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Epigalocatechin-3-gallate (EGCG) downregulates PEA15 and thereby augments TRAIL-mediated apoptosis in malignant glioma

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ABSTRACT

EGCG is a flavonoid that exhibited therapeutic activity in cancer. In this study three glioblastoma cell lines (U87, A172 and U251) were treated with EGCG, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or the combination of both. Treatment with subtoxic doses of EGCG in combination with TRAIL induces rapid apoptosis in TRAIL-resistant glioma cells, suggesting that this combined treatment may offer an attractive strategy for treating gliomas. EGCG treatment down-regulated phosphoprotein-enriched in astrocytes (PEA15) through an Akt (PKB)-dependent mechanism. In addition, over-expression of PEA15 attenuated cytotoxicity induced by the combination of EGCG and TRAIL. In summary, PEA15 is a key regulator in TRAIL–EGCG-mediated cell death in malignant glioma.

Malignant gliomas are the most commonly diagnosed malignant adult primary brain tumors. Median survival for glioblastoma is ~12 to 15 months. Surgical resection or diagnostic biopsy is usually the first step in therapy, followed by adjuvant radiation and chemotherapy [17]. Thus developing of new treatment strategies is necessary.

The use of flavonoids as anticancer agents has gained considerable importance in recent years. Several studies suggested that green tea, especially its constituent polyphenols possess chemopreventive and therapeutic potential against tumor cells [14]. Much of the anticancer and/or cancer chemopreventive effects of green tea are attributed to be mediated by its major polyphenol, epigallocatechin-3-gallate (EGCG) [14].

Targeting death receptors to trigger apoptosis in tumor cells is an attractive concept for cancer therapy. To this end, TRAIL appears to be a relatively safe and promising death ligand for clinical application since TRAIL induces almost selectively apoptosis in cancer cells. However, many tumors remain resistant to treatment with TRAIL, and this resistance may be caused by deregulated expression of anti-apoptotic molecules [14]. Inhibitor of apoptosis proteins (IAPs) including survivin are also capable of inhibiting TRAIL-mediated apoptosis in malignant glioma cells [14,2].

One of the causes of cell-death resistance could reside in altered expression of apoptosis inhibitory molecules belonging to the death-effector-domain (DED)-containing protein family, e.g. c-FLIP and phosphoprotein enriched in astrocytes (PEA15). c-FLIP and PEA15 are recruited to the DISC and inhibit the activation of caspase 8 [5]. The expression of both proteins is increased in several human glioma cell lines and the expression of PEA15 correlates with TRAIL resistance in a human glioma-derived cell line [5].

In the present study we investigated the effects of EGCG on the expression of several anti-apoptotic proteins in three human glioma cell lines, U87, U251 and A172 and demonstrated that the down-regulation of PEA15 and survivin is regulated by the AKT-pathway. In addition we provided evidence that the suppression of PEA15 by EGCG enhanced TRAIL-mediated apoptosis.

Human glioblastoma cell line U87 (*p53-wild type*), U251 and A172 (*p53 mutant*) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM Glutamax-I 4500 g/l glucose (Invitrogen, Karlsruhe, Germany) with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and was incubated at 37 °C in a humidified atmosphere containing 10% carbon dioxide.

EGCG, LY294002 and wortmannin were obtained from Axxora (Loerrach, Germany) and malignant glioma cell lines were treated with the indicated amounts of EGCG as individually indicated.

Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IAPs, inhibitor of apoptosis proteins; PEA15, phosphoprotein-enriched in astrocytes; EGCG, epigallocatechin-3-gallate; DISC, death-inducing signalling complex; ph-Akt, phosphorylated Akt; PKB, protein kinase B.

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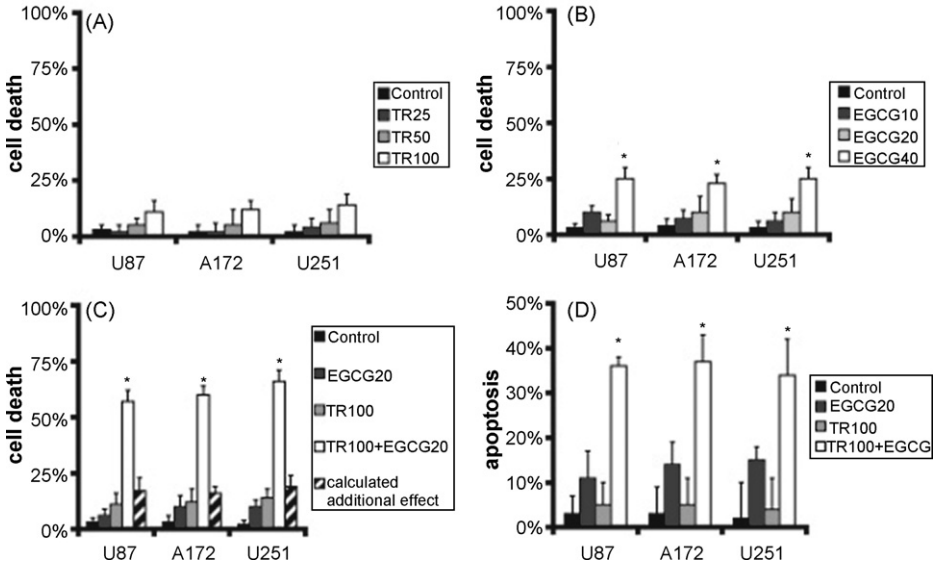

Fig. 1. Crystal-violet viability assay and flow cytometry after treatment with TRAIL, EGCG or the combination of both after 24 h of treatment. (A) Crystal-violet-assay in U87, A172 and U251 cells upon treatment with TRAIL. (B) Crystal-violet-assay in U87, A172 and U251 cells upon treatment with EGCG. (C) Crystal-violet-assay in U87, A172 and U251 cells upon combined treatment with TRAIL and 17 AAG. For determination of the calculated additive effect. (D) Effect of EGCG on TRAIL-induced apoptosis in U87, A172 and U251 cells analysed by flow cytometry. Control, not treated, EGCG 10/20/40–EGCG 10/20/40 μM, TR25/50/100–TRAIL 25/50/100 ng/ml. Experiments were performed at least three times. Asterisk (*) outline values which are different from the respective control (*t*-test, $^*p < 0.05$).

Recombinant human TRAIL/Apo2L was purchased from Peprotech (Rocky Hill, New York, USA). Transient transfection of U87 cells was achieved by Fugene Transfection reagent (Roche Deutschland Holding GmbH, Mannheim, Germany) or by electroporation, using Nucleofector I, programme U29 (Amaxa AG, Cologne, Germany). Using electroporation up to 80% transfection efficiency was achieved. The survivin-wild-type plasmid pcDNA3-survivin was a gift from Dario Altieri (University of Massachusetts, Worcester, USA). Empty pcDNA3 was used as a negative control in our experiments. The plasmid, pcDNA-HA-PEA15 was provided by Michael Ginsberg **(**University of California, San Diego, USA).

Cells were seeded into 96-well plates at a density of 3×10^4 cells/well in 100-μl tissue culture medium in triplicate. After 24 h incubation to allow cells to adhere U87, A172 and U251 glioma cells were treated for 24 h either with EGCG and TRAIL separately or in different combinations, as described in individual experiments. Cell death was determined as described [5]. The fractional product method was used to evaluate synergy. The effect of two independently acting agents is defined as the product of the unaffected fractions after treatment with either drug alone: $f_x(1,2) = f_x(1) \times f_x(2)$ reviewed in [22]. This formula allows the predicted effect of cotreatment to be calculated on the basis of the assumption that the two agents do not interact or cooperate in inducing their effects. If the relative percentage of surviving cells after cotreatment with the two drugs is below the calculated product, then the two drugs show synergy. Apoptotic cells were assessed by flow cytometry with PI-method. For detection of apoptotic cells a FACS Calibur flow cytometer equipped with a 488 nm air cooled argon laser (Becton & Dickinson, Cytometry Systems, San Jose, CA) was used with filter combinations for propidium iodide. For analyses and calculations the Cellquest program (Becton & Dickinson, Cytometry Systems, San Jose, CA) was used. For each measurement 10,000 cells were analysed. After cell preparation according to Nicoletti with modifications [3,4,11] measurements were acquired in Fl-3 in logarithmic mode and calculated by setting gates (M-1) over the first three decades to detect apoptotic cells.

Twenty micrograms of protein diluted in NuPAGE-sample buffer and reducing reagent (Invitrogen, Carlsbad, Germany) were denatured at 95 °C for 5 min and electrophoretically separated on ready-to-use 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, Germany). Proteins were blotted onto nitrocellulose membranes at 1.5 mA/cm^2 for 1.5 h (Invitrogen, Carlsbad, Germany). After blocking in 0.5 M Tris–base, pH 7.4, 5% milkpowder, 1.5 M NaCl, 0.05% Tween, the membranes were incubated with rabbit anti-human survivin antibody diluted

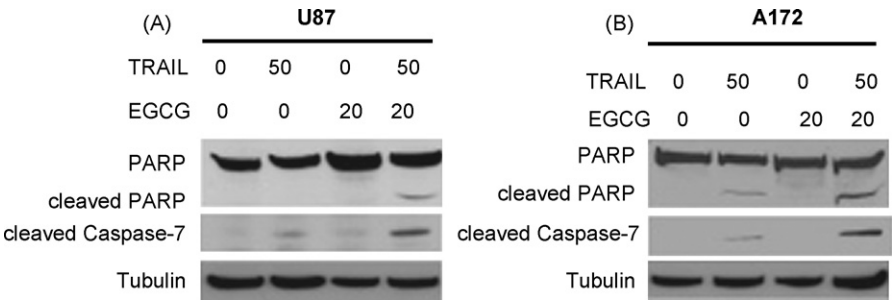

Fig. 2. Immunoblot demonstrating the effect of EGCG on TRAIL-induced proteolytic cleavage of PARP and caspase-7 in U87 and A172. (A) U87 cells were treated for 6 h with EGCG (20 μM) in the presence or absence of TRAIL (50 ng/ml). (B) A172 cells were treated for 6 h with EGCG (20 μM) in the presence or absence of TRAIL (50 ng/ml). EGCG–EGCG 5 μM, TR50–TRAIL 50 ng/ml. Immunoblots are representative of at least three independent experiments.

1:1000 (R&D Systems, Minneapolis, USA), rabbit anti-human PEA15 (CST Inc., Danvers, MA, USA) anti-human phosphorylated Akt (Ser 473) antibody diluted 1:100 (CST Inc., Danvers, MA, USA), rabbit anti-human cleaved PARP (CST Inc., Danvers, MA, USA), rabbit anti-human DR4/DR5 (R&D Systems, Minneapolis, USA) and rabbit anti-human caspase-7 (CST Inc., Danvers, MA, USA) overnight at 4 °C. Staining with secondary horseradish peroxidise conjugated anti-rabbit or anti-mouse antibodies at dilutions of 1:10,000 or 1:2000, respectively (Amersham Biosciences, Buckinghamshire, UK) was followed by immunodetection with Western Blotting Detection System ECL (Amersham Biosciences, Buckinghamshire, UK). Protein signals were analyzed semiquantitatively, using a computer-assisted image analysis system and the NIH gel analysis software (http://www.rsb.info.nih.gov/nihimage/download.html).

The data were expressed as mean ± standard error of mean (S.E.M.) of separate experiments ($n > 3$) and compared by the two-tailed paired Student's *t*-test. Differences between two treatments was considered significant at $p < 0.05$ and 0.01, respectively.

U87 (*p53-wildtype*), A172 (*p53-mutant*) and U251 glioma (*p53-mutant*) cells were resistant to low-dose TRAIL treatment (Fig. 1A). Treatment with TRAIL (i) 25 ng/ml, (ii) 50 ng/ml and (iii) 100 ng/ml for 24 h alone did not have a significant effect on cell death in U87 ((i) 2 ± 6%; (ii) 5 ± 4%; (iii) 11 ± 3%), A172 ((i) 2 ± 5%; (ii) 5 ± 4%; (iii) 12 ± 6%) and U251 ((i) 2 ± 5%; (ii) 4 ± 3%; (iii) 14 ± 5%) (Fig. 1A).

After 24 h treatment with low dose EGCG (i) 10 μM and (ii) 20 μM cell viability was not altered significantly in U87 ((i) 10 ± 3%; (ii) 6 ± 7%), A172 ((i) 14 ± 3% and (ii) 15 ± 6%) and U251 ((i) 7 ± 5%; (ii) 12 ± 6%) (Fig. 1B). However, treatment with (i) 40 μM increased cell death in U87 ((i) 25 ± 5%, $p < 0.05$), in A172 ((i) 23 ± 3%, $p < 0.05$) and in U251 ((i) 25 ± 3%, $p < 0.05$) (Fig. 1B).

Combining 20 μM EGCG with 100 ng/ml of TRAIL increased cell death in U87 (57 ± 7%, $p < 0.05$), A172 (60 ± 4%, $p < 0.05$) and U251 (66 ± 4%, $p < 0.05$) (Fig. 1C). The calculated additional effect was determined and yielded in U87 (17 ± 3%, $p < 0.05$), A172 (16 ± 4%, $p < 0.05$) and U251 (19 ± 6%, $p < 0.05$), suggesting that EGCG and TRAIL act in synergy. It has been reported that various cancer cells are resistant to the cytotoxic effects of TRAIL [7,8]. These include various glioma cells, although they express the TRAIL receptor DR5 [7]. Cell sensitivity to TRAIL can be affected by TRAIL-receptor expression at the cell membrane, DR4/DR5 ratio and functionality of TRAIL-receptors. Additional intracellular factors leading to TRAIL-resistance affect the caspase 8/c-FLIP ratio, such as loss of caspase-8 and caspase-10 due to mutations or gene methylation, degradation of active caspase-8 and changes in caspase 8 or c-FLIP expression levels. Further downstream in the TRAIL apoptotic pathway increased expression of IAP family members, in particular XIAP and survivin, also cause resistance [8].

To elucidate that TRAIL–EGCG-mediated cell death occurs by apoptosis we employed flow cytometry and determined the percentage of specific apoptotic cells (Fig. 1D). U87, A172 and U251 cells treated either with 100 ng/ml TRAIL or 20 μM EGCG alone for 24 h did not reveal a significant increase in apoptosis compared with that of untreated controls. Combined treatment with 100 ng/ml TRAIL and 20 μM EGCG augmented apoptotis in U87 (36 ± 4%; $p < 0.05$), in A172 (37 ± 5%; $p < 0.05$) and in U251 (34 ± 4%; $p < 0.05$) (Fig. 1D). *TP53* mutations are a common feature of malignant glioma [12]. Inactivation of p53 which has been reported to be involved in the intrinsic and extrinsic apoptotic pathways [21] seems not to be of major importance to TRAIL–EGCG-mediated apoptosis. Notably, both A172 and U251 cell lines harbour *TP53* mutations and both cell lines were efficiently sensitized to TRAIL-mediated apoptosis by EGCG.

We employed Western blotting to elucidate the proteolytic mechanism in TRAIL and TRAIL–EGCG-induced apoptosis (Fig. 2).

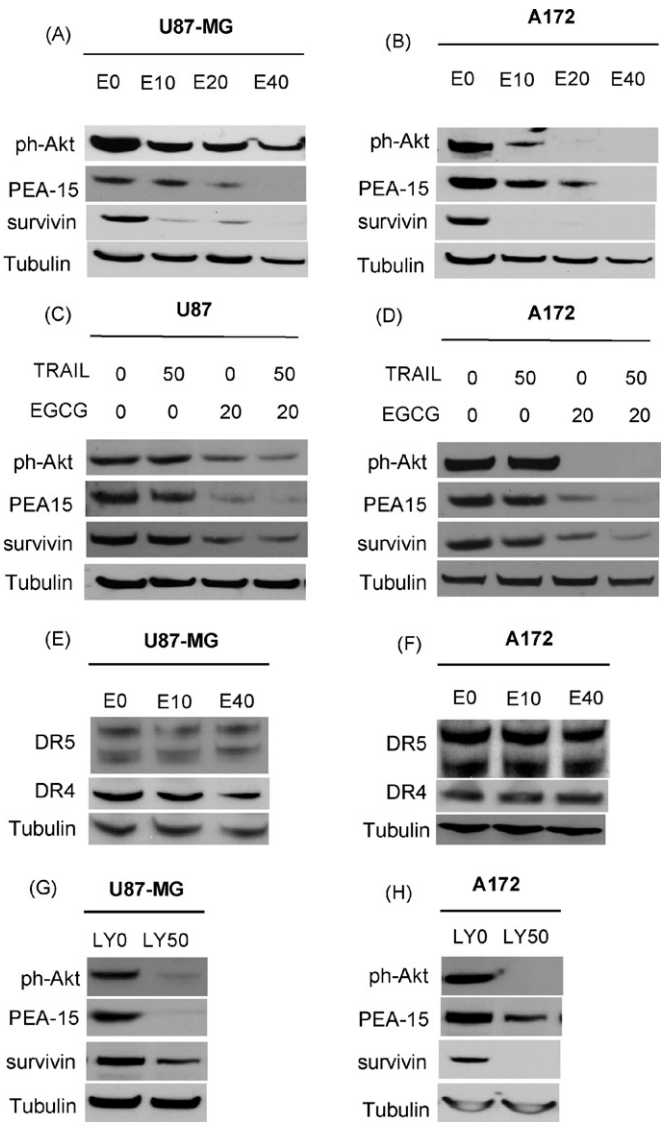

Fig. 3. Immunoblot showing expression of phosphorylated Akt, PEA15 and survivin after treatment with different concentrations of EGCG for 24 h. U87 (A) and A172 (B) cells were treated with increasing concentrations of EGCG for 24 h and harvested. Immunoblots show expression of phosphorylated Akt, PEA15 and survivin. U87 (C) and A172 cells (D) were treated for 24 h with EGCG (20 μM) in the presence or absence of TRAIL (50 ng/ml). Immunoblots show expression of phosphorylated Akt, PEA15 and survivin. (E and F) Western-blot showing DR4 and DR5 expression in U87 and A172 cells treated with increasing concentrations of EGCG. (G and H) Western-blot showing expression of ph-AKT, PEA15 and survivin in U87 and A172 cells treated with 50 μM of LY294002. EGCG 0/10/20/40–EGCG 0/10/20/40 μM, LY0/50–LY294002 0/50 μM.

U87 and A172 cells were treated with EGCG, TRAIL or the combination of both for 6 h (Fig. 2A and B). Since EGCG augments TRAIL-induced apoptosis, we examined the activation/cleavage of caspase-7 and poly-ADP-ribose-polymerase (PARP). Treatment of U87 and A172 cells with 20 μM EGCG alone did not induce cleavage of PARP or effector-caspase-7 (Fig. 2A and B). Exposure of cells to 50 ng/ml TRAIL alone yielded an undetectable signal of cleaved fragment of PARP sized 89 kDa and active cleaved caspase-7 sized 20 kDa in U87. Combining TRAIL with EGCG led to a significant increase in cleaved fragment of PARP and active cleaved caspase-7 in both U87 and A172 (Fig. 2A and B). Taken together, these data support that combined EGCG–TRAIL treatment induces apoptosis and EGCG efficiently augments TRAIL-mediated apoptosis.

24 h after treatment with EGCG we detected a significant down-regulation of PEA15 and survivin in U87 and A172 (Fig. 3A and B). In addition the protein levels of phosphorylated Akt (ph-Ak) were also suppressed (Fig. 3A and B). Single treatment with 50 ng/ml TRAIL for 24 h did not change the protein levels of PEA-15, ph-Akt and survivin significantly in U87 and A172 glioma cells (Fig. 3C and D). The combination of 50 ng/ml TRAIL and 20 μM EGCG resulted in a significant down-regulation of PEA15 in U87 and A172 cells and survivin in A172 cells compared to single treatment with EGCG (Fig. 3C and D). The protein levels of phosphorylated Akt in U87 glioma cells were also suppressed by the combined treatment with TRAIL and EGCG compared to single treatment with EGCG (Fig. 3C and D). Furthermore we analysed the protein levels of TRAIL-R1 (death-receptor 4; DR4) and TRAIL-R2 (death-receptor 5; DR5). After 24 h treatment with different concentrations of EGCG the protein levels of DR4 and DR5 did not change significantly in U87 and A172 glioma cells, respectively (Fig. 3C and D). This contrasts to the results of Siddiqui et al. who reported that in a prostate cancer cell line EGCG regulated DR4. They claimed that the up-regulation of DR4 was due to demethylation activity of EGCG. As a strategy of survival many cancers epigenetically repress the expression of death-receptors hence bypassing apoptosis [6] and EGCG has also been reported to modulate DNA methylation [16].

Since EGCG is a potent inhibitor of the AKT-pathway, we were interested in the importance of the AKT-pathway in the regulation of both protein levels of PEA15 and survivin. For that reason, U87 and A172 glioma cells were treated with the specific AKT-inhibitor, LY294002 (Fig. 3E and F). Treatment with 50 μM of LY294002 for 24 h resulted in a significant reduction of phosphorylated-Akt in both U87 and A172 cells, respectively. Importantly, the protein levels of PEA15 and survivin were also significantly reduced, suggesting that the AKT-pathway is involved in the regulation of both PEA15 and survivin protein levels. In addition we performed these experiments with an additional AKT-inhibitor, wortmannin, and the results were almost identical (data not shown). Our results are in line with previous reports since it has been demonstrated that PEA15 protein levels in breast cancer cells are dependent on the AKT-pathway [15]. PEA15 interacts *in vitro* and in intact cells with the serine-threonine kinase AKT [19]. AKT is also able to phosphorylate PEA15 at Ser^{116} [19]. AKT-mediated phosphorylation of PEA15 increases its stability, thus determining an increase in the content of this protein within the cell [19].

Regulation of survivin by the AKT-pathway has already been reported by other research groups [10,13]. Survivin is a highly evolutionarily conserved protein and has been reported to inhibit both caspase-dependent apoptosis and caspase-independent cell death [1]. Furthermore, it has been demonstrated that survivin also antagonizes mitochondrial and death receptor-mediated (TRAIL) apoptosis [1]. Survivin is one of the most tumor-specific molecules [20]. It also promotes tumor-associated angiogenesis [18] and acts as a resistance factor to various anticancer therapies [9].

Given that PEA15 is significantly suppressed by EGCG we were interested in the functional role of PEA15 in TRAIL–EGCG-mediated cytotoxicity. That is why we determined if ectopic over-expression of PEA15 is capable of rescuing glioma cells from TRAIL–EGCG-mediated cytotoxicity. Transfection efficiency was 90% and PEA15 protein levels were confirmed by Western-blot (Fig. 4A). 24 h after treatment with the combination of 100 ng/ml TRAIL and 20 μM EGCG cell death was reduced from 57 ± 7% in U87 cells without ectopic over-expression of PEA15 to 15 ± 3% in PEA15-transfected U87 cells ($p < 0.05$; Fig. 4B). After treatment with either 20 μM EGCG or 100 ng/ml TRAIL there was no significant difference

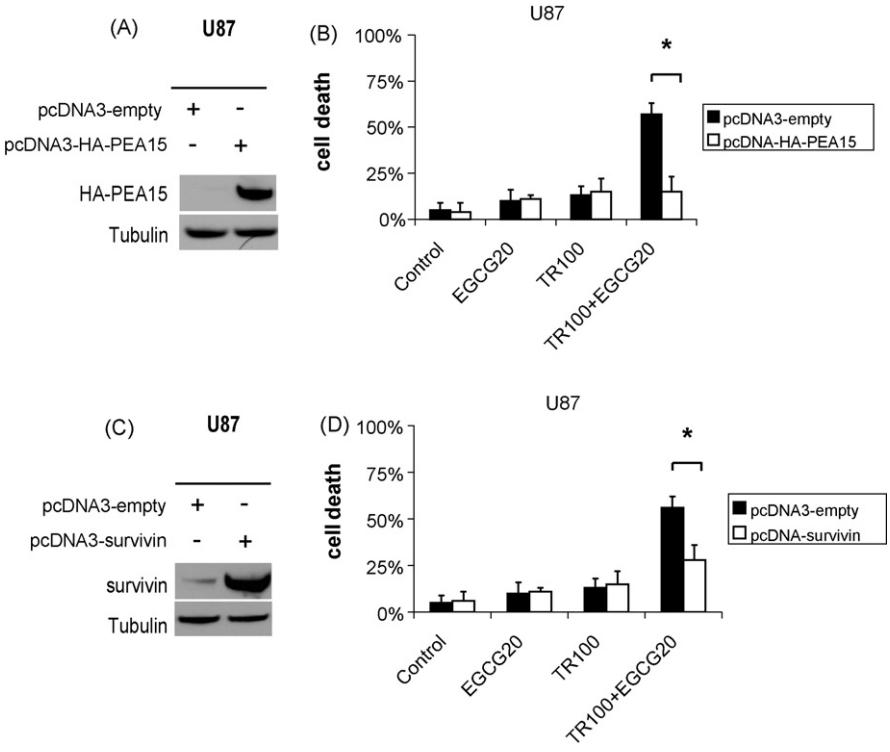

Fig. 4. Over-expression of PEA15 and survivin attenuate TRAIL–EGCG-mediated cytotoxicity. (A) Western-blot demonstrating PEA15 expression in U87 cells 48 h after transfection with pcDNA3-HA-PEA15 or pcDNA3-empty. pcDNA3-HA-PEA15 contains a HA-tag. (B) Crystal-violet-assay in U87 cells 72 h after transfection with pcDNA3-HA-PEA15 or pcDNA3-empty and 24 h after treatment with 100 ng/ml TRAIL and 20 μM EGCG. (C) Western-blot demonstrating survivin expression in U87 cells 48 h after transfection with pcDNA3-survivin or pcDNA3-empty. (D) Crystal-violet-assay in U87 cells 72 h after transfection with pcDNA3-survivin or pcDNA3-empty and 24 h after treatment with 100 ng/ml TRAIL and 20 μM EGCG. Cont.: Control, not treated, EGCG 20–EGCG 20 μM, TR100–TRAIL 100 ng/ml. Asterisk (*) indicates values which are different from the respective control (*t*-test, $^*p < 0.05$).

between cellular viability of vector transfected U87 cells and PEA15-transfected U87 cells (Fig. 4B).

Next it was determined if ectopic over-expression of survivin is also capable of inhibiting TRAIL–EGCG-mediated cytotoxicity. Transfection efficiency was 90% and survivin protein levels were confirmed by Western-blot (Fig. 4C). 24 h after treatment with the combination of 100 ng/ml TRAIL and 20 μM EGCG cell death was reduced from 57 ± 4% in U87 cells without ectopic over-expression of survivin to 28 ± 3% in survivin-transfected U87 cells ($p < 0.05$; Fig. 4D). After treatment with either 20 μM EGCG or 100 ng/ml TRAIL there was no significant difference between cellular viability of vector transfected U87 cells and survivin-transfected U87 cells (Fig. 4B). Importantly, over-expression of survivin led to a weaker attenuation of TRAIL–EGCG-mediated cytotoxicity than PEA15.

Taken together, we demonstrated that EGCG suppressed both PEA15 and survivin levels by inhibition of the Akt-pathway and thereby contributed to enhanced TRAIL-mediated cytotoxicity in glioma cells.

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