APO010, A Synthetic Hexameric CD95 Ligand, Induces Death of Human Glioblastoma Stem-like Cells

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Abstract. *The treatment of glioblastoma remains a major challenge in the field of neuro-oncology. There is emerging evidence that glioblastomas consist of heterogeneous cell populations with a small subset of cells with stem cell-like properties which might be resistant to conventional therapy and are thus crucial for tumor recurrence. These glioma-initiating cells (GICs) are therefore an attractive therapeutic target. Death receptor activation is one promising approach of cancer therapy. The synthetic hexameric cluster of differentiation 95 (CD95) agonist APO010 exhibits strong antiglioma activity towards human glioma cell lines, as well as in cell cultures of primary glioblastoma. Here, we investigated the ability of APO010 to induce cell death in a panel of previously well-defined GIC lines. The GIC lines and their derived differentiated cultures expressed CD95 on the cell surface and were sensitive towards APO010-mediated cell death to a variable extent. Temozolomide enhanced sensitivity of GICs to APO010. APO010 warrants being further evaluated as a tool to target GICs.*

The treatment of glioblastoma remains a major challenge in the field of neuro-oncology. Despite the current standard-of-care, including surgery, radiotherapy and alkylating chemotherapy, the median survival time is approximately 12.6 months according to a population-based study (1) and approximately 15 months within the clinical trial defining the current standard-of-care (2).

For a refinement of treatment strategies, a population of cells within glioblastomas with stem cell-like properties may be of particular interest. In recent years, there has been growing evidence for the presence of glioma-initiating cells (GIC) with stem cell properties within glioblastoma (3), also called glioma stem cells. In this hierarchical tumor model, stem cells might have important functions in the initiation and maintenance of glioblastoma and are therefore an attractive therapeutic target. Putative glioma stem cells are characterized by stemness properties of self-renewal, multipotency and tumorigenicity at low cell numbers in immunodeficient mice, forming tumors resembling the initial human tumors (4). Current treatments aim largely at the bulk population of tumor cells. These therapies might, however, spare enough stem cells to allow for regrowth of these tumors. Resistance of stem cells to conventional radiotherapy has already been suggested (5-7). To investigate their biological properties, stem cells can be isolated from gliomas *ex vivo* and propagated under serum-free stem cell culture conditions including fibroblast and epidermal growth factors (8). Other approaches to isolate glioma stem cells use functional assays or cell surface markers such as cluster of differentiation 133 (CD133) (4). CD133, or prominin-1, a cholesterol-binding molecule of unknown biological function, was introduced as a candidate cell surface marker for glioma stem cells (3) and is still widely used. However, the role of CD133 in identifying stem cells remains controversial and is challenged by the characterization of CD133-negative glioblastoma cells with stem cell properties (9), and the up-regulation of CD133 under stress conditions (10). A definite cell surface marker to identify glioma stem cells is lacking.

Death receptors are a promising target for the treatment of gliomas. The death receptor pathway is activated via receptors on the cell surface belonging to the family of tumor necrosis factor receptors (TNFR). Following binding of the ligands, the receptors are trimerized and the death-inducing signaling complex (DISC) is formed, leading to the subsequent activation of a killing cascade. The death receptor ligand systems include TNFR-α with TNF-α; cluster of differentiation 95 (CD95/FAS/APO-1) and CD95 ligand (CD95L/APO-1L/FASL); and TNF-related apoptosis-inducing ligand receptor (TRAIL-R/APO-2) and TRAIL/APO-2L, with CD95L and TRAIL having the most promising therapeutic impact for

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glioblastoma (11). Agonistic antibodies against CD95 (12, 13), as well as CD95L (14), are cytolytic for malignant glioma cells. A further development in the search for a potent death ligand with an acceptable safety profile is APO010, a hexameric protein consisting of two CD95L extracellular domain trimers and the collagen domain of adiponectin, designated adipocyte complement-related protein of 30 kDa (ACRP30) (15). Compared with a cross-linked soluble CD95L or a CD95-agonistic antibody, APO010 exhibited superior activity against glioma cell lines expressing CD95 and triggered caspase-dependent cell death (16). APO010 reduced glioma cell viability in synergy when combined with temozolomide and the locoregional administration of APO010 induced glioma cell death *in vivo* and prolonged the survival of tumor-bearing mice (16).

In the present work, we investigated the activity of APO010 in cell cultures kept under stem cell culture conditions in a panel of previously well-defined GIC lines exhibiting stem cell properties (8, 17, 18). The GIC lines express CD95 on the cell surface and are sensitive towards APO010-mediated apoptosis to a variable extent. The response to APO010 is probably dependent on the level of CD95 expression and downstream intracellular targets.

Materials and Methods

Materials and cell lines. We here used a panel of GIC lines with stem cell properties. Those GIC lines were designated with the acronym "GS" (GS-2, GS-4, GS-5, GS-7 and GS-9). We obtained these GS lines from Professor K. Lamszus, MD (University Hospital Eppendorf, Hamburg, Germany). Each "GS" line was derived from an individual patient and they have all been characterized in detail for stemness properties elsewhere (8): in summary, the GS lines express stem cell markers sex determining region Y-box 2 (Sox2) and nestin. Multipotency was demonstrated with the expression of glial (glial fibrillary acidic protein), neuronal (microtubule-associated protein 2 (MAP2) and neurofilament) or oligodendroglial (galactocerebroside C) markers after differentiation. The stemness-defining characteristics of self-renewal *in vitro* and tumorigenicity *in vivo* have also been verified in long-term use of these GS lines (8, 18).

The culture of the GS lines has been described elswhere (8, 19). Briefly, for maintenance of stem cell properties, GS lines were cultured in stem cell permissive neurobasal medium with B-27 supplement (20 μl/ml) and Glutamax (10 μl/ml) from Invitrogen (Basel, Switzerland), fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, PA), and heparin (32 IE/ml; Ratiopharm, Ulm, Germany). To differentiate GS cells, they were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), containing 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), penicillin (100 IU/ml)/streptomycin (100 mg/ml) (Gibco) and 10% fetal calf serum (FCS; PAA, Vienna, Austria). The GS-derived differentiated cells were designated with the suffix "d". Following differentiation the GS-X"d" cells are per definition no GIC anymore. The broad spectrum caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (zVAD-fmk) was obtained from Bachem (Heidelberg, Germany). APO010 was provided by Topotarget (Copenhagen, Denmark).

Flow cytometry for CD95 and CD133 expression. Cells were detached and singularized using Accutase (PAA) and blocked with 2% fetal calf serum in phosphate-buffered saline (PBS). Cell surface expression was assessed using the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-labeled antibodies to CD95 (clone UB2; Beckman Coulter, Fullerton, CA, USA; and clone DX2; Biolegend, San Diego, CA), to CD133 (clone AC133; phycoerythrin (PE)-conjugated; mouse IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany). FITC-labeled IgG1 (clone MOPC21, Biolegend) and PE-conjugated IgG1 (clone MOPC 21; Sigma, St Louis, MO, USA) were used as isotype control antibodies. Flow cytometry was performed with a Dako flow cytometer (Dako, Glostrup, Denmark). Signal intensity was calculated by dividing the median fluorescence obtained with the specific antibody by signal intensity obtained with the isotype control antibody (specific fluorescence index, SFI).

Following staining with PE-conjugated monoclonal antibody to CD133, GS-2 cells were separated by fluorescence-activated cell sorting (FACS) using FACSAria III and FACSDiva software (both Becton Dickinson, San Diego, CA, USA) for the presence of CD133 on the cell surface. The purity of the sorted cells was ascertained by subsequent analysis by flow cytometry using a CyAn ADP Analyzer (Beckman Coulter).

Detection of apoptosis by annexin V binding. Apoptotic cell death was analyzed by staining with FITC-labeled annexin V (BD Bioscience, Heidelberg, Germany) and propidium iodide (PI) (Sigma). The cells were treated as indicated. Treatment with temozolomide (Schering Plough, Kenilworth, NJ, USA) for 72 h was followed by addition of APO010 for another 20 h where indicated. The cells were detached or singularized using accutase (PAA), washed with PBS, and resuspended in a buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCL_2$. Annexin V-FITC and PI were then added. After incubation for 30 min, the cells were analyzed by flow cytometry (Dako CyAn ADP 7). Cells positive for annexin V binding and negative for PI staining were considered as early apoptotic. Cells positive for annexin V binding and positive for PI staining were considered as late apoptotic cells.

Immunoblot analysis. The general procedure has been described elsewhere (16). To induce differentiation in GIC lines, the cells were kept under serum-containing culture conditions for three to five passages. The GIC and the differentiated cells were treated with APO010 as indicated, 20 μg of protein per lane were separated on 12% acrylamide gels (Biorad, Munich, Germany). After transfer to a nitrocellulose membrane, the blots were pre-treated for 1 h with PBS containing 5% skim milk and 0.05% Tween 20. The following primary antibodies were used: Caspase-3 (No. 610322) from BD Biosciences (Heidelberg, Germany), cellular FAS associated death domain-like interleukin-1 beta-converting enzyme (FLICE)-inhibitory protein (c-FLIP) (ALX-804-428) from Enzo Life Sciences (Lausen, Switzerland), X-linked inhibitor of apoptosis protein (XIAP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Chemicon (Billerica, MA, USA), caspase-8, B-cell lymphoma (BCL-2), BCL-2-associated X protein (BAX) all from Santa Cruz Biotechnology (Santa Cruz, CA, USA), poly(ADP-ribose) polymerase (PARP) from BD Pharmingen (Heidelberg, Germany) and actin from Santa Cruz Biotechnology. Visualization of protein bands was accomplished using horseradish peroxidase-coupled IgG secondary antibody (Santa Cruz) and enhanced chemoluminescence (Amersham, Little Chalfont, UK).

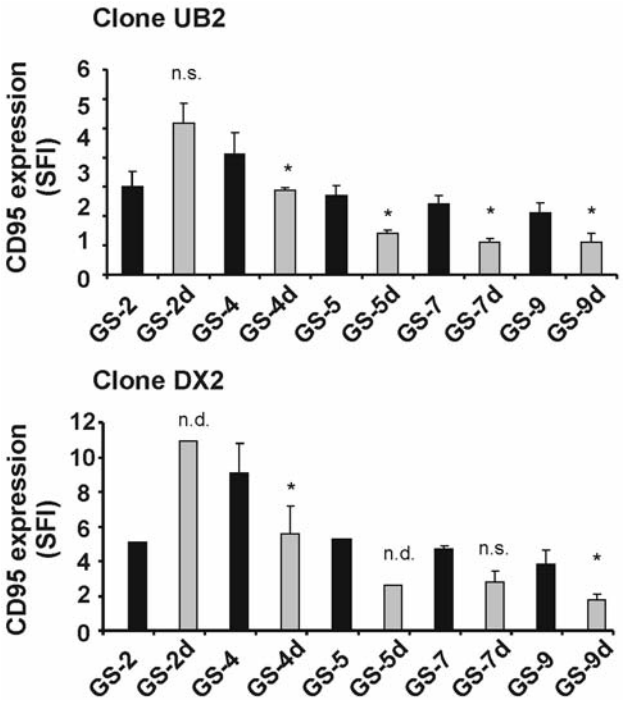


Figure 1. *Glioma-initiating cells (GICs) express cluster of differentiation 95 (CD95) on the cell surface. The GICs were assessed for expression of CD95 by flow cytometry following staining with antibody clones UB2 and DX2 to CD95 under stem cell (GS) and differentiating (GSd) culture conditions, respectively. Data are expressed as the mean and SD. Analysis of statistical significance was performed by ANOVA (n=3; *p<0.05; n.s. not significant; n.d. not determined). SFI: specific fluorescence index.*

Statistics. Statistical significance was assessed by ANOVA as indicated (SPSS 17; SPSS, Chicago, IL, USA). Synergy of APO010 and temozolomide was evaluated using the fractional product method. Here, predicted values correspond to an additive action of two compounds that can be compared to the observed effect (20). If the observed activity exceeded the predicted activity by 10%, then synergy was assumed.

Results

Expression of CD95 on the cell surface of GICs. The expression of CD95 on the cell surface of GIC and derived differentiated cells was assessed by flow cytometry following staining of the cells with antibodies to CD95 (clones UB2 and DX2 respectively) (Figure 1). In four GS cell lines (GS-4, GS-5, GS-7 and GS-9) CD95 expression was higher in the undifferentiated cultures compared to the differentiated cells. In GS-2, the result was opposite, with a higher CD95 expression on the differentiated cells. The results were similar for both antibody clones, with clone DX2 leading to higher SFI values. The surface expression of CD95 qualifies these lines for treatment with APO010.

Cytotoxicity of APO010 to GIC lines is limited. The induction of cell death by APO010 in GICs and derived differentiated cells was characterized by flow cytometry using annexin V/PI labeling for the assessment of apoptosis. The GICs and differentiated cells were treated with increasing concentrations of APO010 (Figure 2).

For GS-2, GS-4 and GS-5, the differentiated cells exhibited, in part, higher sensitivity towards APO010 than the GIC. For GS-7, the GICs were more sensitive than the differentiated cells and for GS-9, no differential sensitivity between the GICs and differentiated cells was apparent. The cells were protected from APO010-mediated cell death by the broad spectrum caspase inhibitor zVAD-fmk (Figure 2).

Overall, the GS lines derived from five patients investigated here differed markedly in their sensitivity towards APO010-mediated apoptosis, with GS-5d and GS-7 being sensitive with more than 50% apoptotic cells following treatment with 1000 ng/ml APO010, and GS-2 and GS-4 GICs being virtually resistant towards APO010. The expression of CD95 on the cell surface is a prerequisite for the action of APO010 (16), however, the degree of CD95 expression alone does not predict sensitivity of GICs in the cell lines tested here.

Expression of apoptosis regulating proteins in GIC. As well as for the cell surface expression of CD95, further downstream molecules might influence the sensitivity of the cell lines towards APO010. Therefore, we assessed the expression levels of a panel of proteins involved in the regulation of apoptosis (caspase-3 and -8, c-FLIP, BCL-2, BAX and XIAP) in GICs and differentiated cells by immunoblot (Figure 3A). The pro-apoptotic proteins caspase-3 and -8 and BAX are expressed in GICs and differentiated cells to a variable extent, as are the anti-apoptotic proteins BCL-2, XIAP and c-FLIP(l), which is presumed to have anti- as well as pro-apoptotic functions. Notably, caspase-8 was expressed in all cell lines investigated here. Next, we performed densitometry to quantify differences in the expression levels of these proteins between GICs and differentiated cells (Figure 3B). Major alterations (change of factor 2 or more) include higher expression levels of BAX and caspase-3 in GS-2 compared to GS-2d and higher expression of BAX and lower expression of caspase-8 in GS-9 compared to GS9d. However, no clear expression pattern towards a more pro- or anti-apoptotic phenotype within one GS line and between the GS lines was apparent. Next, we assessed the cleavage of apoptosis-regulating proteins following treatment with APO010 (Figure 3C). We observed cleavage of caspases-8 and -3, and of PARP and a reduction of XIAP levels following treatment of two GS lines with APO010 to a similar extent under stem cell as well as under differentiating conditions.

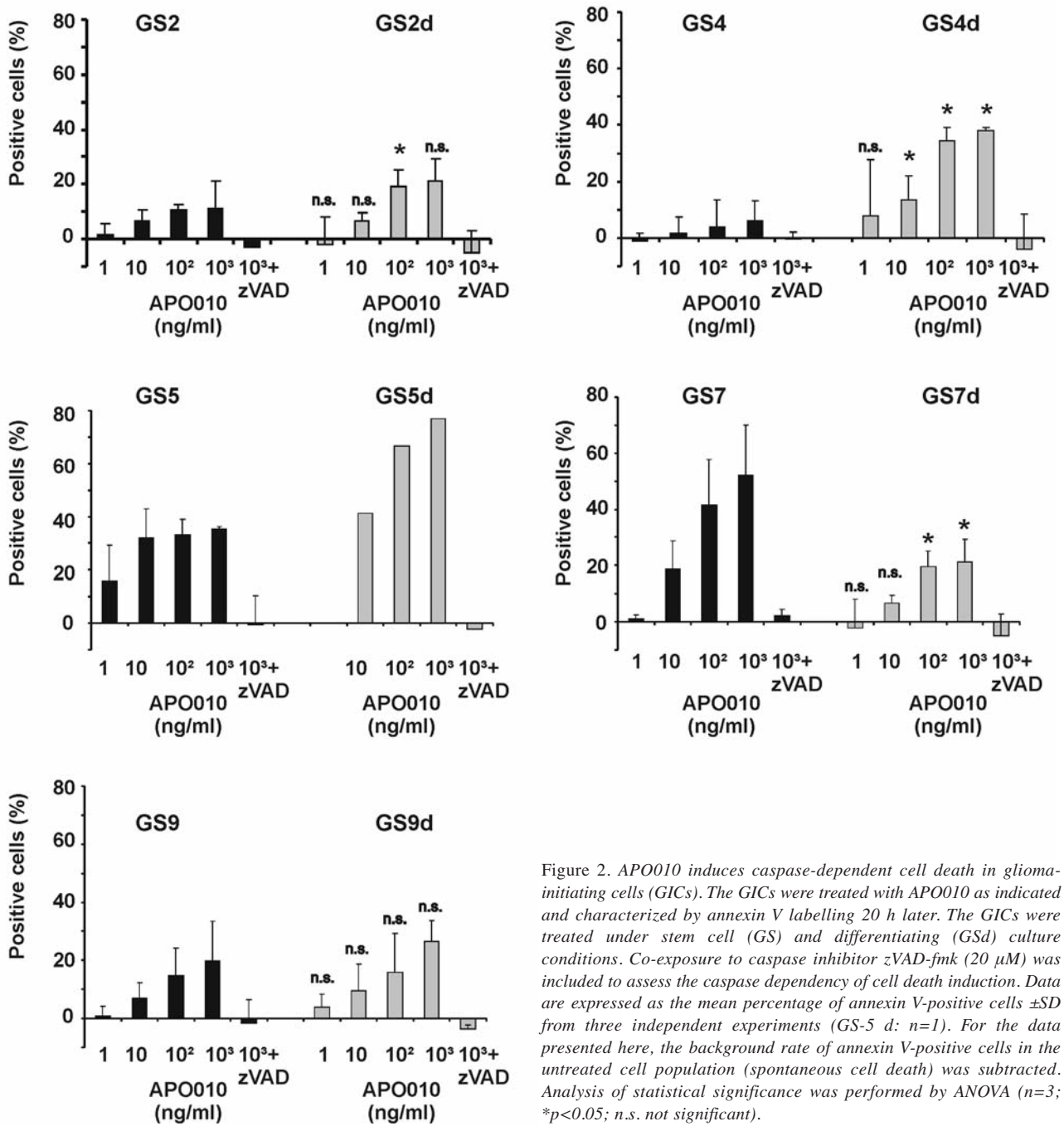
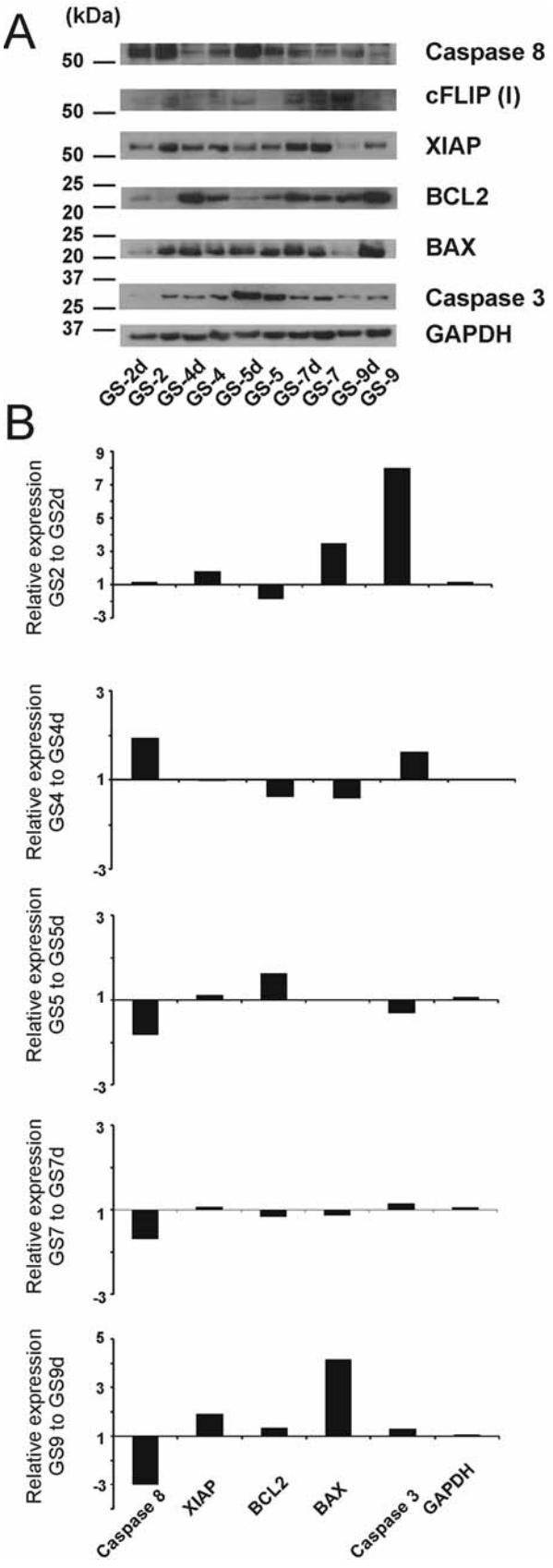


Figure 2. *APO010 induces caspase-dependent cell death in glioma-initiating cells (GICs). The GICs were treated with APO010 as indicated and characterized by annexin V labelling 20 h later. The GICs were treated under stem cell (GS) and differentiating (GSd) culture conditions. Co-exposure to caspase inhibitor zVAD-fmk (20 μM) was included to assess the caspase dependency of cell death induction. Data are expressed as the mean percentage of annexin V-positive cells ±SD from three independent experiments (GS-5 d: n=1). For the data presented here, the background rate of annexin V-positive cells in the untreated cell population (spontaneous cell death) was subtracted. Analysis of statistical significance was performed by ANOVA (n=3; *p<0.05; n.s. not significant).*

Sensitization of GICs towards APO010 by temozolomide. Since the sensitivity of GICs towards APO010 is limited in some GS lines, we next assessed the possibility of sensitizing GS-2, GS-5 and GS-9 GICs to APO010 by pre-treatment with temozolomide, the most commonly used drug for the treatment of glioblastoma. According to the fractional product method (20), the combination of APO010 and temozolomide had a more than additive, that is, synergistic cytotoxic effect on the GIC lines tested here. The combined action of temozolomide and APO010 led to significantly more cell death compared to either agent alone (Figure 4).

Effect of APO010 and temozolomide in CD133$^+$ cells. Despite some limitations, CD133 is still used as a putative cell surface marker for glioma stem cells. GS-2 GICs kept under stem cell culture conditions were sorted by FACS into



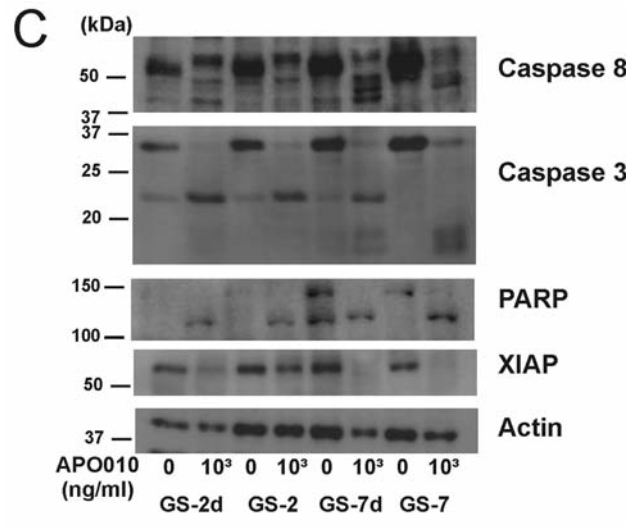


Figure 3. *Expression of apoptosis-regulating proteins in glioma-initiating cells (GICs). A: Cellular lysates from GICs under stem cell and differentiating culture conditions were examined for the levels of caspase-3 and -8, cellular FAS associated death domain-like interleukin-1 beta-converting enzyme (FLICE)- inhibitory protein (c-FLIP), B-cell lymphoma 2 (BCL-2), BCL-2-associated X protein (BAX), X-linked inhibitor of apoptosis protein (XIAP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins by immunoblot. B: Relative band densities from immunoblots are shown. The band densities from GS cells are shown in relation to band densities in differentiated cultures. The x-axis at relative expression value "1" means equal band densities in GS cells and differentiated cells. C: GS-2 and GS-7 cells maintained under stem cell culture conditions or as derived differentiated cells were treated with APO010 (1000 ng/ml) for 20 h. Cellular lysates were examined for the levels of caspases-3 and -8, poly(ADP-ribose) polymerase (PARP), XIAP and actin by immunoblot.*

a $CD133^+$ and a $CD133^-$ fraction. The purity of the respective fractions was ascertained as about 95% by flow cytometry. The expression of CD95 was similar on $CD133^+$ and $CD133^-$ GS-2 cells as assessed by flow cytometry following staining with FITC-conjugated antibody to CD95 (Figure 5A). The induction of cell death by APO010 in $CD133^+$ and $CD133^-$ GS-2 cells was characterized by flow cytometry using annexin V/PI labeling (Figure 5B). There was a trend for enhanced induction of cell death in $CD133^+$ cells compared to the $CD133^-$ fraction.

Discussion

In the present study, we used a panel of GIC lines, maintained under stem cell culture conditions, that have been extensively characterized before (8, 17, 18). Recently, prognostically relevant subgroups in glioblastoma have been defined according to gene expression profiles (21, 22). Gene expression analysis revealed two different subtypes of the

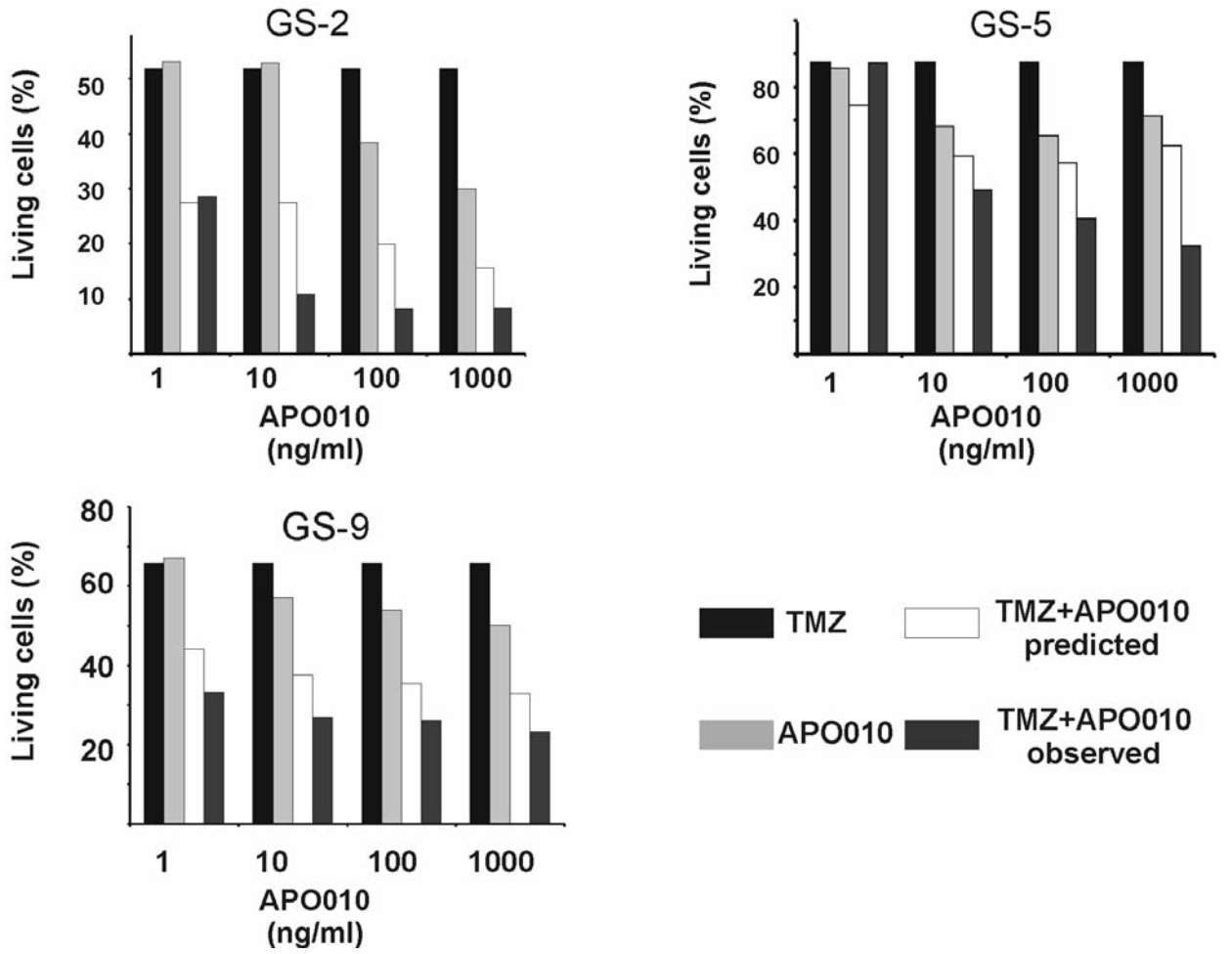


Figure 4. *Synergistic induction of cell death by APO010 and temozolomide. GS-2, GS-5 or GS-9 GICs were treated with temozolomide (GS-2: 500 μM; GS-5: 250 μM; GS-9: 100 μM) for 72 h followed by APO010 for another 20 h. The graphs show the results of treatment with either agent alone, the predicted effect assuming independent (additive) effects, and the truly observed effect. The bars express the percentage of living cells as assessed by flow cytometry after annexin V and propidium iodide (PI) staining. If the observed activity exceeded the predicted activity by 10%, then synergy was assumed (n=2).*

GIC lines used here. GIC cluster 1 included GS-5 and 9 and was characterized by the expression of neurodevelopmental genes, thus resembling the pro-neural subtype. Cluster 2 included GS-2, 4 and 7, and exhibited an expression signature enriched for extracellular matrix-related genes, resembling the mesenchymal subtype (8, 18). We characterized five GIC lines in comparison to derived differentiated cells for the expression of CD95 and a panel of downstream molecules regulating apoptosis and for their sensitivity towards APO010, a hexameric CD95 ligand.

All GS lines tested here, the GICs and the differentiated cells, express CD95 on the cell surface (Figure 1). Except for GS-2, the GICs exhibit enhanced CD95 expression compared to the differentiated cells. We observed differences in the sensitivity of the GS lines towards APO010-mediated cytotoxicity. While GS-7 and GS-4d are rather sensitive towards APO010, GS-2, GS-4 (GIC) and GS-9 do not respond well to APO010-induced cell death, with GIC from GS-4 being virtually resistant (Figure 2).

The cell surface expression of CD95 is a prerequisite for the cytotoxic action of APO010 and the expression level of CD95 is a gross indicator for the sensitivity and the susceptibility of the cell lines, which are likely modulated additionally by down-stream intracellular targets (16). However, the investigation of the baseline expression levels of the pro-apoptotic proteins caspase-3 and -8 and BAX, and of the antiapoptotic proteins BCL-2 and XIAP, and of c-FLIP(l) revealed neither major differences between the GICs

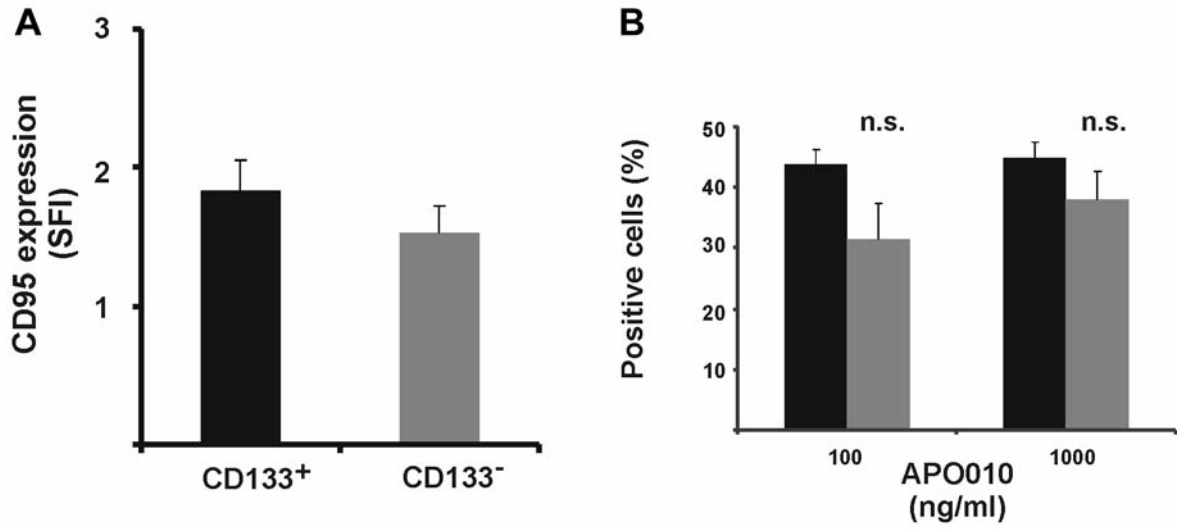


Figure 5. *Effect of APO010 and temozolomide in cluster of differentiation 133 (CD133+) cells. A: GS-2 cells were sorted for the expression of CD133 on the cell surface by FACS. The CD133+ and CD133– fractions were assessed for the expression of CD95 by flow cytometry. B: The CD133+ and CD133– fractions were treated with APO010 as indicated and characterized by annexin V labelling 20 h later. Data are expressed as the mean percentage of annexin V-positive cells ±SD from two independent experiments. Analysis of statistical significance was performed by ANOVA (*p<0.05; n.s. not significant).*

and differentiated cells, nor a clear expression pattern pointing towards a more pro- or anti-apoptotic phenotype (Figure 3B). However, the involvement of other anti- or pro-apoptotic proteins in the differential sensitivity towards APO010 cannot be excluded. The assessment of cleavage of caspases-3 and -8, and of PARP, a classic caspase substrate, provides evidence for death receptor-mediated apoptosis in GICs kept under stem cell culture conditions, as well as in derived differentiated cells (Figure 3C). The reduced level of XIAP following treatment with APO010 indicates that this regulator of apoptosis might also be cleaved. XIAP can be cleaved by caspases and the cleavage product may represent a dominant-negative form of XIAP, which interferes with the function of the active, full-length form of XIAP by promoting caspase activation (23). Again, no clear pattern explaining differences in the susceptibility of GIC lines towards APO010 was revealed. Further investigations using high-throughput gene expression assays to reveal distinctive expression patterns of apoptosis-relevant genes might help to identify key regulatory elements.

The proper killing of glioma cells upon activation of the CD95 pathway is crucial for the use of APO010 or other CD95 activators as therapeutic agents since binding of CD95 ligand to CD95 on glioblastoma cells has also been suggested to promote invasion of glioma cells via the glycogen synthase kinase 3-β pathway and subsequent expression of matrix metalloproteinases following recruitment of the SRC family member YES and the p85 subunit of phosphatidylinositol-3-kinase to CD95 (24). In a previous report, we found that APO010 was superior to both soluble CD95L and the agonistic CD95 antibody CH11 in killing CD95-expressing long-term glioma cell lines, as well as primary glioma cells (16). The latter is in contrast to human TRAIL/APO2L, which failed to kill freshly-isolated glioma cells (25). Therefore, APO010 is a potent cytotoxic CD95 activator which might circumvent possible unwanted invasion-promoting properties of the CD95 pathway by effectively killing the cells. Moreover, combination therapies with agents blocking phosphatidylinositol-3-kinase (*e.g.* enzastaurin) or SRC kinase (*e.g.* dasatinib) might help to avoid these unwanted tumor-promoting effects (26).

In a previous report, stem-like cells isolated from the long-term cell line U-87 MG were described as being relative resistant towards CD95-mediated apoptosis, suggesting a possible escape mechanism from CD95-targeting therapies (27). In contrast to the cells used in the latter publication, here we investigated five well-described GIC lines established from human glioblastomas, kept under stem cell conditions and verified for the core stem cell characteristics of self-renewal, multipotency and tumorigenicity *in vivo* (8). Nevertheless, we also found only restricted sensitivity of our GIC lines towards APO010 (Figure 2).

To further enhance the action of APO010, GICs might be sensitized towards CD95-mediated cytotoxicity. APO010 exhibited enhanced activity when combined with platinum in ovarian cancer cells (28), and with imatinib in gastrointestinal stromal tumors (29). In glioma cell lines, APO010 exhibited synergistically enhanced activity when

combined with temozolomide, the current standard-of-care chemotherapy for glioblastoma (2). Here we provide evidence that GICs, too, might be sensitized towards APO010 by temozolomide (Figure 4).

Although controversially discussed (4, 9), the use of CD133 as a cell surface marker for glioma stem cells is still common. In the GIC line GS-2, the expression level of CD95 was similar in $CD133^+$ and $CD133^-$ GS-2 cells (Figure 5A) and there was a tendency towards higher susceptibility of $CD133^+$ cells towards APO010-mediated cell killing (Figure 5B).

Overall, APO010 still warrants evaluation for locoregional treatment of glioblastoma. However, regarding the fraction of cells within the tumor with stem cell characteristics, APO010 may need to be combined with an agent sensitizing these cells to CD95-mediated cytotoxicity.

Acknowledgements

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