Epigallocatechin-3-gallate exhibits anti-tumor effect by perturbing redox homeostasis, modulating the release of pro-inflammatory mediators and decreasing the invasiveness of glioblastoma cells

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Abstract. Polyphenol epigallocatechin-3-gallate (EGCG) induced apoptosis in glioma cells by elevating oxidative stress through increased reactive oxygen species (ROS) generation. Signs of apoptosis included altered mitochondrial membrane potential and elevated expression of caspase-3 and cytochrome c. The increase in ROS was concomitant with the decrease in expression of thioredoxin (TRX-1) and ceruloplasmin (CP), mediators associated with protection against oxidative stress. EGCG downregulated the levels of pro-inflammatory cytokine interleukin (IL)-6 and chemokines IL-8, monocyte-chemoattractant protein (MCP)-1 and RANTES. EGCG also decreased the invasive potential of gliomas, possibly by affecting the urokinase plasminogen activator (uPA) and cytoskeletal architecture. Our study indicates that EGCG might serve as an effective therapeutic strategy against glioma as it not only promotes cell death through redox perturbation, but also downregulates the release of pro-inflammatory mediators while concomitantly decreasing the invasive potential of glioma cells.

Introduction

Polyphenol epigallocatechin-3-gallate (EGCG), which is present in green tea, has cancer chemopreventive effects in many animal tumor models (1). Epidemiological studies have moreover shown that the consumption of green tea can reduce the incidence of cancer (2). Glioblastoma multiforme (GBM) remains one of the most challenging solid cancers to treat due to its highly proliferative, angiogenic and invasive nature. Heightened oxidative stress appears to be characteristic of GBMs, with reactive oxygen species (ROS) increasing resistance to chemotherapy in gliomas (3). We and others have reported that human cancer cells with intrinsic oxidative stress

are highly sensitive to ROS stress, and that promoting ROS generation can effectively bring about cell death (4,5). As EGCG induces the apoptosis of malignant cells via the production of ROS (6,7), we investigated whether this catechin could be used as a redox-modulating agent to induce apoptosis in glioma cells by elevating oxidative stress.

Inflammation is also a contributing factor in cancer development (8). Increased expression of pro-inflammatory mediators has been implicated as a possible prognostic indicator in GBM (9). EGCG not only inhibits the release of pro-inflammatory mediators in human prostate carcinoma cells (10), but is also known to prevent glioblastoma migration by downregulating matrix metalloproteinase (MMP) activities (11). We therefore investigated whether EGCG could affect the release of pro-inflammatory mediators from glioma cells, altering their invasive potential.

Materials and methods

Cell culture and treatment. Glioblastoma cell line U87MG, obtained from American Type Culture Collection (Manassas, VA), was cultured in DMEM supplemented with 10% FBS. On attaining semi-confluence, cells were switched to serum-free media (SFM). After 12 h, cells were treated with 25, 50 and 100 μM EGCG (Sigma, St. Louis, MO) in SFM for 48 h. Following treatment, the conditioned media from the cells were collected and stored at -80˚C for cytokine bead array, whereas cells were processed for immunoblot analysis, apoptosis bead array and ROS measurement. To determine the involvement of ROS in apoptosis, the ROS-specific inhibitor N-acetylcysteine (NAC) was added to cells either alone or in combination with EGCG. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Measurement of ROS. Intracellular ROS generation in the control and EGCG-treated cells was monitored using H_2O_2-sensitive probe 5 (and 6)-chloromethyl-2',7'-dichlorodi-hydrofluorescein diacetate (CM-H_2DCFDA), as previously described (5). Briefly, cells were incubated with H_2DCFDA (5 μM) for 30 min at 37˚C and washed twice with PBS. Then, the fluorescent intensity of 5x10^5 cells was measured using a spectrofluorometer (excitation 500 nm; emission 530 nm).

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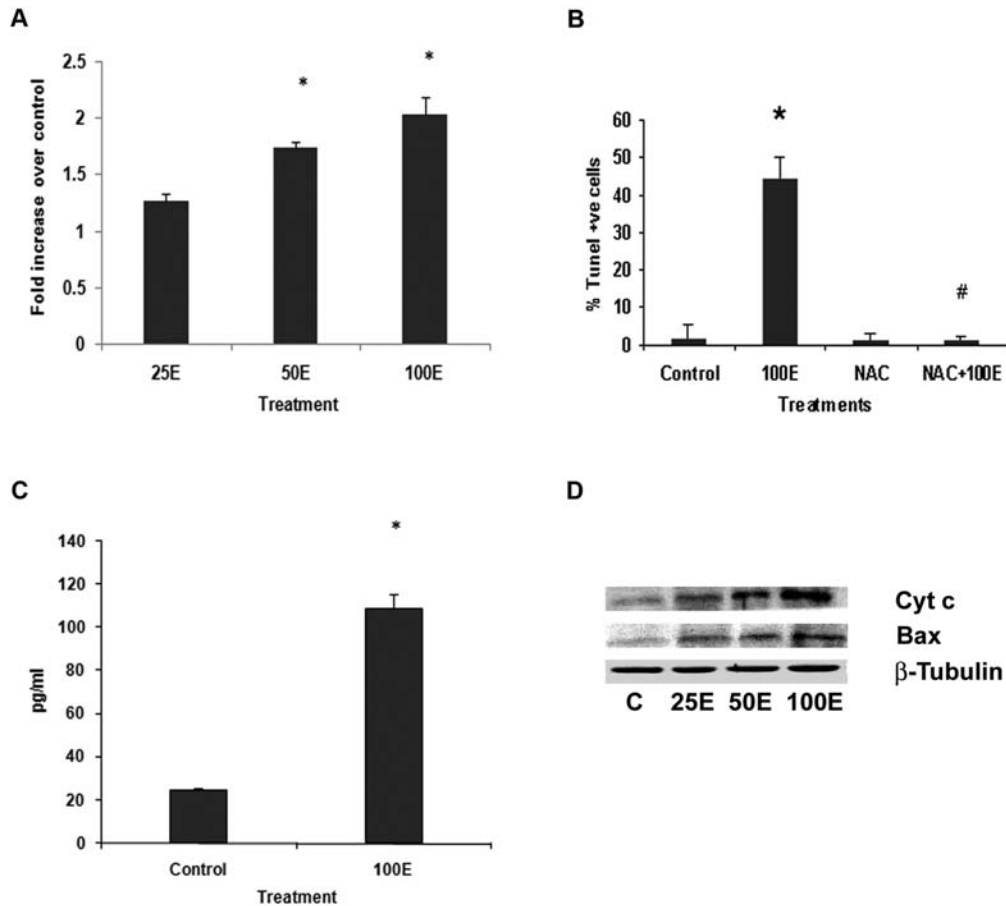*Key words:* epigallocatechin-3-gallate, homeostasis, glioblastoma

Figure 1. EGCG increases ROS production in glioma cells to induce apoptosis. (A) ROS levels were measured in cells treated with EGCG for 48 h using H_2O_2-sensitive probe DCFDA. ROS levels following treatment with increasing concentrations of EGCG are indicated. (B) The ROS inhibitor NAC abolishes EGCG-induced cytotoxicity. Cell death was determined by TUNEL assay. The graph represents the percentage of TUNEL-positive cells treated with either 100 μM EGCG, NAC, or a combination of both for 48 h, as calculated from multiple fields. (C) Levels of active caspase-3 in the control and EGCG-treated U87MG cells, as observed by Apoptosis bead array. Values represent the means ± SEM of 3 independent experiments. *Significant increase compared to the control; #significant decrease compared to EGCG-treated cells (p<0.05). (D) Expression of cytochrome c and Bax in EGCG-treated cells as analyzed by Western blot analysis. A representative blot from three independent experiments with identical results is shown. Blots were reprobed for ß-tubulin to establish equivalent loading. E, EGCG; C, control.

TUNEL assay. U87MG cells (10^4) were treated with EGCG or 1 mM NAC (a ROS-specific inhibitor) or a combination of both for 48 h in 8-well chamber slides (Nunc, Denmark). Following treatment, apoptotic cells were identified using the In situ Cell Death Detection Kit, TMR red (Roche, Germany) as previously described (5). Cells fixed with 4% paraformaldehyde in PBS were blocked with 4% BSA containing 0.02% Triton X-100. Fixed cells were then incubated in the TUNEL mix (terminal deoxynucleotidyl transferase in storage buffer and TMR red-labeled nucleotide mixture in reaction buffer) for 1 h at room temperature. The slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories Inc., CA). TUNEL-positive cells (red) that co-localized with DAPI (blue) were counted from multiple fields.

Western blot analysis. Protein (20 μg) isolated from untreated and EGCG-treated U87MG cells was electrophoresed on 6-10% polyacrylamide gel. Western blotting was performed as previously described (5) using the following antibodies: ceruloplasmin (CP) (Dako, Carpinteria, CA), Bax, cytochrome *c* and thioredoxin (TRX-1) (Lab Frontier, Seoul, Korea). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise mentioned. Secondary anti-

bodies were purchased from Vector Laboratories. Following the addition of chemiluminescence reagent (Amersham, Buckinghamshire, UK), blots were exposed to the Chemigenius Bioimaging System (Syngene, Cambridge, UK) for developing, and images were captured using GeneSnap software (Syngene). The blots were stripped and reprobed with anti-ß-tubulin to determine equivalent loading as previously described (5).

Determination of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using JC-1 probe as previously described (5). Control and EGCG-treated cells were labeled for 10 min with 10 μM JC-1 at 37°C, then cells were examined by fluorescence microscopy. JC-1 accumulates as J-aggregates (590 nm, red) in metabolically active mitochondria only, while the depolarization of mitochondrial membranes leads to JC-1 monomer formation (527 nm, green).

Cytometric bead array (CBA). The Human Inflammation Kit (BD Biosciences, NJ) was used to quantitatively measure cytokine/chemokine levels in the media obtained from untreated and EGCG-treated cells as previously described (5). The Human Apoptosis Kit (BD Biosciences) was used to
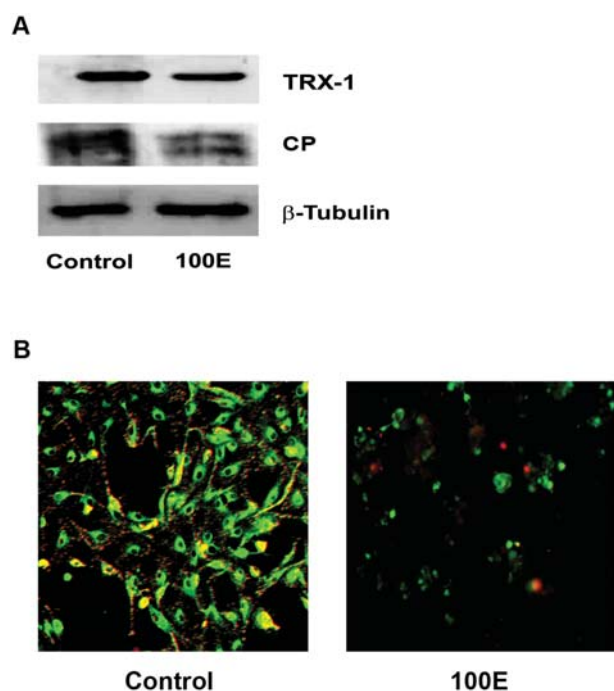
Figure 2. EGCG alters expression of the molecules associated with redox homeostasis and decreases the mitochondrial membrane potential of glioma cells. (A) Western blot showing the expression of TRX-1 and CP in the control and in cells treated with 100 μM EGCG for 48 h. Data are representative of results obtained from 3 independent experiments. Blots were reprobed for ß-tubulin to establish equivalent loading. (B) Fluorescence micrographs of cells stained with JC-1 show the decrease in mitochondrial membrane potential in cells treated with 100 μM EGCG. Micrographs show that JC-1 accumulates within active mitochondria and exhibits orange fluorescence. E, EGCG; TRX-1, thioredoxin; CP, ceruloplasmin.

quantitatively measure caspase-3 levels in the protein lysates of untreated and EGCG-treated cells. The assay was performed according to the manufacturer's instructions and analyzed on FACSCalibur (Becton Dickinson) using CBA software as previously described (5).

Matrigel invasion assay. *In vitro* invasion of U87MG cells grown in the absence or presence of EGCG for 48 h was measured by the invasion of cells through Matrigel-coated (Collaborative Research Inc., Boston, MA) transwell inserts (Costar, Cambridge, MA) as previously described (12). Briefly, 2×10^5 cells were added to transwell inserts with an 8-μm pore size coated with a final concentration of 0.7 mg/ml Matrigel. Following treatment, cells that passed through the filter on the bottom side of the membrane were fixed, stained with crystal violet, photographed and quantitated as described (12).

Urokinase plasminogen activator assay. The assay was performed according to the manufacturer's instructions (Chemicon). Cell lysates from treated and untreated cells and the positive control (urokinase plasminogen activator (uPA)-containing sample) were added to each well of a 96-well plate, and the volume was adjusted with deionized water. Following the addition of assay buffer and chromogenic substrate, the samples were incubated for 24 h at 37˚C, following which the absorbance was read at 405 nm. Optical density values obtained were compared with known standards to obtain relative activities.

Visualization of actin cytoskeleton. Cells (10^4) were treated with EGCG in 8-well chamber slides. After 48 h, cells were washed with PBS and fixed with 4% formaldehyde for 10 min at 37˚C. Fixed cells were then incubated in PBS containing 1% BSA for 30 min, then in staining solution (6.25 μl rhodamine-labeled phalloidin, 2.5 mg BSA in 250 μl PBS) for 20 min at room temperature. Cells were washed with PBS, mounted, and immunofluorescence was recorded using a fluorescence microscope.

Statistical analysis. All comparisons between groups were performed using the two-tailed paired Student's t-test. Values of p<0.05 indicated significant differences.

Results

EGCG enhances ROS production in glioblastoma cells to induce apoptosis. While treatment of U87MG cells with 25 μM EGCG had no effect on ROS production, a significant 1.7- and 2-fold (p<0.05) increase in ROS production was observed in cells treated with 50 and 100 μM EGCG respectively, as compared to the control cells (Fig. 1A). To determine whether increased ROS production induced apoptosis in glioma cells, TUNEL staining was performed on cells treated with the ROS inhibitor NAC in the presence and absence of 100 μM EGCG (this dose was selected as maximal ROS production was observed at this concentration). No significant difference in the percentage of apoptotic cells was observed between untreated and NAC-treated cell (Fig. 1B). However, the 22-fold increase in apoptotic cells observed when U87MG cells were treated with 100 μM EGCG was significantly reduced to that of the control level when EGCG treatment was supplemented with NAC (Fig. 1B). Thus, the cytotoxicity of EGCG was abolished in the presence of ROS inhibitor.

Since significant apoptosis (42%, Fig. 1B) was observed in U87MG cells treated with 100 μM of EGCG, we performed FACS analysis to detect the levels of pro-apoptotic molecule caspase-3 in EGCG-treated cells. A significant 5-fold increase in the expression of caspase-3 was observed in EGCG-treated cells as compared to the control cells (Fig. 1C). Bax, a pro-apoptotic protein, promotes the release of cytochrome *c* from the mitochondria, in turn activating caspase-3, one of the key executioners of apoptosis (13). Since increased caspase-3 levels were observed in EGCG-treated cells, we determined Bax and cytochrome *c* levels in EGCG-treated cells by Western blot analysis. A significant increase in both Bax and cytochrome *c* expression was observed in cells treated with 50 and 100 μM EGCG (Fig. 1D).

EGCG decreases expression of the molecules associated with the maintenance of redox homeostasis. Since a major cellular defense against ROS is provided by the antioxidant TRX-1, we evaluated the status of redox protein TRX-1 in EGCG-treated glioma. Additionally, the level of CP, an antioxidant that protects astrocytes from sustaining damage after injury, was investigated. Treatment of glioma cells with 100 μM of EGCG dramatically decreased the expression of both TRX-1 and CP as compared to the untreated control cells (Fig. 2A).

EGCG decreases mitochondrial membrane potential in human glioma cells. As ROS generation is inversely correlated with
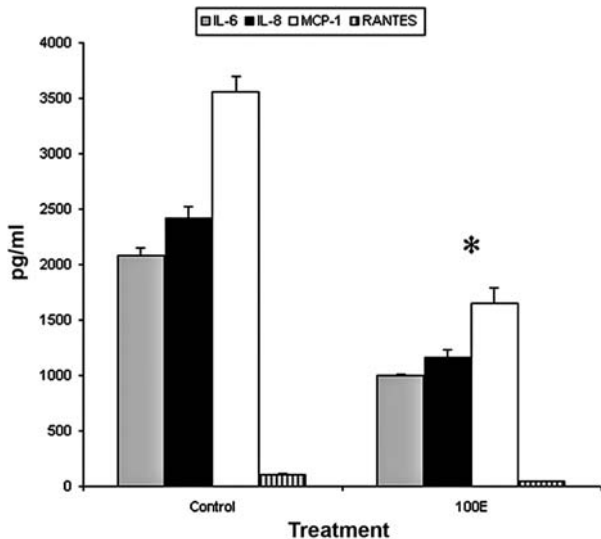
Figure 3. EGCG modulates the release of cytokines/chemokines from glioblastoma. Expression of the cytokine IL-6 and chemokines IL-8, MCP-1 and RANTES in untreated and EGCG-treated U87MG cells, as observed by CBA. Values represent the means ± SEM of 3 individual experiments. *Significant decrease compared to the control (p<0.05). E, EGCG.

mitochondrial membrane potential ($\Delta\Psi_m$) (14), and since increased release of cytochrome *c* in EGCG-treated cells indicates mitochondrial involvement, we investigated mitochondrial changes in terms of alterations in $\Delta\Psi_m$. Intact mito-

chondrial function, as indicated by the accumulation of JC-1 orange fluorescence, was observed in untreated cells (Fig. 2B). The absence of detectable JC-1 orange fluorescence observed in cells treated with 100 μM EGCG indicated diminished $\Delta\Psi_m$ (Fig. 2B).

EGCG decreases the release of cytokines/chemokines from glioma cells. Pro-inflammatory cytokine interleukin (IL)-6 plays a role in the malignant progression of astrocytoma (15), and chemokines such as IL-8, monocyte-chemoattractant protein (MCP-1) and RANTES are crucial regulators of cancer cell invasion (16). Since EGCG inhibits pro-inflammatory mediator release in human prostate carcinoma cells (10), we performed CBA to investigate the profile of cytokines/chemokines in EGCG-treated cells. A significant ~2-fold decrease in IL-6, IL-8, MCP-1 and RANTES levels was observed in EGCG-treated cells as compared to the control cells (Fig. 3).

EGCG decreases the invasiveness of glioma cells. EGCG prevents GBM migration by downregulating MMP activity (11). Since EGCG treatment decreases levels of IL-8, MCP-1 and RANTES, which are associated with cancer cell invasion (16), Matrigel invasion assay was performed to determine the effect of EGCG on glioma cell invasiveness. As a concentration of 100 μM EGCG was most effective in inducing ROS-mediated apoptosis, this dose was chosen for the Matrigel invasion assay. A 60% decrease in the invasive potential of glioma was observed upon treatment with 100 μM of EGCG (Fig. 4A).
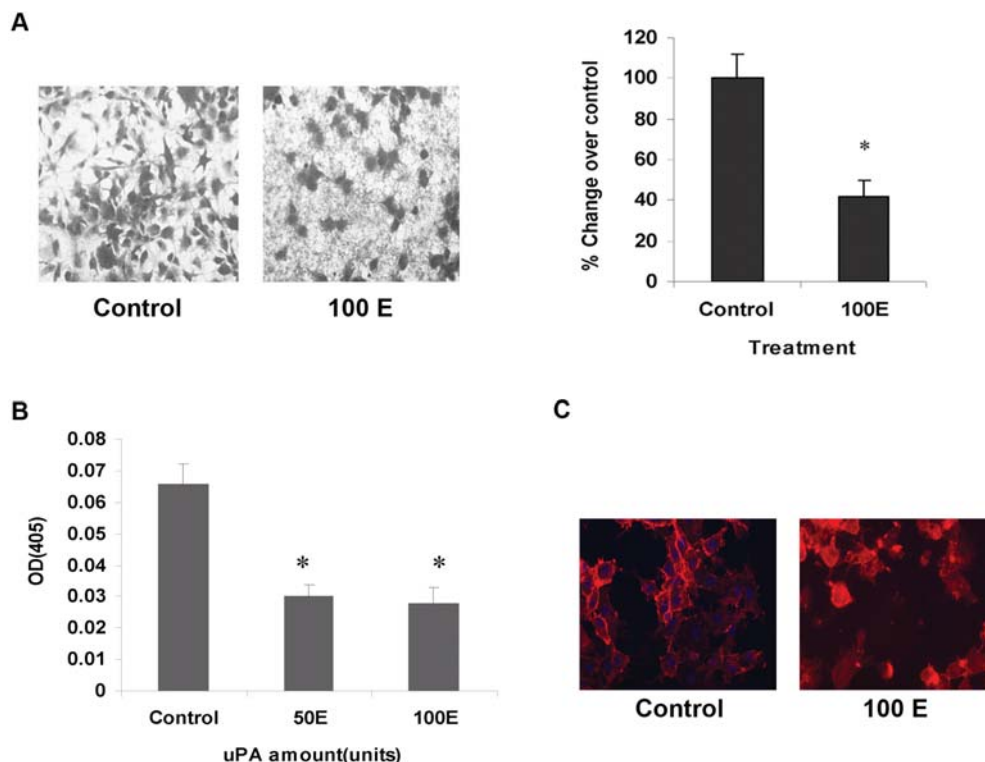

Figure 4. EGCG decreases the invasiveness of glioma cells. (A) EGCG decreases the invasiveness of glioma cells as determined by Matrigel invasion assay. Control and EGCG-treated cells were allowed to pass through a Matrigel chamber; the cells that invaded the bottom side of the membrane were fixed, stained with crystal violet and photographed. The graph represents the percentage of glioma cells treated with EGCG that invaded the Matrigel. (B) EGCG decreased the uPa activity of glioma cells. The uPa activity of cell lysates obtained from EGCG-treated U87MG cells was measured, and the absorbance was read at 405 nm. The optical density values were compared with known standards to obtain relative activities. The graph represents optical density as a measure of uPa activity in the control and EGCG-treated cells. Values represent the means ± SEM of 3 independent experiments. *Significant decrease compared to the control (p<0.05). (C) EGCG alters the cytoskeletal architecture of GBM by affecting F-actin condensation as visualized after staining cells with rhodamine-labeled phalloidin. E, EGCG.

The binding of urokinase plasminogen activator (uPA) to its receptor (uPAR) initiates a proteolytic cascade facilitating the activation of MMP, which in turn degrades the extracellular matrix (12). As EGCG suppressed uPA expression in human fibrosarcoma (17), and since EGCG decreased the invasiveness of U87MG cells (Fig. 4A), we determined uPA activity in glioma cells treated with EGCG (Fig. 4B). A 40-50% decrease in uPa activity was observed in glioma cells upon treatment with 50 and 100 μM of EGCG (Fig. 4B).

Actin-binding proteins influence microfilament dynamics to facilitate the invasiveness of malignant cells (18). Since EGCG downregulated the invasive potential of U87MG cells, we stained glioma cells with phalloidin (which binds specifically to F-actin) to investigate the distribution of F-actin in the presence and absence of EGCG (Fig. 4C). A decrease in the actin network was observed in EGCG-treated cells, as evidenced by decreased staining with phalloidin as compared to the control. The disassembly of the network indicated that EGCG affects cytoskeletal architecture to decrease the invasiveness of glioblastoma cells.

Discussion

Despite recent advances in understanding the molecular pathways involved in GBM aggressiveness, the prognosis for this most malignant of brain tumors continues to be dismal. As EGCG suppresses the growth of a number of human malignancies, we investigated the molecular mechanism of the action of EGCG on the human GBM cell line U87MG. It is known that intrinsic ROS stress renders cancer cells highly vulnerable to further oxidative insults by exogenous agents (5,6,19). Our study indicated that EGCG induces apoptosis in glioma cells through increased oxidative stress. Increased apoptosis in glioma with increased ROS levels and downregulated TRX-1 are consistent with previous reports that the inhibition of TRX enhances the sensitivity of tumor cells to ROS-generating anti-cancer agents (5,20). An increase in ROS with a concomitant decrease in antioxidant TRX-1 and ferroxidase CP possibly enhances oxidative stress in EGCG-treated glioma cells by decreasing their ability to detoxify excess free radicals.

ECGC-mediated depolarization of the mitochondrial membrane likely facilitated the release of apoptogenic protein cytochrome *c*, as previously observed in pancreatic cancer cells (7). We speculate that EGCG-mediated downregulation of IL-6, IL-8, RANTES and MCP-1 possibly prevents the establishment of the feed forward cycle of inflammation that promotes disease progression. The ability of EGCG to decrease the invasive potential of glioma cells was concurrent with a decrease in the uPa activity associated with invasiveness.

Our study demonstrates that the ability of EGCG to (i) induce oxidative stress in glioma cells by disrupting the redox homeostasis and (ii) downregulate the expression of pro-inflammatory mediators may be an attractive anti-tumor strategy, given the role of inflammation and oxidative stress in GBM progression

Acknowledgements

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