CD95-mediated Apoptosis of Human Glioma Cells: Modulation by Epidermal Growth Factor Receptor Activity

Joachim P. Steinbach[1]; Petra Supra[1]; H.-J. Su Huang[2]; Webster K. Cavenee[2]; Michael Weller[1]

[1] Laboratory of Molecular Neuro-Oncology, Department of Neurology, University of Tübingen, School of Medicine, Tübingen, Germany
[2] Ludwig Institute for Cancer Research, San Diego Branch, La Jolla, Calif

Apo2L/TRAIL	Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand
CD95L	CD95 ligand
CHX	cycloheximide
crm-A	cytokine response modifier-A
ECL	enhanced chemiluminescence
EGFR	epidermal growth factor receptor
FLIP	FLICE-inhibitory protein
XIAP	X-linked inhibitor-of-apoptosis protein
MAPK	mitogen activated protein kinase
DISC	death-inducing signaling complex
FADD	Fas-associated death domain adapter protein
PI3K	phosphatidylinositol-3 kinase

The death ligands CD95L and Apo2L/TRAIL are promising investigational agents for the treatment of malignant glioma. EGFR is overexpressed in a significant proportion of malignant gliomas in vivo. Here, we report that CD95L-induced cell death is enhanced by EGFR inhibition using tyrphostine AG1478 in 7 of 12 human malignant glioma cell lines. Conversely, CD95-mediated and Apo2L-induced cell death are both inhibited by overexpression of EGFR in LN-229 cells. CD95L-induced cell death augmented by AG1478 is accompanied by enhanced processing of caspase 8. LN-229 cells overexpressing the viral caspase inhibitor, crm-A, are not sensitized to CD95L-induced cell death by AG1478, indicating that EGFR exerts its antiapoptotic properties through a caspase 8-dependent pathway. These data define a modulatory effect of EGFR-activity on death ligand-induced apoptosis and indicate that EGFR inhibition is likely to improve the efficacy of death ligand-based cancer therapies. Furthermore, it is tempting to speculate that EGFR amplification protects tumor cells from death ligand-mediated host immune responses in vivo and that EGFR's effects on death receptor-mediated apoptosis may explain the anti-tumor effects of non-cytotoxic, unarmed anti-EGFR family antibodies.

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Introduction

The death ligands CD95L (FasL/Apo-1L) and Apo2L/TRAIL are members of the TNF cytokine family (2). By activation of the corresponding death receptors (DR), CD95, and DR4 or DR5 respectively, apoptotic cell death is triggered in susceptible target cells. While human malignant glioma cell lines are highly resistant to currently available cytostatic drugs (24), they express these death receptors, like many other cancer cells, and are sensitive to CD95L- and Apo2L-induced cell death (23). Therefore, these death ligands are promising investigational agents for the treatment of malignant glioma. However, high concentrations of these death ligands, or augmentation of CD95L- and Apo2L-induced cytotoxicity by inhibitors of RNA or protein synthesis, are necessary for cell death in many cell lines, because the efficacy of death ligands is counteracted by proteins with antiapoptotic properties. Among these are proteins of the Bcl-2 family (35) and endogenous inhibitors of caspase activation (FLIP, IAP) (19, 30).

Signaling through growth factor receptors of the receptor tyrosine kinase class can also mediate antiapoptotic effects. For instance, amplification or overexpression of EGFR is common in human malignant gliomas and occurs in more than 50% of primary glioblastomas (32). EGFR overexpression results in resistance to apoptosis induced by cytotoxic drugs and enhanced survival of glioma cells in vivo (15, 17). Increased amounts of BCL-X_L (15) and hyperphosphorylation of pkB/Akt (13) have been hypothesized to mediate these antiapoptotic effects. Several strategies for targeting and inhibiting EGFR are currently being developed, among these are the pharmacological inhibition of the tyrosine kinase domain (8, 14), antibodies to the extracellular domain (26), or gene therapy using dominant negative EGFR constructs (18, 22). Here, we

Corresponding author:
J. P. Steinbach, Laboratory of Molecular Neuro-Oncology, Department of Neurology, University of Tübingen, Medical School, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany (e-mail: joachim.steinbach@uni-tuebingen.de)

have investigated whether EGFR is involved in the resistance of human malignant glioma cell lines to CD95L- and Apo2L-induced apoptosis.

Methods

Reagents. AG1478 and EGF were purchased from Calbiochem (San Diego, Calif). PD153035 was from Tocris Cookson (Bristol, UK). All other chemicals not specified below were purchased from Sigma (Deisenhofen, Germany). Acetyl-Asp-Glu-Val-Asp-chloromethylcoumarin (Ac-DEVD-AMC) was obtained from Biomol (Plymouth Meeting, Pa). CD95L was obtained from the supernatant of CD95L-transfected N2A murine neuroblastoma cells (24). Apo2L was kindly provided by A. Ashkenazi (Genentech South, San Francisco, Calif).

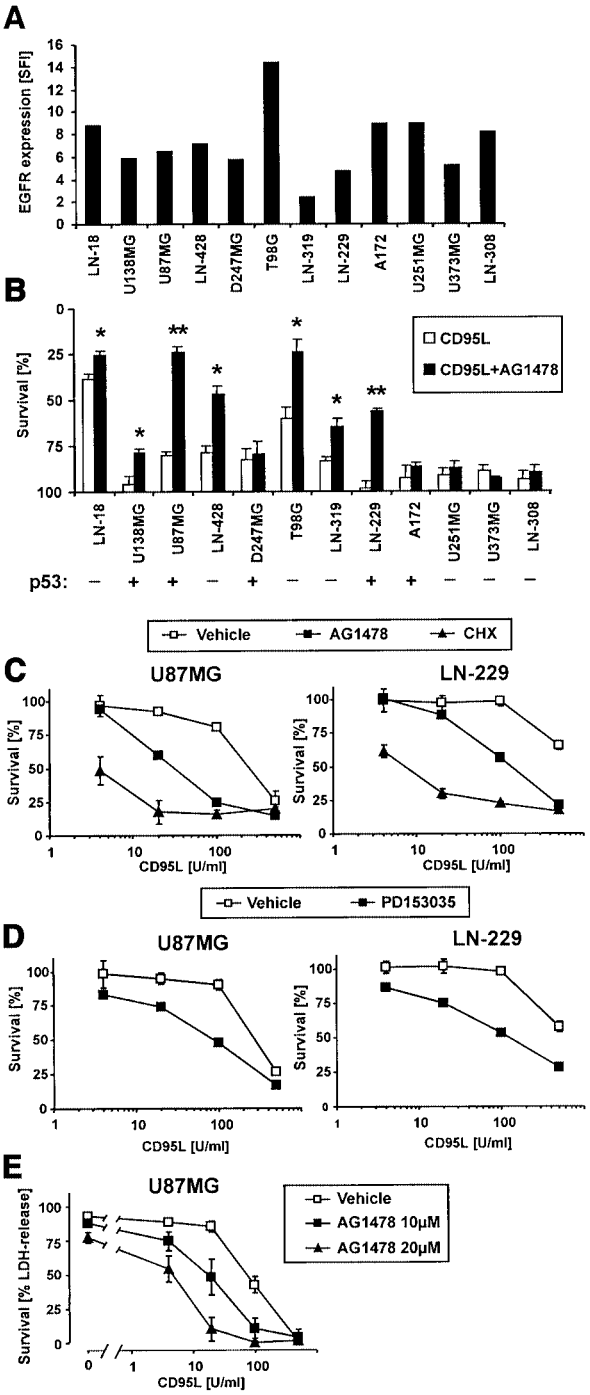
Cell lines. The human malignant glioma cell lines employed in this study have been characterized previously (36). LN-229 crm-A cells were obtained using the Flag-crm-A-puro construct and were compared with puromycin-resistant control cells transfected with the empty vector (31). LN-229 cells were transduced with wild-type (pLWERNL) and kinase-deficient (pLERNL) EGFR cDNA (17) by retroviral transfer from transiently transfected Phoenix ampho packaging cells. Following selection with G418 (1 mg/ml), pooled clones were subjected to FACS sorting for EGFR-overexpressing cells as described below. Because of a possible dominant-negative effect of the kinase-deficient EGFR construct, all experiments were also performed with LN-229 cells transfected with an empty neo vector as controls. Selected experiments repeated with LN-229 cells transduced with kinase-deficient EGFR yielded similar results as the neo control cells (data not shown). A431 cells were purchased from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany) . The cells were maintained in DMEM containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

Viability assays. Cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and were allowed to attach in medium containing 10% FCS for 24 hours. Then the medium was removed and the cells were incubated in serum-free medium with AG1478, PD153035 or EGF and serial dilutions of CD95L or Apo2L. Vehicle controls with solvents were employed as appropriate (DMSO for AG1478 and PD153035, 10 mM acetic acid for EGF). Glioma cell viability was determined after 16 hours. For crystal violet staining, the cell culture medium was removed and the surviving cells were stained with 0.5% crystal violet in 20% methanol for 10 minutes at room temperature. The plates were washed extensively under running tap water, air-dried and optical density values read in an ELISA reader at 550 nm wave length. For LDH-assays, plates were centrifuged, and the LDH concentration in the supernatant was assessed employing the cell death detection kit II (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer.

DEVD-AMC-cleaving caspase activity. The cells were seeded in 96-well plates (10^4 cells per well) and allowed to attach for 24 hours. The cells were treated as indicated and lysed in lysis buffer containing 25 mM TRIS-HCl (pH 8.0), 60 mM NaCl, 2.5 mM EDTA, and 0.25% NP40 for 10 minutes. Ac-DEVD-AMC (12.5 μM), diluted in PBS, was added and incubated at 37°C for 10 minutes. Caspase activity was measured for 1 hour using a CytoFluor 2350 Millipore fluorimeter at 360 nm excitation and 480 nm emission wave lengths.

Immunoblot analysis. Soluble protein lysates were obtained from subconfluent glioma cell cultures and SDS-PAGE with electroblotting was performed as described (34). The following antibodies were used: mouse monoclonal anti-caspase 3, mouse monoclonal anti-human BCL-X_L (Transduction Laboratories, Lexington, KY), mouse monoclonal anti-human BCL-2 (PharMingen, San Diego, Calif), mouse monoclonal anti-caspase 8 (kindly provided by P.H. Krammer, Heidelberg, Germany), rabbit polyclonal anti-EGFR, goat polyclonal anti-human actin, rabbit polyclonal anti-BAX, and mouse monoclonal anti-p53 (Santa Cruz, San Diego, Calif). The secondary antibodies, protein A and anti-mouse IgG, were purchased from Amersham (Braunschweig, Germany), anti-goat antibody was from Santa Cruz. Enhanced chemiluminescence (ECL+, Amersham) was used for detection.

Flow cytometry. The cells were washed in PBS and then incubated in flow cytometry buffer (1% bovine serum albumin, 0.01% sodium azide in PBS) containing 10% sheep serum for 20 minutes at 4°C. After centrifugation, the cells were resuspended in flow cytometry buffer with anti-CD95 antibody (1 μg/ml; mouse IgG_1, Immunotechnology, Hamburg, Germany) or anti-EGFR mouse monoclonal antibody ab-1 (2.5 μg/ml; Oncogene Science, Cambridge, Mass). Mouse IgG_1 was used for isotype controls. After 1 hour incubation, the cells were



washed and then incubated with FITC-labeled sheep anti-mouse IgG (Sigma) diluted 1:256 for 20 minutes at 4°C. The cells were washed and analyzed for CD95 or EGFR cell surface expression by flow cytometry. The level of expression was calculated as the specific fluorescence index (SFI) derived from the ratio of fluorescent signal obtained with the specific antibody and the isotype control.

Immunoprecipitation and phospho-tyrosine blot. A431 cells were grown in 15 cm dishes and serum-starved overnight. Following treatment with EGF and AG1478 for 5 minutes at 37°C, the cells were rinsed with ice-cold PBS and harvested in lysis buffer containing 100 mM HEPES, 300 mM NaCl, 20% Glycerol, 3 mM $MgCl_2$, 2% Triton X-100, 2 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. The lysates were immunoprecipitated with anti-EGFR antibody 1005 (Santa Cruz; 0.5 μg per 5 mg cell extract) separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were probed with anti-phosphotyrosine antibody (4G10, Upstate Biochemicals, Inc., Lake Placid, NY), stripped and reprobed with anti-EGFR antibody 1005.

Statistical analysis. The quantitative data are from triplicate experiments repeated three times with similar results. The levels of CD95L- and Apo2L-induced cell death in different cell lines were compared by unpaired student's *t*-test. Correlations of the sensitization by AG4178 with EGFR expression levels were calculated by Pearson correlation analysis, correlations with p53 status were calculated by χ^2- test with the systat for windows software.

Figure 1. *EGFR expression and sensitization to CD95-mediated cell death by EGFR inhibition.* **A.** The levels of EGFR expression at the cell surface were determined by flow cytometry. **B.** The cells were exposed to CD95L (100 U/ml) in the absence or presence of AG1478 (10 μM). Survival was assessed by crystal violet staining after 16 h. Data are expressed as mean percentages of survival and SD (n=3; *P<0.05, **P<0.01, CD95L plus AG1478 compared with CD95L plus vehicle, student's t-test.). The functional p53 status (transactivating property) of the cell lines as reported elsewhere (27) is also indicated. **C, D.** U87MG or LN-229 cells were exposed to CD95L in the absence or presence of AG1478 (10 μM) or CHX (10 μg/ml) (**C**) or PD153035 (**D**). Survival was assessed at 16 h by crystal violet staining. **E.** U87MG cells were treated as in **C**-**D**, and survival was assessed by LDH release assay. In **C**-**E**, data are expressed as mean percentages of survival and SD (n = 3; note that some of the error bars are too small to be visualized).

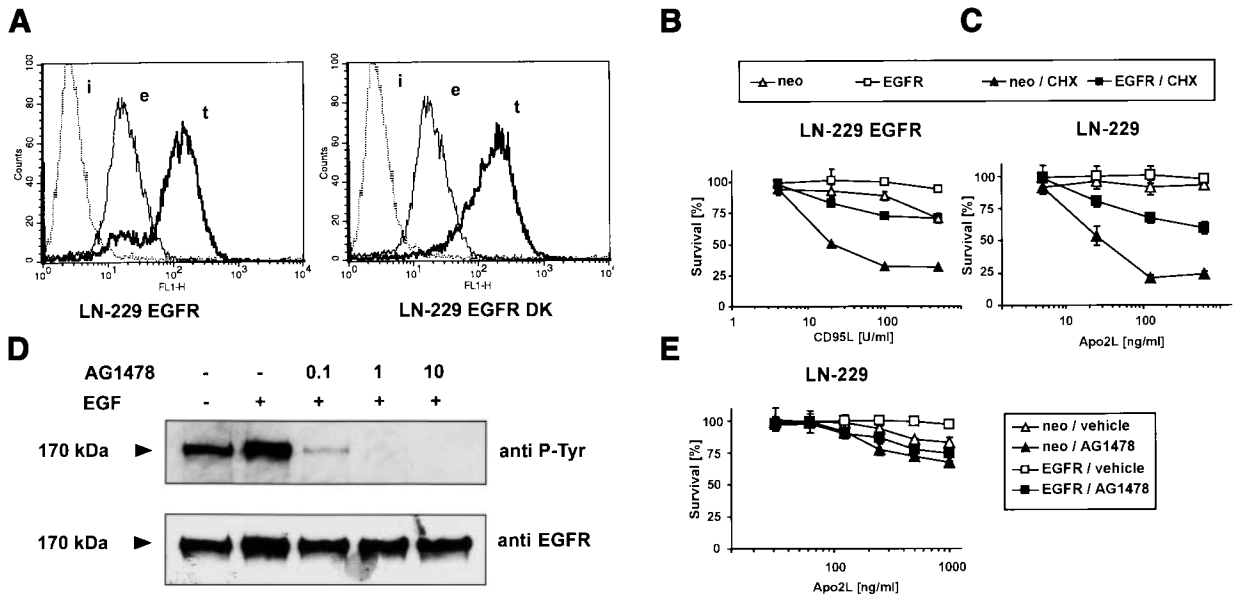


Figure 2. *EGFR overexpression mediates resistance to CD95L- and Apo2L-induced apoptosis.* **A.** LN-229 glioma cells were infected with retrovirus carrying wild-type EGFR or kinase-deficient mutant EGFR (DK) and FACS-sorted for EGFR overexpression. EGFR expression of sorted cells was verified by flow cytometry of LN-229 EGFR cells (left) or LN-229 EGFR DK cells (right); t, transduced cells; e, endogenous EGFR; i, isotype control, log fluorescence intensity. **D.** A431 cells were incubated with AG1478 (0.1-10 μM) for 10 min., immunoprecipitated with anti-EGFR antibody, separated by 8% SDS-PAGE and blotted onto PVDF membranes. Blots were probed with anti-phosphotyrosine antibody (upper panel) or anti-EGFR antibody (lower panel). **B.** LN-229 EGFR or neo control cells were treated with CD95L in the absence or presence of CHX as in Figure 1C, **C,E**. LN-229 EGFR or neo control cells were treated with Apo2L in the absence or presence of CHX (D) or AG1478 (E) as in Figure 1C.

Results

EGFR inhibition enhances CD95-mediated apoptosis in glioma cells. The levels of EGFR expression at the cell surface were determined in 12 human malignant glioma cell lines known to differ widely in their sensitivity to CD95L-induced cell death (23). EGFR levels were rather homogenous among the cell lines, T98G cells exhibiting the highest levels and LN-319 and LN-229 cells exhibiting rather low levels (Figure 1A). No relation of EGFR expression to CD95 expression or sensitivity to CD95-mediated cell death (23) or p53 functional status (27) became apparent. Next, we examined whether inhibition of EGFR activity by AG1478 facilitated CD95-mediated cell death. As shown in Figure 1B, 7 of the 12 cell lines were sensitized significantly to CD95L-induced cell death by AG1478. Concentration-response curves for U87MG and LN-229 are shown in Figure 1C. For comparison, the cells were also co-treated with CHX, a known sensitizer to death receptor-mediated apoptosis in glioma cells (23, 27). There was no significant cytotoxicity caused by EGFR inhibition with AG1478 alone at concentrations up to 10 μM (Figure 1E). Similar results to AG1478 were obtained with the structurally unrelated tyrosine kinase inhibitor, PD153035 (Figure 1D). Among the 12 cell lines (Figure 1B), sensitization to CD95-mediated cell death by AG1478 did not correlate with EGFR expression levels (coefficient = -0.270, Pearson correlation analysis) or p53 status ($P = 0.92$, χ^2- test).

EGFR overexpression inhibits CD95L- and Apo2L-mediated apoptosis. After retroviral infection of LN-229 cells and FACS sorting, LN-229 EGFR cells overexpressed EGFR 6.2-fold, and LN-229 EGFR DK cells overexpressed EGFR 8.7-fold, compared with parental LN-229 cells, as calculated from SFI ratios as outlined in Methods (Figure 2A). These levels are comparable to A431 cells, which are known to highly overexpress the EGFR constitutively. EGFR overexpression was accompanied by enhanced EGFR autophosphorylation in LN-229 EGFR but not in LN-229 DK EGFR cells (data not shown). Stable expression in culture was observed when maintained in selection media for over 1 year. The inhibition of EGFR tyrosine kinase activity by AG1478 was verified by phosphotyrosine blot. At 10 μM, autophos-

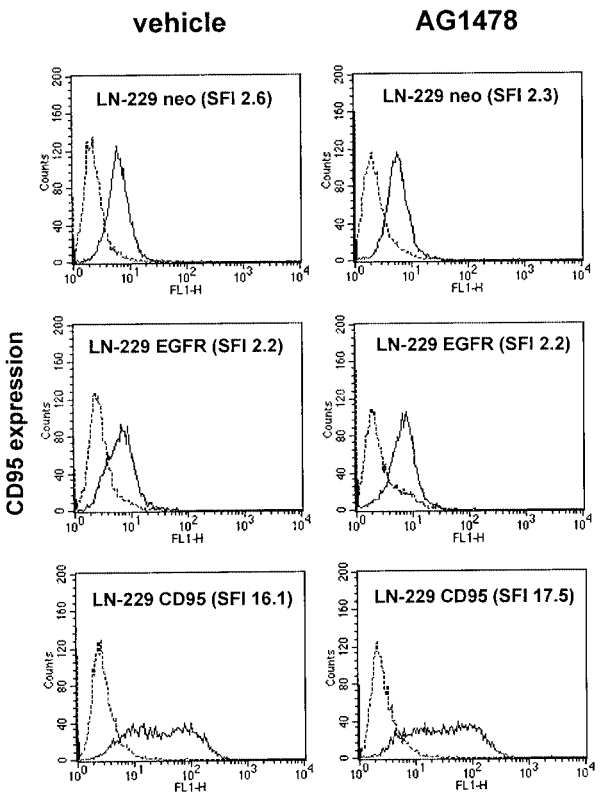


Figure 3. *Altered EGFR signaling does not modulate CD95 expression.* CD95 cell surface expression was studied by flow cytometry in the absence or presence of AG1478 (10 μM, 24h): in LN-229 neo cells (upper panel), in LN-229 EGFR cells (middle panel) and in CD95-transfected LN-229 cells (lower panel). Data are expressed as SFI indexes.

phorylation was abolished whereas total EGFR protein levels were unaltered (Figure 2D). This assay was performed on A431 cells which have high levels of EGFR. Due to the limited sensitivity of assay and the low levels of autophosporylation in the glioma cell lines, a quantitative analysis of the efficacy of EGFR-Inhibition by AG1478 in the 12 glioma cell lines by IP-Western blot was not feasable. 10 μM AG1478 also abolished the proliferative response to EGF in the LN-229 neo and LN-229 EGFR cell lines, thus offering biological proof for effective inhibition of the EGFR during the assay (data not shown).

LN-229 cells engineered to overexpress EGFR were protected from CD95L-induced cytotoxicity, both in the absence and in the presence of CHX (Figure 2B). We further investigated whether the principle of modulation of death ligand-induced cytotoxicity by EGFR would also extend to Apo2L. Similar to CD95L, LN-229 EGFR cells were protected from Apo2L-induced cytotoxicity in the absence or presence of CHX, and EGFR inhibition by AG1478 sensitized the cells to Apo2L-induced cell death (Figure 2C, E).

EGFR activity modulates CD95L-induced cell death at the level of caspase processing. Flow cytometric measurements revealed that the influence of EGFR activity on CD95-mediated cell death did not involve changes in CD95 expression at the cell surface: AG1478 enhanced CD95 expression neither in neo control cells nor in LN-229 cells engineered to overexpress CD95 (38), nor were CD95 levels decreased in LN-229 cells engineered to overexpress EGFR (Figure 3). To further investigate how EGFR inhibition enhances CD95L-induced cell death, we explored modulation of caspase cleavage by EGFR inhibition and overexpression. First, LN-229 or U87MG cells were exposed to vehicle, AG1478 or CHX alone or co-exposed to CD95L. CD95L-induced caspase 8 cleavage was enhanced by AG1478. AG1478 alone did not induce caspase processing. Co-exposure to CD95L and CHX, as a positive control for augmentation of the caspase cascade (31), also resulted in the potentiation of caspase processing compared with CD95L alone (Figure 4A).

Next, the influence of EGFR overexpression on CD95L-induced caspase cleavage was studied by a fluorometric DEVD-AMC cleavage assay as a surrogate marker for caspase activity. LN-229 EGFR cells displayed significantly less DEVD-AMC cleavage compared with LN-229 neo controls. Co-exposure to AG1478 increased CD95L-induced DEVD-AMC cleavage in both LN-229 sublines, whereas treatment with AG1478 alone did not result in DEVD-AMC cleavage (Figure 4B). Finally, we took advantage of LN-229 cells overexpressing the viral caspase inhibitor crm-A. Crm-A is a selective caspase 8 inhibitor which interferes with the caspase-dependent killing cascade triggered by CD95L in glioma cells (31). Figure 4C shows that these cells were refractory to AG1478-mediated sensitization to cell death, indicating that EGFR exerts its antiapoptotic properties through a caspase 8-dependent pathway.

Upregulation of BCL-X_L has been suggested to mediate EGFR-induced protection from apoptosis in U87MG cells (15). We find that the levels of the antiapoptotic proteins, BCL-X_L and BCL-2, are unaltered by ectopic EGFR expression or EGFR inhibition with AG1478 (Figure 4D). As previously reported (11), co-exposure to CD95L and CHX induced a loss of BCL-X_L, but not of BCL-2 protein. This loss of BCL-X_L was prevented by EGFR overexpression, consistent with the idea that the

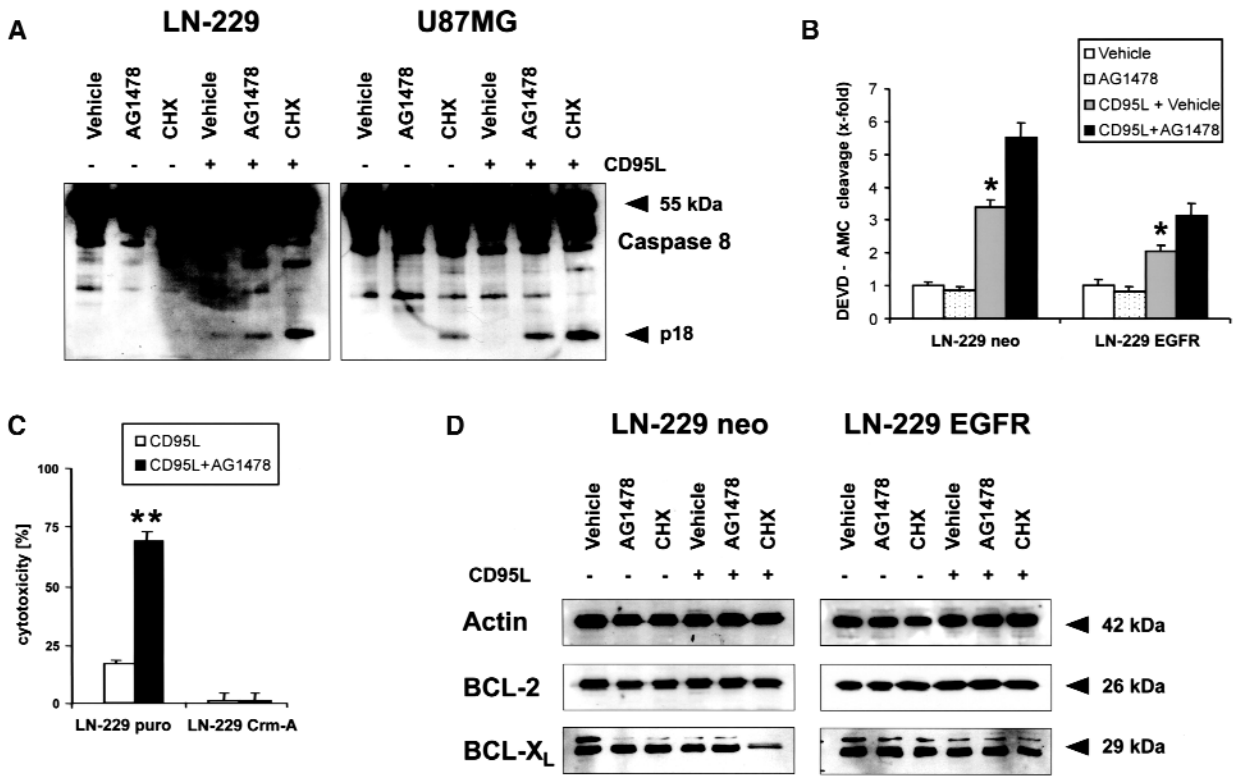


Figure 4. *Altered EGFR activity modulates CD95L-mediated caspase processing and activity.* **A.** LN-229 or U87MG cells were exposed to vehicle, AG1478 (10 μM) or CHX (10 μg/ml) alone or co-exposed to CD95L (100 U/ml) for 6 h. The processing of caspase 8 was assessed by immunoblot as reflected by the formation of p18. **B.** LN-229 neo or EGFR cells were exposed to vehicle or AG1478 (10 μM) or CD95L (100 U/ml), or combinations thereof, for 6 h. DEVD-AMC cleavage was assessed by fluorimetry (**P* <0.05, CD95L plus vehicle compared to CD95L plus AG1478, student's *t*-test). **C.** LN-229 cells transfected with the viral caspase inhibitor crm-A, or puromycin-resistant control cells (31), were treated with CD95L (100 U/ml) in the absence or presence of AG1478 (10 μM) (*n* = 3; ***P*<0.01, CD95L plus AG1478 compared with CD95L plus vehicle, student's *t*-test.). **D.** LN-229 neo or EGFR cells were treated as in A and analyzed for the levels of BCL-2, BCL-X_L or β-actin.

loss of BCL-X_L is caspase-mediated (11) and that EGFR overexpression inhibits apoptosis at the level of caspase activation (Figure 4B).

Discussion

Enhanced EGFR expression is thought to confer a survival advantage to various types of human cancers, particularly glioblastomas. Sensitization to cytotoxic drug-induced cell death by inhibition of EGFR or ErbB2/Neu signaling in epithelial cancer cell lines of various origin has been reported (1, 5, 7, 15). Also, inhibition of CD95L-mediated apoptosis by EGF stimulation has been observed in T47D breast adenocarcinoma cells and embryonic kidney epithelial cells (10). Furthermore, amplification of EGFR deletion mutants (ie, de 2-7, caused by the deletion of exons 2-7 and resulting in truncation of the extracellular domain with ligand-independent constitutive activity) has been shown to exert particularly strong antiapoptotic effects in vivo, and AG1478 preferentially inhibits (de 2-7) EGFR signaling and reverts its antiapoptotic effects (12, 15).

Here, we report that EGFR signaling modulates susceptibility to CD95L- and Apo2L-induced cell death. This is because inhibition of EGFR function by the tyrphostine AG1478, sensitized more than half of the investigated malignant glioma cell lines to CD95L-induced apoptosis (Figure 1B) and because gene transfer-mediated EGFR overexpression inhibited CD95L- and Apo2L-induced apoptosis (Figure 2). The facilitatory effect of EGFR inhibition on CD95L-induced death signaling did not correlate with the levels of cell surface EGFR expression (Figure 1), suggesting that even low level EGFR expression has functional significance.

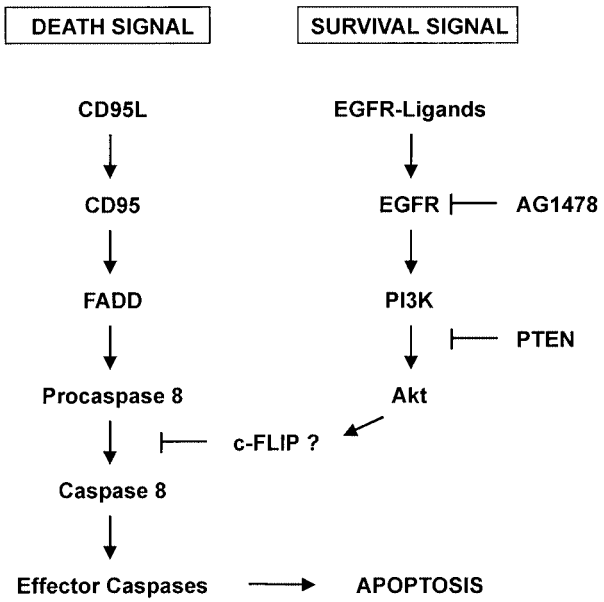


Figure 5. *Schematic drawing of the putative mechanisms underlying the modulation of CD95L-induced apoptosis by EGFR activity.* CD95L binds to its trimerized receptor CD95, which interacts with the Fas-Associated Death Domain adapter protein (FADD) and other molecules to form the Death-Inducing Signaling Complex (DISC), resulting in cleavage of procaspase 8, activation of effector caspases and apoptosis. EGFR-signaling, which is inhibited by AG1478, leads to phosphorylation of Akt through the phosphatidylinositol-3 kinase (PI3K) pathway,which is modulated by PTEN. One of the antiapoptotic consequences of Akt phosporylation is enhanced expression of c-FLIP, an endogenous inhibitor of procaspase 8 cleavage, thus suggesting a link between EGFR-activity and the CD95L-dependent death pathway.

Glioma cell lines commonly lose EGFR amplification and overexpression during in vitro passaging. Glioma cells maintained as xenografts in mice maintained 10 to 20-fold the mRNA expression compared with the respective cell lines established in vitro (3). In 11 of the 12 cell lines investigated here, there is no evidence of EGFR amplification. D247MG cells display amplification of the whole chromosome 7 which contains the *ErbB1* locus (33). The levels of EGFR expression in the cell lines examined here are therefore likely to be considerably lower than those expressed in many malignant gliomas in vivo. The possible effect of inhibition of EGFR signaling in the treatment of patients with malignant glioma may thus be underestimated by in vitro studies where the EGFR expression levels are uniformly rather low. This notion is supported by the dependence of the clinical efficacy of Trastuzumab (Herceptin), a humanized anti-ErbB2/Neu antibody, on high expression levels of ErbB2/Neu in breast adenocarcinomas (20).

Upregulation of CD95 expression has been described in response to stimulation of the mitogen activated protein kinase (MAPK) pathway in other cell types (21). In contrast, we did not observe regulation of CD95 expression after EGFR inhibition with AG1478 or in cells overexpressing EGFR (Figure 3). Therefore, the enhanced susceptibility to CD95L in cells with impaired EFGR signaling cannot be accounted for by enhanced levels of CD95. The mechanism by which inhibition of EGFR signaling sensitizes glioma cells to CD95L- and Apo2L-induced cell death operates in a caspase 8-dependent pathway since glioma cells overexpressing crm-A were not sensitized to CD95-mediated cell death by EGFR inhibition (Figure 4C). Also, EGFR activity controlled cell death upstream of or at the level of caspase processing as confirmed by immunoblot analysis and enzyme activity assays (Figure 4A,B). Overexpression of the EGFR was associated with elevated levels of BCL-X_L in U87MG cells in an independent study (15). Although no such effect was seen here in EGFR-overexpressing LN-229 cells, EGFR overexpression nevertheless maintained BCL-X_L levels in CD95L/CHX-challenged cells whereas BCL-X_L was lost in neo control cells (Figure 4A). How EGFR interacts with the caspase cascade remains unclear.

Mechanisms mediating the antiapoptotic effects of EGFR include enhanced activity of phosphoinositol 3-kinase (PI3-kinase) which activates protein kinase B (pkB/Akt) by phosphorylation. The antiapoptotic properties of pkB/Akt in turn may be mediated by phosphorylation of proapoptotic molecules such as caspase 9 and BAD, by induction of the FLIP protein (19), an endogenous inhibitor of caspase 8, and by phosphorylation of transcription factors which modulate antiapoptotic cellular programs (4). Loss of the tumor suppressor gene PTEN, is a common event in glioblastoma, resulting in enhanced pkB/Akt phosphorylation through disinhibition. Accordingly, transfer of PTEN into PTEN-negative glioma cell lines facilitates CD95L-induced cell death in glioma cells (37). It might thus be speculated that EGFR inhibition decreases the unrestrained phosphorylation of pkB/Akt in PTEN-negative cell lines. However, EGFR inhibition by AG1478 was also effective in the three cell lines with PTEN wild-type status (LN-18, LN-229, LN-428) (Figure 1B) (9), and there was no correlation between the effect of EGFR inhibition (Figure 1B) and the PTEN status as reported previously (37).

EGFR amplification and p53 mutations are almost mutually exclusive genetic alterations in human glioblastomas in vivo (32). Given the known loss of EGFR amplification in glioma cell lines in vitro (3), it is not surprising that there was no difference in EGFR expression between p53 mutant and p53 wild-type cell lines. Accordingly the sensitizing effects of EGFR inhibition on CD95L-induced cell death were also independent of the p53 status of the cell lines.

Death ligands are promising investigational agents for the therapy of malignant glioma. Apo2L appears to be better suited for systemic administration in clinical trials since it is less toxic than CD95L. Recently eradication of intracranial human glioma xenografts in nude mice by intratumoral injection of Apo2L has been demonstrated in the absence of acute or delayed neurotoxicity (25). Additionally, systemic co-administration of Apo2L with *cis*-diamminedichloroplatinum (II) (CDDP) resulted in regression of subcutaneous tumors and extended survival of mice bearing intracerebral human malignant glioma xenografts without relevant systemic toxicity or neurologic sequelae (16).

Novel inhibitors of the EGFR which potently reduce tumor cell EGFR phosphorylation in vivo are now available. In a mouse xenograft paradigm, once-daily oral administration of the irreversible EGFR and ErbB2 inhibitor PD0169414 caused regression of subcutaneous grafts of several human cancer cell lines or significant growth delay in the absence of relevant systemic toxicity (29). Our results suggest that sensitizing glioma cells to death ligand therapy via EGFR inhibition appears to be a feasible strategy to improve the efficacy of this experimental therapy.

Since Apo2L and CD95L have been attributed a role in tumor surveillance (28), our results may also explain anti-tumor effects of unarmed, non-cytotoxic antibodies to EGFR (26) or pharmacological inhibitors of the EGFR (29), at the level of enhancing susceptibility to death receptor-mediated anti-tumor immune responses. Conversely, we assume that anti-tumor immune responses may constitute an additional selective pressure for EGFR amplification and overexpression. This mechanism may also be relevant for the therapeutic effect of ErbB2 antibodies such as Trastuzumab (Herceptin) in clinical studies of breast cancer (6).

Acknowledgments

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