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Achievable Central Nervous System Concentrations of the Green Tea Catechin EGCG Induce Stress in Glioblastoma Cells in Vitro

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ABSTRACT

The polyphenolic compounds present in green tea are preventative against cancer in several animal tumor models. However, direct cytotoxic effects on cancer cells have also been reported. In order to determine whether drinking of green tea has chemopreventive or cytotoxic effects on brain cancer cells, we investigated the effect of the major green tea polyphenol EGCG as a pure substance and as tea extract dietary supplement on primary human glioblastoma cell cultures at the CNS-achievable concentration of 100 nM reported in the literature. We compared this with the effect of the cytotoxic concentration of 500 μM determined to be specific for the investigated primary glioblastoma cultures. After treatment with 500 μM EGCG, strong induction of autophagy and apoptosis was observed. Under treatment with 100 nM EGCG, glioblastoma cells proliferated over the entire observation period of 6 days without any detectable signs of cell death. Only within the first 12 h of treatment was increased accumulation of autophagic vacuoles and increased reactive oxygen species production as a stress response demonstrated. Mild forms of stress, such as treatment with 100 nM EGCG, activate different endogenous repair mechanisms to protect cells. Our data imply that drinking of green tea may have chemopreventive effects, but no direct cytotoxic properties.

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Introduction

Glioblastoma, the most common primary malignant brain tumor, is characterized by rapid proliferation and destructive invasion of surrounding neural structures. Therapeutic options are focused on gross total surgical resection, followed by radiation therapy with concurrent temozolomide therapy. The resulting median overall survival of 14.6 months remains dismal (1). New therapeutic strategies are required to target the dysregulated molecular pathways in this disease and to tackle evolving treatment resistance.

Green tea (*Camellia sinensis*) is one of the most popular beverages worldwide and has long been associated with health-related benefits, including chemopreventive effects (2). It contains four major polyphenolic compounds: (−)-epigallocatechin, (−)-epigallocatechingallate (EGCG), epicatechin-gallate, epigallocatechin, and epicatechin (3). The most abundant polyphenol in green tea is EGCG, representing 16.5% of the weight of the water extractable fraction of green tea leaves (4). It has been suggested as a potential chemotherapeutic agent given its inhibitory effects on cell proliferation (5,6) and on key proteins involved in cell cycle regulation (7,8). Furthermore, induction of cancer cell apoptosis by EGCG has been shown in two in vitro studies (9,10).

Several mechanisms have been proposed to explain the chemopreventive activity of EGCG, including direct or indirect blocking of multiple signaling pathways by nonspecific protein binding and the generation of reactive oxygen species (ROS) (9), which is purported to suppress ubiquitous processes that can lead to the initiation of carcinogenesis. ECGC-modulated signaling pathways include the nuclear factor-κB pathway (11,12), the epidermal growth factor receptor (EGFR)-mediated pathway (11,13), the insulin-like growth factor 1 (IGF-1)-mediated signal transduction pathway (14,15), the mitogen activated protein (MAP)

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kinase-dependent pathway (11,14,16,17), and the ubiquitin/proteasome degradation pathway (18).

The cytotoxic effect of EGCG on glioblastoma cells has been investigated in the past, using concentrations of between 25 µM and 1.1 mM (19–21). Although these data have demonstrated its anti-tumor activity on glioblastoma cells, to date there exist no data concerning the properties of EGCG at low, central nervous system-attainable concentrations.

This study examines the cytotoxic effect of EGCG, the main constituent of green tea, at supranormal concentrations of 500 µM, on primary glioblastoma cell cultures. Furthermore, it demonstrates that neither EGCG, nor the tea extract dietary supplement (GTE), have inhibitory effects on glioblastoma cell proliferation in CNS-achievable concentrations of 100 nM (22,23). In addition, the data indicate that catechins at CNS-achievable concentrations act as mild stressors with short-term effects on signal transduction cascades, regulating autophagy and generation of reactive oxygen species in glioblastoma cells. These effects are in turn compensated for by regulation of gene expression in the EGCG-modulated signaling pathways. As a result, a hormetic effect (24) may be induced by long-term application, as achieved through the regular consumption of green tea.

Materials and methods

Cell culture and active compounds

GBM02, GBM15, GBM16, and GBM17 are primary cell cultures derived from intraoperatively obtained glioblastoma tissues, cultivated as previously described (25). After disaggregation by mincing and incubation in an enzyme mix [2.5 U/ml Pronase E, 250 U/ml Collagenase Type IVa in 1 × phosphate-buffered solution (PBS), Sigma-Aldrich, St. Louis, MO] for 1 h, cells were resuspended in DMEM +10% FBS (Thermo Fisher Scientific, Waltham, MA) and maintained in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. The glioblastoma cell line A172 was provided by the ATCC (Manassas, VA). Tumor cells cultivated on poly-D-lysine hydrobromide (PDL, Sigma-Aldrich) coated cover slips were stained immunocytochemically using the anti-laminin-R (A-7) antibody (Santa Cruz Biotechnology, Dallas, TX; 1:100) to visualize expression and cellular distribution of the 67 kDa laminin receptor 67LR. The active compounds EGCG (Sigma-Aldrich) and GTE (commercially available tea extract dietary supplement) were dissolved in 1 × PBS (Thermo Fisher Scientific) at 55 mM stock concentrations, and added to the cultures to achieve final concentrations of 100 nM, as CNS-attainable concentration, and 500 µM, determined by dose-response analysis (see below). Temozolomide (TMZ) and lomustine (CCNU; both Sigma-Aldrich) were dissolved in DMSO (Merck Millipore, Darmstadt, DE) at 50 mM stock concentrations.

Determination of MGMT promoter methylation

DNA extraction and bisulfite treatment were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, DE) and the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Quantitative PCR was performed using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific) with methylation-specific primers in a Rotor-Gene Q (Qiagen). Following methylation-specific qPCR, the resultant Ct values and melt curves were reviewed to assess for MGMT promoter methylation by analyzing the amplification of both the methylated and unmethylated sequences.

Viability assay

The half-maximal cytotoxic concentration (IC_{50}) was determined through dose-response analysis using the CyQuant Cell Proliferation Assay Kit (Thermo Fisher Scientific) on GBM15, 16, 17, and A172 cells (10,000 cells per well in microtiter plates) after 24 h of incubation with increasing concentrations of EGCG and GTE (0.11–11,000 µM) and calculated using a four-parameter logistic model (26). Viability was defined using the PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific). Glioblastoma cells (GBM02, GBM15, and GBM16) were plated at a density of 5,000 or 2,000 cells per well in microtiter plates for 48 h with one change of medium. They were treated with the active compounds for another 48 h and 6 days (with daily change of medium and active compound) with simultaneous non-treated controls. GBM02 and GBM15 were treated with TMZ and CCNU (50 and 100 µM, described as reachable serum concentration; (27) alone or in combination with 100 nM EGCG (with or without pretreatment for 6 days) for 48 h. To study the influence of 67LR on EGCG action, cells were pretreated for 60 min with the rabbit monoclonal anti-laminin-R (A-7) antibody or isotype control mouse IgG_2 (52G1; Santa Cruz Biotechnology; both 20 µg/ml) corresponding to the figures reported in the literature (28), incubated with 500 µM EGCG for 24 h and measured using the

PrestoBlue Cell Viability Reagent. Fluorescence intensity and absorbance were measured on an Infinite M1 000 PRO microplate reader (Tecan Group, Männedorf, CH).

Autophagy/cytotoxicity assay and nuclear size determination

GBM15 and 16 cells were plated at a density of 1,000 cells per well on PDL coated cover slips in 24-well plates for 48 h with one change of medium. They were treated with EGCG (500 and 100 nM) and GTE (100 nM) for 6, 12, and 24 h with simultaneous non-treated controls. Autophagy and cytotoxicity at the cellular level were studied using monodansylcadaverine (MDC, Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich). Cells were stained without any fixation firstly with 20 µg/ml PI for 5 min and then with 50 µM MDC for 10 min to visualize cell death or accumulation of autolysosomes (29). To analyze nuclear size, the fluorescence dye Hoechst 33258 (Sigma-Aldrich) was used. Cells were fixed with 4% PFA (Carl Roth, Karlsruhe, DE), Hoechst 33258 (1 µg/ml) was added and incubated for 15 min. Cells were examined by fluorescence microscopy and photographed using an AxioPlan microscope system (Zeiss, Oberkochen, DE). Fluorescence intensity of MDC was analyzed densitometrically and the size of nuclei was calculated via the ImageJ analyze particles command (ImageJ 1.46v, NIH, Bethesda, MD) (30).

Measurement of intracellular reactive oxygen species

For measurement of intracellular reactive oxygen species (ROS), cells were incubated with indicated EGCG concentrations for 5 h, then 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Thermo Fisher Scientific) in cell culture medium was added. After one further hour of incubation, fluorescence intensity was measured on an Infinite M1 000 PRO microplate reader (Tecan Group, CH).

Western blot analysis

Treated cells were harvested and resuspended in 500 µl modified RIPA buffer. Twenty micrograms of lysate were separated in polyacrylamide gels and transferred to a PVDF membrane (Carl Roth). The blots were probed with anti-Caspase-3 (Cell Signaling, MA; 1:1,000), anti-LC3B (D11) XP Rabbit mAb (Cell Signaling; 1:1,000), anti-laminin-R (A-7) antibody (Santa Cruz Biotechnology; 1:1,000), and anti-GAPDH antibody (loading control, Abcam, Cambridge, UK; 1:2,000) at 4 °C overnight. Antigen was detected by enhanced chemiluminescence (Immobilon Western HRP Substrate, Merck Millipore).

Quantitative polymerase chain reaction

Total RNA was extracted from treated biopsy-derived primary cultures (GBM15 and 16) or from frozen tissue (normal, $n = 5$; high grade glioma, $n = 37$) and reverse transcribed (RNeasy Mini Kit, Qiagen; First Strand cDNA Synthesis Kit, Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed in a Rotor-Gene Q (Qiagen) using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific). Primers for the analyzed genes are listed in Table 1. Quantitative analysis of mRNA expression in relation to the reference genes *RPL13A* and *CYC1* was estimated according to the Pfaffl method (31).

Statistical evaluation

Comparisons based on data from viability and autophagy assays and from nuclear size determination were performed using one-way analysis of variance. Differences in mRNA expression between normal and high grade glioma were analyzed by Wilcoxon rank-sum test. Results are show as mean ± SD. Alpha was set at $P \leq 0.05$ for all analyses. All experiments were done in triplicate.

Results

Cell death induction by EGCG and GTE in supranormal concentrations

For dose-response analysis, primary glioblastoma cell cultures were incubated with increasing concentrations of EGCG and GTE (0.11–11,000 µM) over 24 h. Catechins began to have an effect, with a statistically significant increase in the level of cell death, at concentrations of 55 µM EGCG (GBM16, $P = 0.001$). The calculated mean half-maximal cytotoxic concentration (IC_{50}) was 489 ± 58 µM catechin (Fig. 1). Compared to other tumor entities, the determined IC_{50} was relatively high (32–34). A concentration of 500 µM EGCG and GTE was used in the later experiments. A subsequent cell viability analysis after treatment with 10 and 500 µM EGCG and GTE over a period of 48 h confirmed the cytotoxic properties of catechins (Fig. 2A). We observed a nearly complete reduction in

Table 1. Primers used for qPCR analysis.

Gene name	Gene symbol	Primer sequence (5′→3′)
Cytochrome c-1	*CYC1*	F: GAGGTGGAGGTTCAAGACGG R: TAGCTCGCACGATGTAGCTG
Ribosomal protein L13a	*RPL13A*	F: CCTGGAGGAGAAGAGGAAAGAGA R: TTGAGGACCTCTGTGTATTTGTCAA
BC12/adenovirus EIB 19 kDa interacting protein 3	*BNIP3*	F: GCTCGCAGACACCACAAGAT R: TCATGACGCTCGTGTTCCTC
Caspase 3	*CASP3*	F: ATGTCGATGCAGCAAACCTCA R: TCTTCACCATGGCTCAGAAGC
Death associated protein kinase 2	*DAPK2*	F: CCTCCCGCCGATTGTATGTT R: GCTCTGCCGCTTCTTGATGA
DNA damage inducible transcript 4	*DDIT4*	F: CCTCACCATGCCTAGCCTTT R: GGTAAGCCGTGTCTTCCTCC
GABA type A receptor-associated protein	*GABARAP*	F: CCTICTGATCTCACAGTTGGTCA R: GCTTCACAGACCGTAGACACT
Inducible nitric oxide synthase	*NOS2*	F: CAGCGGGATGACTTTCCAAG R: AGGCAAGATTTGGACCTGCA
Ribosomal protein SA (67-kDA laminin receptor)	*RPSA*	F: GCCTGTCTTTTCCGTGCTAC R: CCTAAGTGGGTTCCTGCTGC
Superoxide dismutase 2, mitochondria	*SOD2*	F: GGTTTTGGGGTATCTGGGCT R: CGGTGACGTTCAGGTTGTTC

Gene names, gene symbols and primer sequences are listed (F: forward, R: reverse).

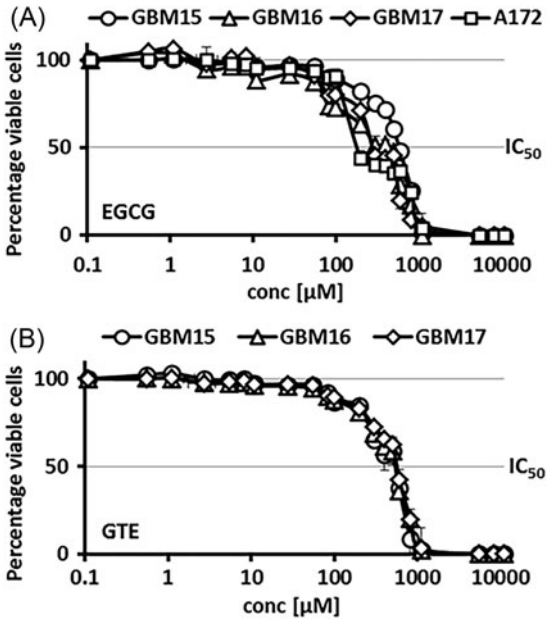

Figure 1. Dose response analysis for the catechins EGCG (A) and GTE (B). After incubation of glioblastoma cell cultures with increasing concentrations of the active compounds (0.11–11,000 µM) over 24 h the determination of the half maximal cytotoxic concentration (IC_{50}) of EGCG and GTE was done using the four-parameter logistic model. The calculated IC_{50} constitutes for EGCG: 565 µM (GBM15), 432 µM (GBM16), 411 µM (GBM17), and 456 µM (A172), and for GTE: 492 µM (GBM15), 523 µM (GBM16), and 543 µM (GBM17).

cell viability ($P < 0.001$) of 98.1% in GBM15 and 94.5% in GBM16 with EGCG, and of 98.5% (GBM15) and 95.2% (GBM16) with GTE, as expected. Using 10 µM EGCG and GTE, viability was reduced slightly compared to untreated cells.

In order to visualize cellular effects of catechin treatment on glioblastoma cells, we assessed nuclear size and autolysosome formation. In both treated cultures (500 µM EGCG for 24 h), upon Hoechst 33258 staining we observed statistically significant nuclear shrinkage, a sign of cell death (GBM15 0.55-fold, $P = 0.000$; GBM16 0.78-fold, $P = 0.000$; Fig. 2B). To analyze the formation of autolysosomes in EGCG-treated GBM15 and 16 cells, MDC/PI live cell staining was performed. Monodansylcadaverine (MDC) incorporates in lipid-rich membranes and is located in a juxta-nuclear region and in a dispersed peripheral pool. In comparison, propidium iodide (PI) permeates dead cells and binds to double-stranded DNA. During the first 6 h of EGCG treatment, significant accumulation and increasing dispersal of MDC occurred (GBM15 3.40 -fold, $P = 0.000$; GBM16 2.13-fold, $P = 0.000$; Fig. 2C). Over the next 18 h of incubation profuse nuclear PI staining (indicating cell death) was detected. These results were in line with the cell viability measurements. An analysis of apoptotic and autophagic markers based on protein levels revealed strong caspase-3 activation and increased LC3B II formation after 6 and 12 h of incubation (Fig. 2D).

After treatment with 500 µM EGCG over 12 h and compared to non-treated controls, mRNA levels of apoptotic markers were differentially expressed between the two cell cultures. Whereas *CASP3* and *BNIP3* mRNA expression was upregulated in primary GBM15 cultures, their expression was downregulated in GBM16 cultures (Fig. 2E,F). In both cell cultures *DDIT4, a* regulator of autophagy, showed a strong increase in its expression, together with increased *DAPK2* and *GABARAP* mRNA levels (Fig. 2G–I). Interestingly, changes in mRNA expression were much more pronounced in GBM15 than in GBM16

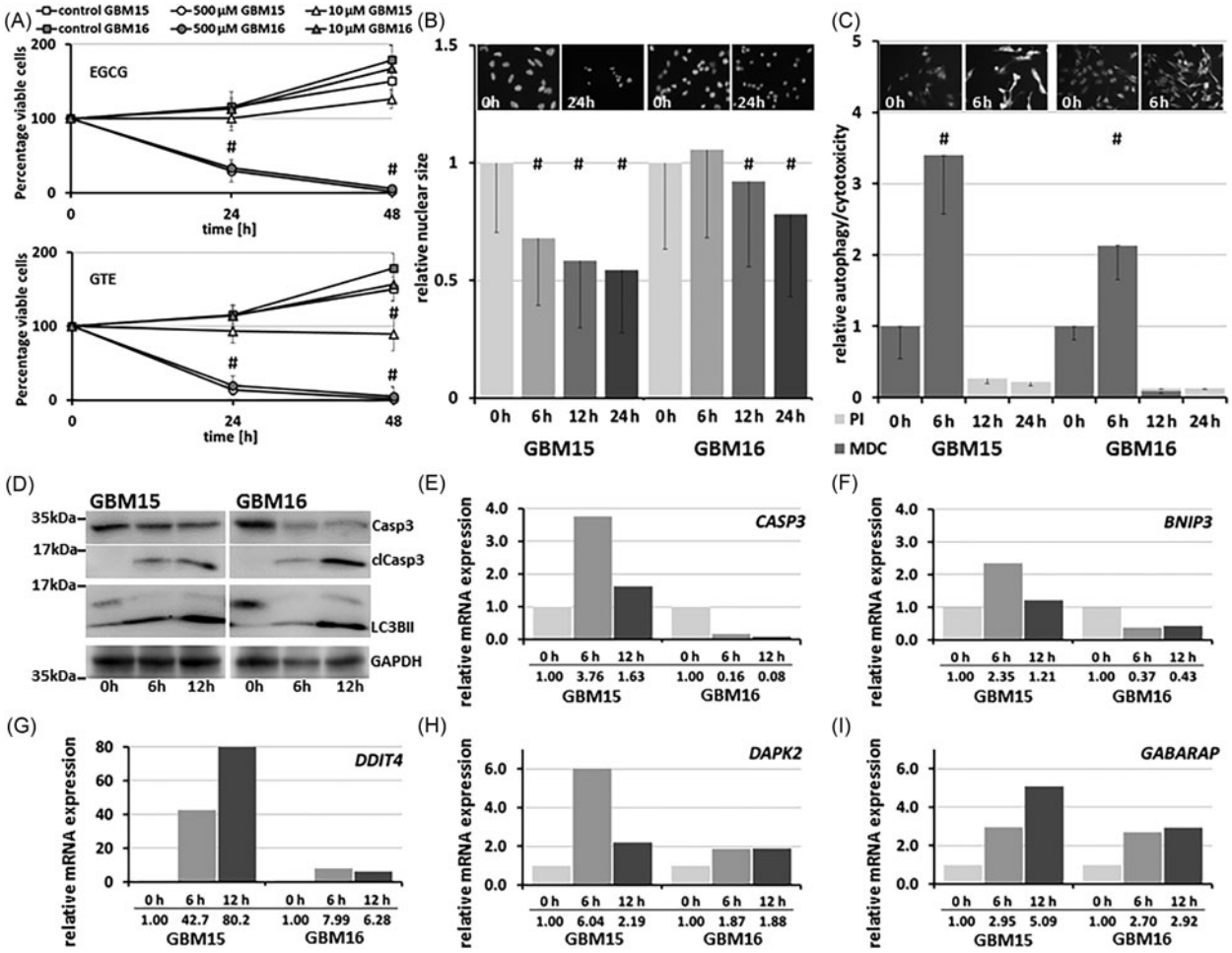

Figure 2. Induction of cell death by incubation of GBM15 and 16 with catechins. (A) Detection of cell viability on GBM15 and 16 after 48 h of incubation with 10 µM EGCG and GTE (triangle, #$P < 0.001$), and 500 µM EGCG and GTE (circle, #$P < 0.001$) in percent. (B) Determination of the nuclear shrinkage in relation to control (0 h) over 24 h of incubation with 500 µM EGCG, stained with Hoechst 33258 (#$P < 0.001$). Images show examples of treated versus untreated cells. (C) Identification of autophagic activity compared to control (0 h) after 24 h of incubation with 500 µM EGCG, stained with MDC/PI (#$P < 0.001$). Images show examples of treated versus untreated cells. (D) Determination of caspase-3 activity and LC3B conversion after 12 h of incubation with 500 µM EGCG using western blotting. (E–I) Detection of the mRNA expression of CASP3 (E) and BNIP3 (F) as apoptotic markers and DDIT4 (G), DAPK2 (H) and GABARAP (I) as autophagic markers after 12 h of incubation with 500 µM EGCG. The change in expression level compared to untreated control for the different time points is shown (values in data table).

cultures. It seems that in GBM16 cells, different protective mechanisms might be active versus GBM15 cells.

In order to analyze the influence of EGCG on cellular oxidative stress, the formation of reactive oxygen species (ROS) was determined by H_2DCFDA fluorescence staining after 6 h of incubation with 500 µM EGCG. The intracellular ROS level, a sign of oxidative stress, was increased 1.68-fold in GBM15 ($P = 0.003$) and 1.48-fold in GBM16 ($P < 0.001$, Fig. 3A). High expression levels of the inducible nitric oxide synthase *NOS2* in GBM15 (2.18-fold, Fig. 3B), and of the mitochondrial superoxide dismutase *SOD2* (GBM15 9.55-fold, GBM16 2.36-fold, Fig. 3C), but not the cytoplasmic superoxide dismutase *SOD1* (Fig. 3D) also indicated elevated ROS production.

Treatment effects with CNS-achievable concentrations of EGCG and GTE on glioblastoma cells

Given that green tea is a widely consumed beverage worldwide, the influence of catechins at CNS-achievable concentrations (100 nM; 22,23) on tumor cell growth was analyzed. Both primary glioblastoma cell cultures were incubated over six days with repeated catechin application. During this treatment period no time-dependent effects on the apoptotic and autophagic markers caspase-3 and LC3B II were detectable through Western blot analysis (Fig. 4A,B). Furthermore there were no influences on cellular effects, as accumulation of autolysosomes and changes in nuclear size were not observed even after long-term catechin exposure (data

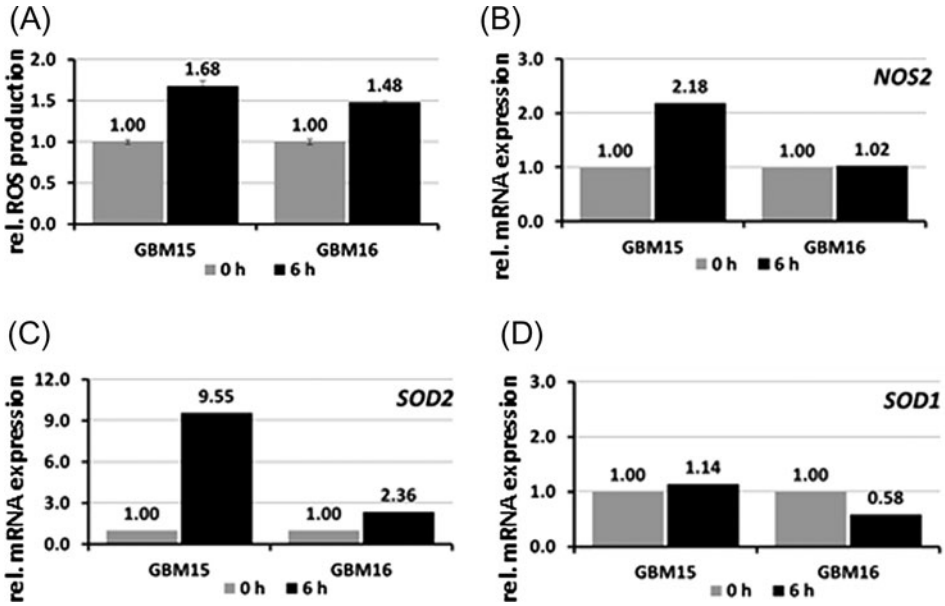

Figure 3. Determination of induced cellular oxidative stress after 6 h incubation with 500 µM EGCG on the glioblastoma cell cultures GBM15 and 16. (A) The relative intracellular ROS production in H2DCFDA stained cells increases in treated cells compared to untreated controls. (B–D) Detection of the relative mRNA expression of NOS2 (B), SOD2 (C) and SOD1 (D) compared to untreated control cells after 6 h of incubation.

not shown). In the analysis of cell viability during this treatment period, no differences were found between catechin-treated and untreated cells, with the continuously growing cell cultures showing no visible changes in cytomorphology (Fig. 4C,D).

It is possible that in CNS-achievable concentrations catechins may have short-term effects, given their half-life of only 5 h in human plasma (35). Acting as a mild stressor, they activate endogenous mechanisms of repair and maintenance to protect cells against subsequent stresses. Investigating the influence of CNS-achievable catechin concentrations on tumor cells over a short incubation time of 2 days, changes in the cell viability were found. Within the 48 h period, in GBM15, 100 nM EGCG reduced cell viability by 11.0% ($P = 0.073$) and GTE by 25.3% ($P < 0.001$). The cell viability of GBM16 was slightly increased by EGCG to 11.4% and by GTE to 1.5% (not statistically significant; Fig. 4E). Within the first 24 h a further influence of EGCG and GTE on autophagy was demonstrated. This was detected at the cytological and expression levels. To visualize possible cellular effects, we looked for potential changes in autolysosome formation and nuclear size. During the first 12 h of treatment with 100 nM EGCG and GTE, accumulation of autolysosomes was significantly increased, in GBM15 by 4.76-fold (EGCG, $P < 0.001$) and 4.16-fold (GTE, $P < 0.001$, Fig. 4F), but not in GBM16. Here, within the first 6 h of incubation, catechins induced initial autolysosome formation (EGCG: 2.04-fold, $P = 0.014$; GTE: 1.23-fold, $P = 0.004$), which decreased rapidly during the following 6 h (EGCG: 0.53-fold; GTE: 0.60-fold). An induction of nuclear shrinkage was not detectable (data not shown). Messenger RNA expression of *DAPK2*, *DDIT4*, and *GABARAP* strongly increased during 24 h of incubation with 100 nM EGCG and GTE (Fig. 4G,H) in both glioblastoma cell cultures. Given that an autophagic cellular response was detectable within the first 6–12 h of incubation with catechins at CNS-achievable concentrations, the formation of ROS was analyzed. Slight increases in cellular ROS production, to 1.15-fold in GBM15 ($P = 0.048$) and 1.25-fold in GBM16 ($P = 0.008$), were detectable after 6 h of catechin treatment (Fig. 5A). In contrast, the mRNA expression of *NOS2* (GBM15: EGCG 3.8-fold, GTE 2.0-fold; GBM16: EGCG 3.7-fold, GTE 7.1-fold, Fig. 5B) and *SOD2* (GBM15: EGCG 8.2-fold, GTE 2.4-fold; GBM16: EGCG 1.2-fold, GTE 19.1-fold, Fig. 5C), but also *SOD1* (GBM15: EGCG 2.3-fold, GTE 0.8-fold; GBM16: EGCG 1.2-fold, GTE 3.9-fold, Fig. 5D) strongly increased. The strength of the observed cellular effects corresponds to the degree observed for the autophagy markers *DAPK2*, *DDIT4*, and *GABARAP*.

Effects of combinatory treatment with CNS-achievable concentrations of EGCG and temozolomide or lomustine on glioblastoma cells

Further the influence of EGCG at CNS-achievable concentrations (100 nM) on the effect of temozolomide

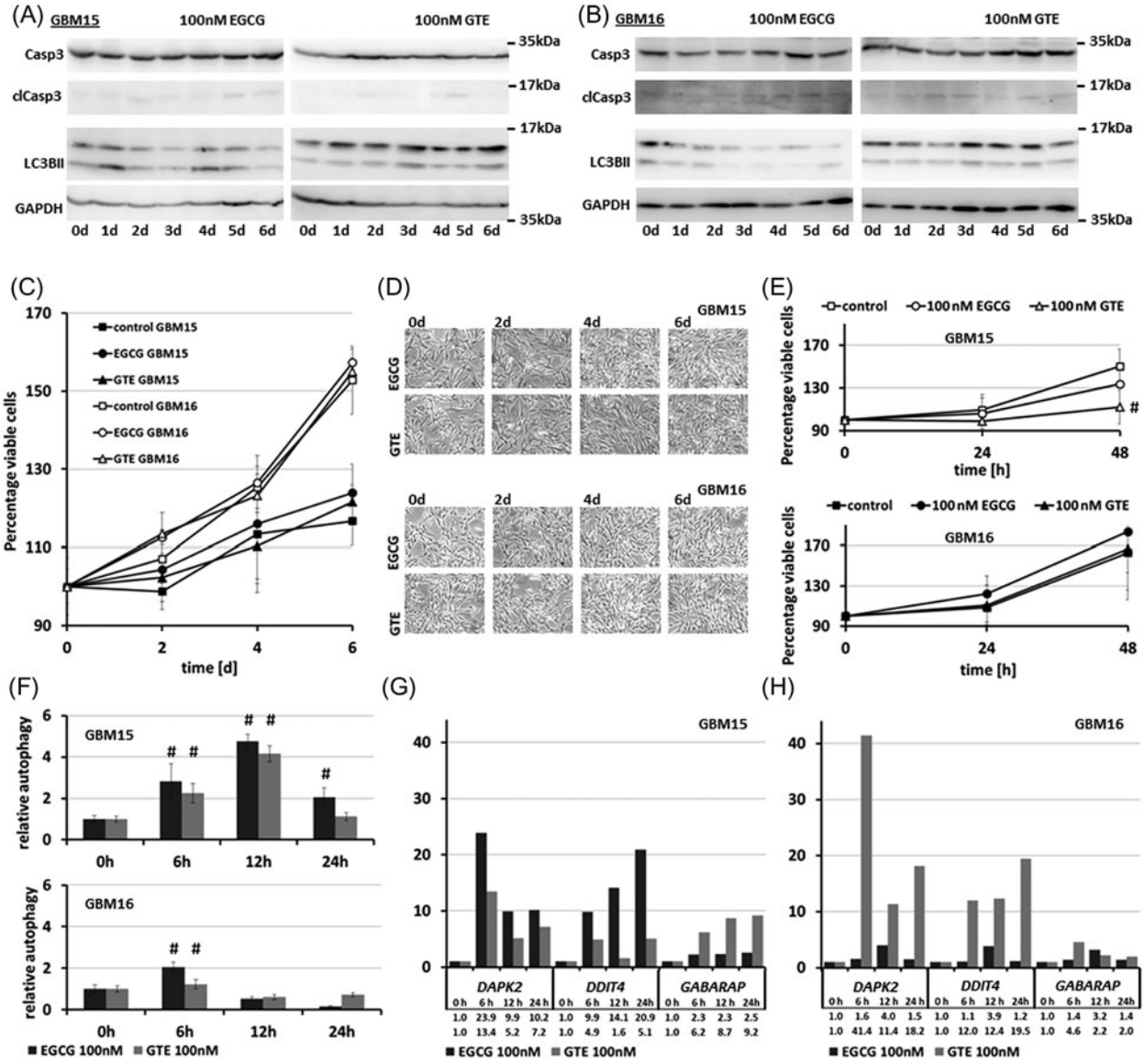

Figure 4. Analysis of the cellular reaction of glioblastoma cell cultures GBM15 and 16 after incubation with the intracranial reachable concentration (100 nM) of EGCG and GTE. (A and B) Detection of caspase-3 activity and LC3B conversion after 6 days of incubation using western blots in GBM15 (A) and GBM16 (B). (C) Detection of cell viability on GBM15 and 16 after 6 days of incubation with 100 nM EGCG (circle) and GTE in percent (triangle). (D) Representative phase contrast images for every time point are shown. (E) Detection of cell viability on GBM15 and 16 after 48 h of incubation with 100 nM EGCG (circle, #$P < 0.001$) and GTE in percent (triangle). (F) Identification of autophagic activity compared to control (0 h) after 24 h of incubation with 100 nM EGCG and GTE, stained with MDC/PI (#$P < 0.05$). (G and H) Detection of mRNA expression of DDIT4, DAPK2, and GABARAP as autophagic markers after 24 h of incubation with 100 nM EGCG and GTE in GBM15 (G) and GBM16 (H). The change in expression level compared to untreated control for the different time points is shown (values in data table).

(TMZ) and lomustine (CCNU; both 50 and 100 µM (27,36) on tumor cell growth was analyzed. Two primary glioblastoma cell cultures were incubated over six days with repeated catechin application. After this pretreatment period the cultures were incubated with 100 nM EGCG in combination with TMZ or CCNU over 48 h. The results were compared with the effects of a combinatory treatment of TMZ or CCNU with 100 nM EGCG and the effects of TMZ or CCNU alone. The cell cultures were chosen depending on their MGMT promoter methylation status. In GBM15 the MGMT promoter was methylated, like the original tumor sample. GBM02 and its corresponding tumor sample showed no methylation of the MGMT promoter. GBM15 cells were more sensitive to TMZ treatment than GBM02 cells, as expected (GBM15: 50 µM 10.47% viability reduction, $P = 0.032$, 100 µM 14.93% viability reduction, $P = 0.007$, Fig. 6A; GBM02: increase in viability, Fig. 6B). In both cultures a negative CCNU response was detected, the cells showed increased

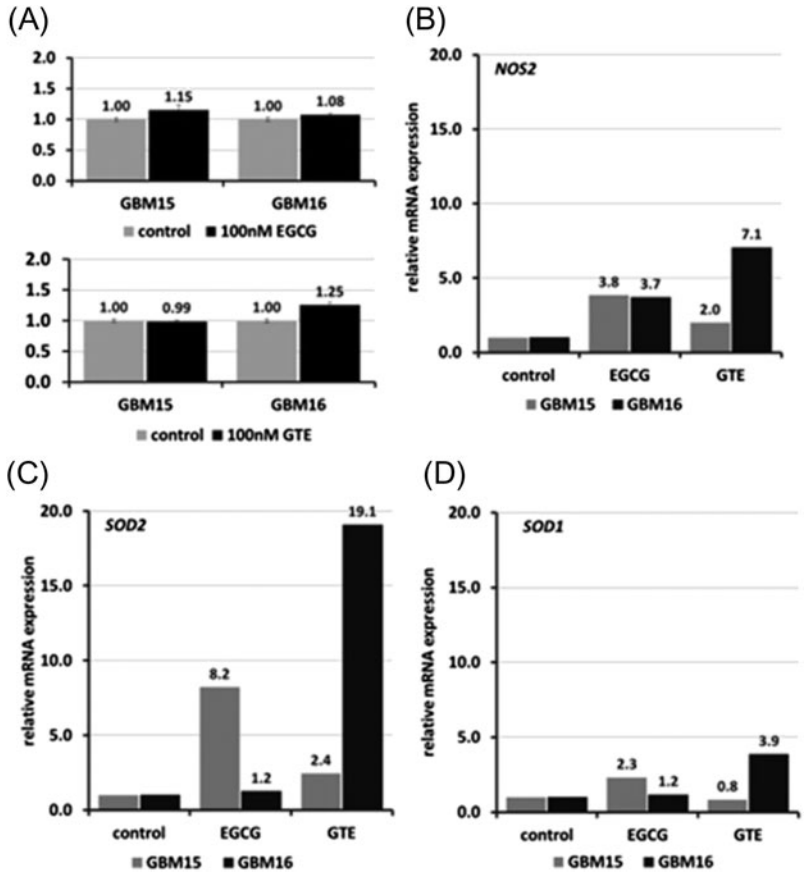

Figure 5. Determination of induced cellular oxidative stress after incubation with 100 nM EGCG and GTE for 6 h on the glioblastoma cell cultures GBM15 and 16. (A) The relative intracellular ROS production, detected by H2DCFDA staining, increases in treated glioblastoma cells compared to untreated control cells. (B–D) Detection of the relative mRNA expression of NOS2 (B), SOD2 (C) and SOD1 (D) compared to untreated control cells.

viability compared to vector control. A combinatory treatment of TMZ together with 100 nM EGCG over 48 h showed a stronger reduction in viability than TMZ alone (GBM15: 50 µM 11.87% viability reduction, $P = 0.023$, 100 µM 17.1% viability reduction, $P = 0.004$; GBM02: increase in viability). On the other hand, after 6 days of pretreatment with 100 nM EGCG the decrease in viability was less pronounced (GBM15: 50 µM 4.25% viability reduction, 100 µM 11.45% viability reduction, not significant; GBM02: increase in viability).

67-kDa laminin receptor binding of EGCG

The 67-kDa laminin receptor (67LR/*RPSA*) represents a confirmed target of EGCG (37). To test whether EGCG acts on glioblastoma cells by binding to 67LR, the receptor was blocked with a specific antibody and cell viability was analyzed. After 24 h of incubation with the supranormal concentration of EGCG (500 µM), GBM15 cells pretreated with the anti-laminin-R-antibody showed a 37.8% higher cell viability compared to the isotype control antibody-treated cells (Fig. 7A, $P = 0.002$).

As *RPSA* expression is enhanced in some tumors and correlates with tumor progression (38), we analyzed the expression of this gene in gliomas. In the high-grade glioma tumor specimens analyzed in this study, a 1.3-fold ($P = 0.043$) increase of *RPSA* gene expression was detected (Fig. 7B), in comparison with normal tissue. In both cell cultures GBM15 and GBM16 the 67LR/*RPSA* expression was demonstrated at mRNA and protein level (Fig. 7C).

Discussion

Green tea is one of the most widely consumed and popular beverages worldwide. In terms of assessment of health benefits it is the best studied tea and its chemistry has been thoroughly investigated (39). Green tea has demonstrated promising cancer-preventative, or chemopreventive, effects in both laboratory experiments and human epidemiological studies (40). In general chemoprevention is understood to be the use of

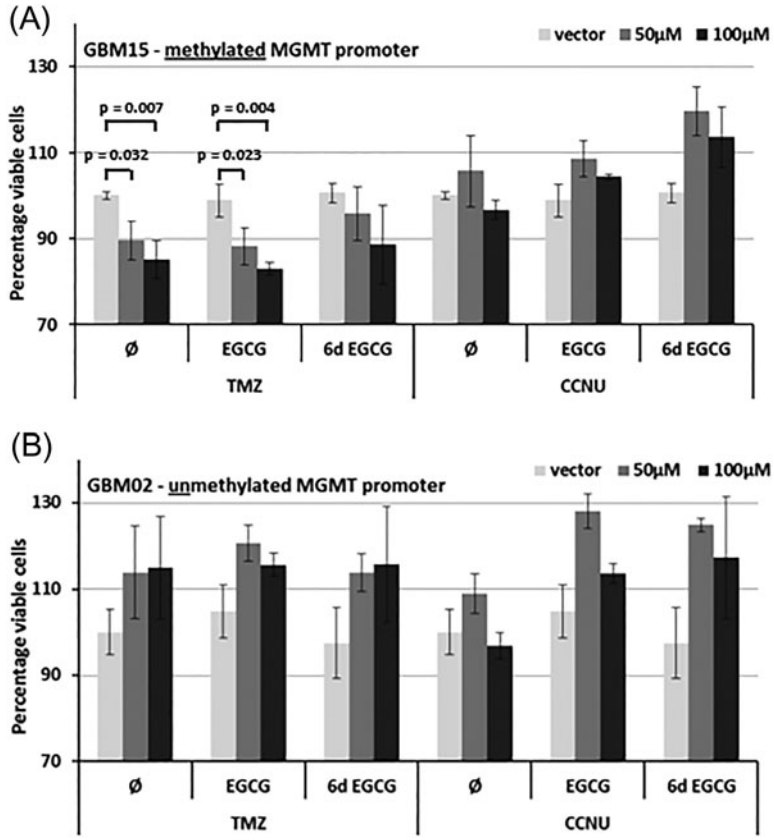

Figure 6. Induction of cell death by incubation of (A) GBM02 (methylated MGMT promoter) and (B) GBM15 (unmethylated MGMT promoter) with TMZ and CCNU. The relative cell viability after 48 h of incubation with 50 µM and 100 µM TMZ and CCNU with and without 100 nM EGCG and after 6 days of incubation with 100 nM EGCG in percent is shown in comparison to vector treated cells.

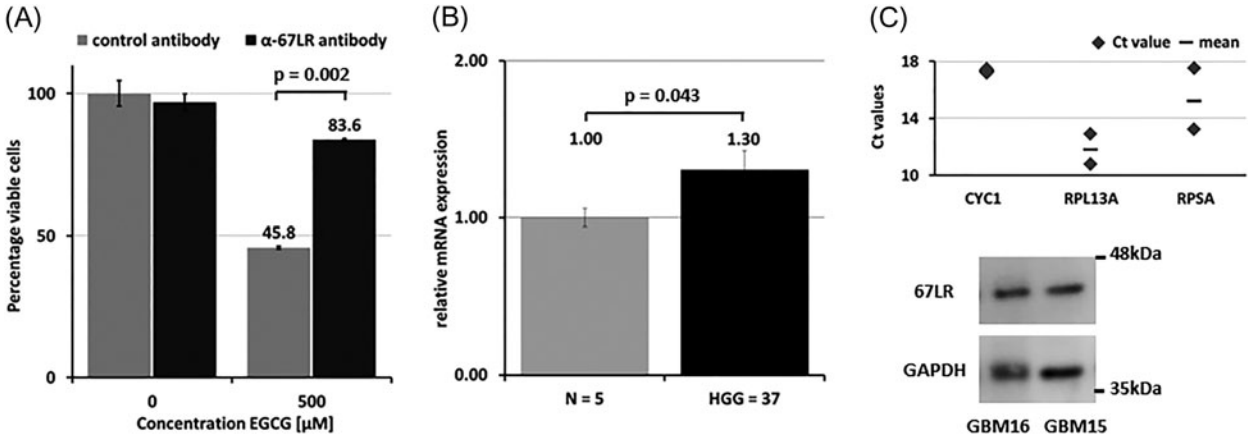

Figure 7. Effects of EGCG treatment on the 67 kDa laminin receptor. (A) Detection of cell viability on GBM15 after 24 h of incubation with or without 500 µM EGCG. The anti-Laminin-R antibody-treated cells are compared to isotype control antibody-treated cells ($P = 0.002$). (B) Relative mRNA expression of RPSA in glioma. Gene expression analysis revealed a 1.3-fold increased RPSA gene expression in high grade tumor specimens ($P = 0.043$). (C) Messenger RNA and protein expression of RPSA/67LR in GBM15 and GBM16. Messenger RNA expression of CYC1, RPL13A (reference genes), and RPSA is shown as Ct values (rhombus) and mean (line) for untreated cells of both cultures. Detection of 67LR expression compared to reference protein GAPDH using Western blots.

drugs, vitamins, or other agents for reduction of the risk of development or recurrence of cancer (41).

EGCG treatment has been shown to reduce tumor weight in mice with breast cancer without adverse effects on healthy tissues (42,43). Tumor growth in Kaposi's sarcoma is similarly restrained (44). Epidemiological studies from Japan have shown cancer-preventative effects (45), with a prolongation of

disease-free survival after cancer treatment (46) where subjects consumed more than five cups of green tea per day.

These findings prompted us to test the influence of EGCG on glioblastoma cells in vitro at two different concentrations: the half-maximal cytotoxic concentration and a lower concentration that approximates that achievable in the central nervous system. The ultimate objective of this study was to determine whether the lower concentration acts as a cytotoxic or chemopreventive molecule at CNS-attainable concentrations.

There are two main modes of action of the inhibitory effect of EGCG on tumor promotion and progression. Firstly, owing to its amphiphilic property, with both hydrophobic and hydrophilic groups in the molecule, EGCG binds nonspecifically to a diverse array of proteins, leading to conformational changes. Thus EGCG directly or indirectly blocks signaling pathways important for carcinogenesis and tumor growth, such as growth factor receptor and MAP-kinase pathways (11,13–17,42). Other examples of EGCG target proteins include 67LR (47), vimentin (48), phosphofructokinase (49), urokinase (50), and MMP-2 (51).

The second mode of action of EGCG involves the induction of cell death via the extra- and intracellular generation of ROS. In aqueous solutions EGCG rapidly dimerizes and auto-oxidizes to generate ROS such as hydrogen peroxide (52). This suggests that the cytotoxic effect of EGCG, especially at supranormal concentrations (e.g., at 500 µM as used in this study), is enhanced owing to ROS generation that triggers cell death pathways, among them caspase-related apoptosis (53).

We demonstrated the strong and wide-ranging cytotoxic effects of a supranormal concentration of EGCG on primary glioblastoma cell cultures. After 6 h of catechin exposure, the cells rounded up and detached. Autophagy was also induced immediately and was already at an advanced stage after 6 h of incubation, confirmed by autolysosome staining, LC3B conversion, and high mRNA levels of *DDIT4*, *DAPK2* and *GABARAP*. DDIT4 is involved in autophagy regulation in response to cellular stress (54), whereas DAPK2 and GABARAP mediate the formation of autophagic vesicles (55,56). Autophagy gave way to cell death, shown by propidium iodide influx in cells, pyknotic nuclei and caspase-3 activation. We were also able to demonstrate a rise in ROS levels and an increase in the expression of the nitric oxide synthase *NOS2* (NO production) and in the expression of the superoxide dismutase *SOD2* (removal of peroxides) as indicators of increased cellular oxidative stress that trigger programed cell death (57–59).

We have shown here that EGCG is cytotoxic to glioblastoma cells at the supranormal concentration of 500 µM. Similar results have previously been presented by other authors using glioblastoma cell lines, whereby an alternative range of concentrations (25 µM to 1.1 mM) was investigated. It was demonstrated that EGCG (50 µM) induced apoptosis in the human glioblastoma cell lines T98G and U87MG, but not in normal astrocytes (19). Furthermore, it has been shown that the growth inhibitory effect of EGCG is associated with the induction of apoptosis in U373 MG, U87MG, and C6 cells (20). Apoptosis is induced through enhanced oxidative stress and disruption of redox homeostasis, shown in U87MG cells (21).

In contrast to most published data, where no less than 20 µM of EGCG was used in cell culture systems, reviewed by Tachibana (4), we tested a lower, CNS-attainable concentration of 100 nM EGCG. EGCG has been shown to penetrate the blood–brain barrier and to be measurable in brain parenchyma 6 h after administration in a mouse model given a plasma concentration of 12% (23). Assuming that the maximal human plasma concentration (C_{max}) of EGCG after ingestion of 2–3 cups of green tea is in the range of 1–0.6 µM (22), a concentration of 100 nM EGCG in cell culture medium could reflect the CNS-achievable concentration. Using cell culture medium supplemented with serum to dissolve EGCG stock solution to a concentration of 100 nM, it is stabilized by albumin through a reversible interaction (60) that prevents auto-oxidation. Furthermore it has been shown that EGCG in concentrations of less than 10 µM, dissolved in cell culture medium, does not produce H_2O_2 (61). Using the potential CNS-achievable concentration of 100 nM EGCG, the resulting level of extracellular oxidized EGCG radicals in the experiments should be negligible. For this reason, only the direct intracellular effects of EGCG itself were measured. Due to the daily administration of EGCG at this concentration and based on its half-life of 5 h (35), the cells were exposed to this stress factor only short-term and at a nontoxic concentration. By forming ROS the autophagic flux can be activated (62), and this contributes to the restoration of cellular homeostasis (41). It prevents cell death by degradation of damaged proteins per se, as already described in various cell-damage states (63). We were able to detect these effects within 12 h after catechin application. The regularly exposure to catechins over a longer period of time could lead to a

constant influence of the stressor with habituation of the cells occurring. We could prove the negative influence of regular exposure to catechins over a longer period of time. After application of EGCG in the potential CNS-achievable concentration of 100 nM for 6 days, the cytotoxic effect of temozolomide on glioblastoma cells was reduced. This cellular response could be interpreted as an adaptive stress response and thus represent hormesis. The concept of hormesis has been widely used in the field of toxicology, meaning that low doses of toxins and other stressors are usually harmless but they activate an adaptive stress response that raises the organism's resistance against higher doses of the same stressor (64). In the case of a cellular stress response, this concept supposes that mild forms of stress activate different endogenous repair mechanisms to protect cells (24). These are mainly autophagic processes, that is, cytoprotective mechanisms relying on the digestion of potentially harmful intracellular structures (65). We hypothesize that regular exposure to physiological oxidative stress by consuming green tea and thereby intaking catechins activates protective pathways involved in stress resistance. The induction of an adaptive response has been shown in rodents after consumption of tea (66). Known examples of transcription factors activated by hormetic phytochemicals include NRF2, CREB, FOXO-3, and NF-kB (67–70). These four transcription factors regulate expression of a wide range of protective proteins, including antioxidant enzymes, protein chaperones, and Bcl-2 family members. Such highly conserved pathways are important for cellular survival and resistance to carcinogens.

It has been reported that the inhibitory effect of EGCG on tumor cell proliferation is exerted through its binding to the 67LR as a cell-surface receptor (37,47). We confirmed this effect for the first time in glioblastoma cells. In 67LR antibody-treated cells, the inhibitory effects of EGCG were significantly lower than those observed in the control cells (isotype antibody-treated). The expression of 67LR/*RPSA* has been shown to be enhanced in different tumor entities and to correlate with tumor progression (38). In this study, it could be proven for glioblastoma. A further examination of data generated by the TCGA Research Network (http://cancergenome.nih.gov/) revealed a 1.68-fold ($P = 0.001$) upregulation of mRNA expression of *RPSA* in glioblastoma, compared to normal tissue (71). In the investigated cell cultures the 67LR/*RPSA* expression was detectable at both mRNA and protein level. These results demonstrate that 67LR has a pivotal role as a cell-surface receptor that mediates the inhibitory action of EGCG on cell growth and apoptosis induction in glioblastoma cells.

Conclusions

In conclusion, we found that at supranormal concentrations EGCG suppressed proliferation of glioblastoma cells by inducing apoptotic and autophagic cell death, as expected. Conversely, a cytotoxic effect of EGCG at concentrations reflecting those that are understood to be achievable in the CNS could not be verified. At this nontoxic concentration EGCG acts as a mild stressor and activates autophagic flux by forming reactive oxygen species. Regular exposure to catechins through consumption of green tea seems to lead to activation of protective pathways that confer stress resistance. These results provide evidence that green tea has nutraceutical potential. In the context of longer-term regular consumption, green tea is likely to induce chemopreventive effects rather than be associated with cytotoxic anticancer activity.

Disclosure statement

The authors declare that they have no conflicts of interest.

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