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Original article

Regulation of the oxidative balance with coenzyme Q10 sensitizes human glioblastoma cells to radiation and temozolomide

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ABSTRACT

Objectives: To investigate how the modulation of the oxidative balance affects cytotoxic therapies in glioblastoma, *in vitro*.

Material and methods: Human glioblastoma U251 and T98 cells and normal astrocytes C8D1A were loaded with coenzyme Q10 (CoQ). Mitochondrial superoxide ion ($O_2^{\cdot-}$) and H_2O_2 were measured by fluorescence microscopy. OXPHOS performance was assessed in U251 cells with an oxytherm Clark-type electrode. Radio- and chemotherapy cytotoxicity was assessed by immunostaining of γH2AX (24 h), annexin V and nuclei morphology, at short (72 h) and long (15 d) time. Hif-1α, SOD1, SOD2 and NQO1 were determined by immunolabeling. Catalase activity was measured by classic enzymatic assay. Glutathione levels and total antioxidant capacity were quantified using commercial kits.

Results: CoQ did not affect oxygen consumption but reduced the level of $O_2^{\cdot-}$ and H_2O_2 while shifted to a pro-oxidant cell status mainly due to a decrease in catalase activity and SOD2 level. Hif-1α was dampened, echoed by a decrease lactate and several key metabolites involved in glutathione synthesis. CoQ-treated cells were twofold more sensitive than control to radiation-induced DNA damage and apoptosis in short and long-term clonogenic assays, potentiating TMZ-induced cytotoxicity, without affecting non-transformed astrocytes.

Conclusions: CoQ acts as sensitizer for cytotoxic therapies, disarming GBM cells, but not normal astrocytes, against further pro-oxidant injuries, being potentially useful in clinical practice for this fatal pathology.

Grade IV astrocytoma or glioblastoma multiforme (GBM) is the most common type of malignant brain tumor in adults [1]. The current therapeutic protocol for this pathology includes maximal safe surgery, radiotherapy and chemotherapy with temozolomide (TMZ), an alkylating agent and radiosensitizer [2,3]. Nevertheless, even patients receiving the standard of care die early with a median survival of 14 months due to recurrence within the radiation field [1,4,5].

Several evidences link GBM radioresistance with the mitochondrial pathophysiology and the over-production of radical oxygen species (ROS) scavengers, which lately determine a shift to an antioxidative balance [6–10]. GBM cells are very glycolytic even in normoxia: the so called "Warburg effect" [11]. In this setting, a high level of $O_2^{\cdot-}$ is produced and rapidly dismutated to H_2O_2, which is translocated to the cytosol, orchestrating multiple mechanisms leading to radioresistance [12–14]. High levels of $O_2^{\cdot-}$ and H_2O_2 are toxic and thus, GBM cells increase their antioxidant

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defenses as a survival mechanism, i.e. the levels of catalase and mitochondrial superoxide dismutase (SOD2) are increased [6,7].

The cytotoxic effect of ionizing radiation is mediated by the induction of ROS, leading to DNA and RNA damage and genomic instability [14]. Thus, the naturally increased level of catalase and SOD2 enzymes ameliorates radiation-induced DNA damage, contributing to resistance to radiation-induced cell damage [6,15]. Moreover, the increased level of reduced glutathione (GSH) also contributes to cell protection against ROS and, thus, participates in the resistance to ionizing radiation [16]. Indeed, there is a negative correlation between the level of GSH and sensitivity to ionizing radiation in a series of brain tumors including GBM [17]. GSH level is also related to resistance to TMZ and mediates several mechanisms of resistance development [18]. Furthermore, high lactate levels produced by aerobic glycolysis are also interconnected with radioresistance. Actually, lactate behaves as an antioxidant [19], being positively correlated with resistance to ionizing radiation [14].

Instead of a single factor, radioresistance is orchestrated by several enzymes and small molecules that are linked to the increased generation of ROS. Therefore, the simultaneous modulation of these ROS scavengers may be useful in GBM therapies. In this work, we explore the role of coenzyme Q_{10} (CoQ) in the mechanisms evoking radioresistance using two human GBM cell lines, U251 and T98, compared to non-transformed astrocytes C8D1A. CoQ is a lipophilic antioxidant that crosses the blood–brain barrier [20]. Besides, it is a component of the mitochondrial electron transport chain (ETC) that dampens the $O_2^{\cdot-}$ generated by complex I in dystrophic mitochondria [12]. Our results show that treatment of human U251 GBM cells with CoQ did not affect OXPHOS, but slightly increased oxygen consumption associated with the mitochondrial complex I, which was paralleled by a decrease in the level of mitochondrial $O_2^{\cdot-}$ and cytosolic H_2O_2. Conversely, these reactive species were increased upon irradiation in GBM cells, but not in non-transformed astrocytes. Indeed, CoQ sensitized cells to radiation-induced DNA damage and apoptosis both in short- and long-term clonogenic assays. These effects are mediated by a shift in the oxidative balance toward pro-oxidant conditions, which relies on the reduction in catalase activity, a decrease in SOD2 level and a cut down in intracellular lactate and key metabolites involved in glutathione synthesis, which could be related to the reduced level of Hif-1α. On the other hand, neither SOD1 nor NQO1 levels were altered. These changes result in a reduction in the total antioxidant cellular capacity and a shift to a pro-oxidant cellular state that disarms GBM cells against further pro-oxidant injuries induced by standard therapies, without affecting non-transformed astrocytes. Our results indicate that CoQ combined with radiation and TMZ has a promising potential for improving the current therapeutic protocol for GBM.

Material and methods

Reagents

Coenzyme Q_{10} was provided by Kaneka Corporation. TMZ, Hoechst, crystal violet, EDTA, methanol, DMSO, dH_2O, Tris–HCl, DTT and other general reagents were acquired from Sigma–Aldrich.

Cell culture and irradiation

Human GBM U251 and T98 and non-transformed astrocytes C8D1A were obtained from ATCC and maintained under normoxia in DMEM (Sigma–Aldrich) at 37 °C and 5% CO_2. For irradiation purposes, cells were seeded in 24 well plates and pre-treated for 24 h with CoQ or vehicle (ethanol; control). Depending on the experiment, cells were treated with TMZ (50 μM) or vehicle (DMSO) and again 24 h later cells were irradiated in a Gulmay D 3150 (NCA) at doses ranging between 1 and16 Gy (1.211 Gy/min) at 100 kV tube potential. Different parameters were evaluated immediately after irradiation ($O_2^{\cdot-}$ and H_2O_2), at 24 h (DNA damage), at 96 h (apoptosis) or at 15d (colonies formation) of irradiation.

OXPHOS performance

Oxygen consumption in intact or digitonin-permeabilized cells was determined with an oxytherm Clark-type electrode (Hansatech Instruments, Norfolk, UK) as previously described [21]. Briefly, U251 cells were resuspended in culture medium or respiration buffer (10 mM $MgCl_2$, 250 mM Sucrose, 20 mM HEPES pH 7.4, 1 mM ADP, 2 mM KH_2PO_4), respectively. Oxygen consumption was recorded at 37 °C with stirring. 7.5 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was used to uncouple respiration in intact cells. For polarographic measurements, cells were permeabilized with 1% digitonin (1.2 ul/10^6 cells) for 10 min at 37 °C and oxygen consumption was recorded after the addition of substrates and inhibitors for Complex I (5 mM Glutamate plus 5 mM Malate, and 2 μM Rotenone), Complex II + III (5 mM Succinate plus 5 mM Gliceraldehide-3-P, and 0.1 μM Antimycin A) and Complex IV (1.2 mM TMPD, and 6 mM KCN). Reactions were terminated by the addition of 6 mM KCN to assess any O_2 consumption not derived from the respiratory chain.

Single-cell determination of $O_2^{\cdot-}$ and H_2O_2

Mitochondrial $O_2^{\cdot-}$ and total H_2O_2 levels were determined with MitoSOX and H_2DCFDA probes, respectively (Life Technologies). Cells were seeded in 96 well plates and incubated for 24 h with vehicle (control) or CoQ (2.5–10 μM) and then irradiated as indicated above. Then, cells where immediately loaded for 30 min with 1 μM MitoSOX or H_2DCFDA, washed in fresh medium and imaged in a Nikon TiU microscope (20× objective). Images were analyzed and processed with ImageJ software (NIH). Results are expressed as the percentage of cell signal *vs.* control (minimum 50 cells, $n = 3$).

Determination of total antioxidant capacity

Cells were seeded in 96 well plates and incubated for 24 h with vehicle (control) or CoQ (5 μM). Total antioxidant capacity was determined in cell lysates and in culture media using a commercial kit, following the manufacturer's instructions (MAK187, Sigma–Aldrich). Results were expressed as nmol/μg of protein ($n = 4$).

Determination of DNA damage, apoptosis and viability

Cells treated with CoQ and irradiated, after 24 and 72 h for DNA damage and apoptosis and viability assays, respectively, were fixed for 2 min in 4% para-formaldehyde (PFA), stained with 1 μg/ml Hoechst and immunolabeled for annexin V (1:500; BD Pharmingen) and γH2AX antibody (1:500; Santa Cruz Biotechnology). For γH2AX staining, cells were permeabilized for 2 min in ice-cold methanol after PFA fixation. Binding of primary antibodies was detected with fluorescence-labeled secondary antibodies conjugated with Alexa-594 (1:2000; Life Technologies). Apoptotic nuclei were determined from Hoechst-stained nuclei images according to morphometric criteria with ImageJ. Those cells exhibiting normal, non-apoptotic, nuclei morphology were considered as viable. Results are shown as percentage of apoptotic *vs.* total cells (at least 100 cells, $n = 4$). DNA damage was determined and represented as the number of γH2AX foci per cell (at least 100 cells, $n = 4$). Apoptotic cells were determined according to annexin V level. Results

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