inhibition of MMe REN cell growth with an IC$_{50}$ value of 100 μM, without effects on MET5A cells. In addition, since SOD and CAT cannot cross the plasma membrane, the significant decrease in H$_2$O$_2$ intracellular levels observed in the presence of 100 μM EGCG plus SOD/CAT suggests that the stabilized EGCG acts as a powerful intracellular antioxidant and that the selective inhibition of MMe REN cell growth by EGCG takes place preventing pro-radical activity of the drug by its stabilization. Of course under this condition, i.e. in the absence of extracellular and cytoplasmic ROS, there is a slower induction of cell death than in the presence of non-stabilized EGCG. But in this latter case we cannot surely affirm that the induction of apoptosis, which occurs also in normal mesothelial MET5A cells, was due to EGCG or to its auto-oxidation products which produce themselves ROS. These results should be considered in future studies attempting to elucidate the mechanisms of action of EGCG in vitro systems also in the light of the observation that oxidation of EGCG does not seem to occur in vivo since none of EGCG oxidation products were found in the plasma samples of mice after treatment with EGCG [48].

It should be noted that, as reported in lung cancer cells treated with EGCG stabilized with SOD/CAT [17], a still evident amount of mitochondrial ROS is observed in stabilized EGCG-treated REN cells, probably due to the mitochondrial dysfunction. Consistently, EGCG treatment of MMe REN cells for 24 h results in a dose-dependent impairment of MRC complex activity. Here we provide indications aiming to account for the impairment of MRC complexes found in EGCG-treated MMe REN cells. In an early phase (3–6 h) of treatment, EGCG promotes a significant general decrease in both activity and protein levels of MRC complexes, suggesting a regulation at a transcriptional/translational level. Accordingly, we found a down-regulation by EGCG of STAT3, a transcription factor known to be involved in the regulation of MRC complex expression and activity [37]. STAT3 is a proto-oncogene constitutively activated in diverse human cancers including malignant mesotheliomas [49,50] and known to play a critical role in tumor cell survival, proliferation, migration, invasion, angiogenesis and inhibition of apoptosis [50,51]. Although some studies reported STAT3 as a negative regulator of MRC complexes [52,53], recently STAT3 was shown to support oncogenic transformation sustaining MRC activity and blocking mitochondrial permeability transition pore [37,54,55]. Consistently with our results, it has also been reported that EGCG induces a decrease in STAT3 protein level inhibiting its downstream signaling pathway and inducing apoptosis in human pancreatic cancer [37]. Here we show that a down-regulation of STAT3 occurs as a very early event following EGCG treatment suggesting STAT3 as a primary target of this molecule in MMe REN cells. Further studies are required to verify whether the EGCG-dependent STAT3 down-regulation correlates with the impairment of MRC complexes.

EGCG-treated REN cells show a significant shortage of mitochondrial ATP production, as well as a marked decline in cellular ATP levels. Given that a decrease in ATP levels likely affects a variety of vital cellular processes, including transcription, translation and signal transduction, it is noticeable to find REN cell cycle arrest. Furthermore, following the cell

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Fig. 6. Energy status in EGCG-treated MMe REN cells. Cells were treated with 100 μM EGCG in presence of SOD and CAT and collected at the indicated time periods. (A) ATP cellular levels were measured in cell extracts and expressed as nmol/10^6 cells. (B) The rate of mitochondrial ATP synthesis was measured spectrophotometrically as described in the Materials and methods, in the presence of either glutamate/malate (GLU/MAL) or succinate (SUCC) and expressed as nmol/min/10^6 cells. (C) Levels of L-lactate were measured in culture medium in both untreated and EGCG-treated REN and expressed as μmol/10^6 cells. The inset shows the ratio between the mean values of L-lactate level found in EGCG-treated and untreated cells. Graphs are representative of four independent experiments. Each bar represents mean ± SD. Significant differences between untreated and treated cells are indicated with asterisks (* = P < 0.05).
cycle arrest, EGCG-treated MMe REN cells show typical signs of apoptosis, such as histone-associated DNA fragmentation and nuclear chromatin margination. Drug-induced G2/M arrest followed by apoptosis occurs in a variety of tumor types of both epithelial and hematologic cell origin [56]. Interestingly, ultrastructural mitochondrial changes, such as cristae opening and mitochondrial swelling, as well as cyt c release by mitochondria, occur only 6 h after EGCG treatment when no signs of apoptosis are detectable, indicating that EGCG induces mitochondrial-mediated apoptosis in MMe REN cells.

The results reported in this paper shed a new light on some aspects of energy metabolism in EGCG-treated MMe cells. In the early phase of EGCG treatment (1–3 h), an increase in the ATP level occurs which derives from both anaerobic glycolysis and oxidative phosphorylation, as revealed from the increased L-lactate levels and the still efficient mitochondrial ATP production. This early cellular ATP surge might be a trigger to initiate the programmed cell death [39,40] or even a cytoprotective mechanism of resistance to chemotherapeutic agents [57]. In the late phase of EGCG treatment (18–24 h), when the oxidative phosphorylation is strongly impaired, cellular ATP level strongly decreases, suggesting that the energy shift towards glycolysis is no more sufficient to maintain the energy demand, and MMe cells proceed towards cycle arrest and then apoptosis.

These data have been confirmed in cells derived from biphasic MMe, the MSTO-211H cell line which appeared to be even more sensitive than REN (epithelioid) MMe cell line to the growth-inhibitory effect of EGCG, probably due to the highly aggressive nature of MSTO-211H cells [41].
Interestingly, MSTM-211H cells showed a very high mitochondrial me-
tabolism being both mitochondrial ATP production and ATP cellular
level in basal condition (i.e. without EGCG treatment) about 4-fold
higher than that observed in REM (compare Figs. 7 and 6) sup-
porting the hypothesis of a correlation between enhancedOXPHOS and tumor
proliferation in MMe as described in other tumor types [2–4].

Our results provide evidences supporting the use of EGCG as adjuvant
in combination with other chemotherapeutic agents with proven efficacy
on MMe cells such as gemcitabine, as proposed in [58], to ob-
test in exper-
imental earlier phases of clinical trials. Moreover, it can be hypothe-
sized that a local infusion of EGCG might help in preventing recurrent
pleural effusion in patients with advanced disease.

In conclusion, the present report suggests that EGCG, as well as other
agents that inhibit MRC complex activities and energy metabolism, may
provide reliable therapeutic options for the management of MMe and
may be useful in the treatment of malignant tumors.

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References


Kalyanaraman, G.M. Marta, G.R. Budinger, N.S. Chandel, Mitochondrial metabol-
ism and ROS generation are essential for Kras-mutated tumorigenesis, Proc. Nat.

Kalan, J. Grinnell, M. Hembree, C. Capen, L.M. Slijper, S. Jacks, V. Lassmig, J.
Yang, B. Torlakovic, S. Martinotti, A. Attardi, A. Bartkova, A. Castagneto, M. Rivk,
B. Saltan, S. Passarella, An increase in the ATP levels occurs in cerebellar granule
cells en route to apoptosis in which ATP derives from both oxidative phosphorylation and anaer-

[4] S. Chambé, J. Foulquier, J. Gagnaire, J. Lespessailles, Cytoprotection by the modula-
tion of mitochondrial electron transport chain: the emerging role of mitochondrial
mitochondrial targets of apoptosis in malignant mesothelioma, Mod. Pathol. 25

depolarization and caspase-dependent apoptosis in pancreatic

[6] I. Kil, K.H. Jung, W.S. Nam, J.W. Park, Attenuated mitochondrial NADP+-
dependent isocitrate dehydrogenase activity enhances EGCG-induced apoptosis,


