APO010, a synthetic hexameric CD95 ligand, induces human glioma cell death in vitro and in vivo

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Death receptor targeting has emerged as one of the promising novel approaches of cancer therapy. The activation of one such prototypic death receptor, CD95 (Fas/APO-1), has remained controversial because CD95 agonistic molecules have exhibited either too strong toxicity or too little activity. The natural CD95 ligand (CD95L) is a cytokine, which needs to trimerize to mediate a cell death signal. Mega-Fas-Ligand, now referred to as APO010, is a synthetic hexameric CD95 agonist that exhibits strong antitumor activity in various tumor models. Here, we studied the effects of APO010 in human glioma models in vitro and in vivo. Compared with a cross-linked soluble CD95L or a CD95-agonistic antibody, APO010 exhibited superior activity in glioma cell lines expressing CD95 and triggered caspase-dependent cell death. APO010 reduced glioma cell viability in synergy when combined with temozolomide. The locoregional administration of APO010 induced glioma cell death in vivo and prolonged the survival of tumor-bearing mice. A further exploration of APO010 as a novel antiglioma agent is warranted.

Keywords: apoptosis, CD95 ligand, glioma, temozolomide.
acceptable safety profile, Holler et al.17 developed the “Mega-Fas-Ligand,” a hexameric protein consisting of 2 CD95L extracellular domain trimers and the collagen domain of adiponectin ACRP30. This molecule is now known as APO010. Here, we characterize the cytolytic properties of APO010 on human glioma cells in vitro and in vivo and compare its potency to a cross-linked soluble CD95L and an agonistic anti-CD95 antibody.

Materials and Methods

Materials and Cell Lines

The human malignant glioma cell lines LN-308, LNT-229 (T for Tubingen for clarification), and U87MG were provided by Dr. N. de Tribolet (Centre Hospitalier Universitaire Vaudois). The simian virus 40–fetal human astrocytic cell line (SV-FHAS) was provided by D. Stanimirovic (Institute of Biological Sciences, National Research Council of Canada). The generation of LNT-229.neo control and LNT-229.MGMT transfectants overexpressing O6-ethylguanine-DNA methyltransferase (MGMT) has been described,18 as well as the generation of LNT-229 cells overexpressing crm-A.19 To stably overexpress CD95, LN-308 cells were transfected with the BCMGS.neo expression vector containing the cDNA for CD95 using Metafectene Pro. The generation of the expression vector has been described.6 The LN-308.CD95 transfectants and the LN-308.neo control cells were selected with G418 (500 μg/mL), and LN-308.CD95 cells were further subcloned. Primary glioblastoma cells were established from freshly resected tumors, cultured in monolayers, and used between passages 4 and 9.20 Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS), 2 mM glutamine, and penicillin (100 IU/mL)/streptomycin (100 mg/mL).

DEVD-amc and zVAD-fmk were obtained from Bachem. Propidium iodide (PI) was purchased from Sigma. Temozolomide (TMZ) was obtained from Schering Plough. APO010 was provided by Topotarget.17 O6-Benzylguanine (O6BG) was a gift from Bernd Kaina.21 sCD95L and enhancer were obtained from Alexis. sCD95L consists of the extracellular domain of human CD95L (amino acids 103–281) fused at the N-terminus to a linker peptide (26 amino acids) and a FLAG-tag. The cross-linking enhancer increases the biological activity by ≏50-fold. sCD95L and enhancer were used at a ratio of 1:5. In the following, the term sCD95L stands for the combination of sCD95L and the enhancer molecule. An agonistic antibody to CD95 (clone CH11) was from Upstate.2

RNA Silencing

To silence endogenous CD95 expression, U87MG and LNT-229 cells were transiently transfected with 50 nM HS_FAS_7_HP validated small-interfering RNA (siRNA) targeting CD95 from Qiagen (Cat No. Si02654463; sense strand 5′-GGAGUACACAGACAAAGCCTT-3′). All Stars nonsilencing siRNA from Qiagen (Cat No. 1027280) was used as a negative control. Glioma cells were seeded in 24-well plates and 24 hours later transfected with siRNA using Metafectene Pro. The extent of gene silencing was verified by the analysis of CD95 expression on the cell surface by flow cytometry.

DEVD-amc Cleavage Assay

The cells were seeded in 96-well plates, treated as indicated, lysed in 25 mM Tris–HCl, pH 8.0, 60 mM NaCl, 2.5 mM EDTA, 0.25% Nonidet-P40 for 10 minutes, and DEVD-amc was added at 12.5 mM. Caspase activity was assessed by fluorescence using a Berthold Mithras fluorimeter (Berthold Technologies) at 355 nm excitation and 475 nm emission wavelengths.

Flow Cytometry for CD95 Expression

Adherent glioma cells were detached using Accutase (PAA Laboratories) and blocked with 2% FCS in phosphate-buffered saline (PBS). The cells were incubated for 30 minutes on ice using the fluorescein isothiocyanate (FITC)–antihuman CD95 monoclonal antibody (clone UB2) or immunoglobulin (Ig) G1 isotype control from Beckman Coulter. Flow cytometry was performed with a Dako flow cytometer (Dako). Signal intensity was calculated by dividing median fluorescence obtained with the specific antibody by signal intensity obtained with the isotype control antibody (specific fluorescence index, SFI).

Detection of Apoptosis by Annexin V Binding

Apoptotic cell death was analyzed by staining with FITC-labeled annexin V (BD Bioscience). Glioma cells were treated as indicated, collected, washed with PBS, and resuspended in a buffer containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2. Then annexin V–FITC and PI were added. After incubation for 30 minutes, 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.

Growth and Viability Assay

The cells were seeded in 96-well plates and allowed to attach for 24 hours. Cells were treated as indicated, and cell density of attached cells was assessed by crystal violet staining. Briefly, the cell culture medium was removed and surviving cells were stained with 0.5% crystal violet in 20% methanol for 20 minutes at room temperature. The plates were washed extensively under running tap water and air-dried, and optical density values were read in an ELISA reader (Mithras LB 940, Berthold Technologies) at a wavelength of 550 nm.
Immunoblot Analysis

The general procedure has been described. The cells were treated as indicated and lysed. Twenty micromgrams of protein per lane were separated on 10%–12% acrylamide gels (Bio-Rad). After transfer to a nitrocellulose membrane, the blots were pretreated for 1 hour with PBS containing 5% skim milk and 0.05% Tween 20 and then incubated overnight with the following antibodies; cleaved caspase 3 (No. 9661) from Cell Signaling, caspase 8 (ALX-804-429-C100) from Alexis, poly(ADP-ribose) polymerase (PARP; 4C10-5) from BD Bioscience, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Chemicon. Visualization of protein bands was accomplished using horseradish peroxidase–coupled IgG secondary antibody (Santa Cruz) and enhanced chemoluminescence (Amersham).

Animal Experiments

CD1<sup>nu/nu</sup> mice were purchased from Charles River Laboratories. The experiments were performed according to the German animal protection law. For the intracranial glioma model, mice aged 6–12 weeks were anesthetized and placed in a stereotaxic fixation device (Stoelting). A burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe was introduced to a depth of 3 mm. U87MG cells (10<sup>5</sup>) resuspended in a volume of 2 µL of PBS were injected into the right striatum. Locoregional treatment with APO010 (40 mg in 2 µL of PBS on days 7 and 14 after tumor implantation) or PBS was performed similarly (6 mice per group). The mice employed in our experiments had body weights between 20 and 30 g. Thus, the mice received 0.0013–0.002 mg/kg body weight APO010 i.c. Systemic treatment was performed by i.p. injections of APO010 (0.015 mg/kg body weight 3 times per week) or PBS (5 mice per group). These doses were selected based on a maximum tolerated dose (MTD) for systemic application according to Verbrugge et al. and by an MTD determination for i.c. application performed in the context of this study. The mice were observed twice daily and killed when developing neurological symptoms or at defined time points for histological analysis as indicated. For the detection of intratumoral APO010, U87MG tumors were inoculated and the mice were treated i.c. or i.p. as described above on day 14 following tumor inoculation. Mice were sacrificed 4 (<i>n</i> = 3) or 24 hours (<i>n</i> = 3), respectively, following treatment. Mice were cardially perfused with cold PBS and brains were snap frozen. The tumors were explanted, homogenized, and brought into lysis buffer containing a protease inhibitor cocktail. Lysates were centrifuged at 6000 turns/min for 5 minutes, and the supernatant extracted for gel electrophoresis and immunoblot using anti-ACRP30 monoclonal antibody (ALX-804-144, clone ne.na) from Enzo Life Sciences, which recognizes the collagen domain of adiponectin ACRP30 as part of APO010.

Immunohistochemistry

For histology, 8 µm cryostat sections were stained with hematoxylin-eosin. Cleaved caspase 3 (AF835; R&amp;D Systems) was detected by immunohistochemistry. The tissue sections were pretreated with 3% H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase and boiled for 10 minutes in citrate buffer. Endogenous biotin activity was blocked with the Dako biotin blocking kit (Dako Cytomation GmbH). Sections were blocked with 3% skim milk and 10% normal swine serum for 30 minutes each at room temperature. Primary antibodies were added and the tissue sections were incubated overnight at 4 °C in a humidified chamber. Purified normal IgG substituted for the specific antibody served as a negative control. The sections were washed 3 times with PBS containing 0.05% Tween 20 (PBST). Biotinylated goat antirabbit secondary antibody (Vector Laboratories) was added for 1 hour. After 3 PBST washes, streptavidin peroxidase conjugate (Zymed) was added, and the tissue sections were incubated for 10 minutes, washed with PBST, and 3-amino-9-ethylcarbazole in N,N-dimethylformamide (Zymed) was added as a substrate. After a further 10 minutes of incubation, the sections were finally washed with water. Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling (TUNEL) was performed using the TUNEL-AP Kit (Roche). Neighboring control sections were stained using Mayer’s hematoxylin. All sections were coverslipped, and staining was assessed by light microscopy.

Statistics

Statistical significance was assessed by one-way analysis of variance (ANOVA) between groups and Bonferroni post hoc testing or Student 2-tailed <i>t</i>-test as indicated (SPSS 17, SPSS). Synergy of APO010 and TMZ was evaluated using the fractional product method. Here, “predicted values” correspond to an additive action of 2 compounds that can be compared with the observed effect.

Results

APO010 Is Cytotoxic to Human Glioma Cells

The human glioma cell lines studied here express different levels of CD95 as assessed by flow cytometry using an antihuman CD95-specific antibody (clone UB2). The SFI for the present culture conditions were 2.3 for U87MG, 2.1 for LNT-229, and 1.3 for LN-308. The SFI for SV-FHAS was 1.1.

The lower level of CD95 expression has been held responsible for the relative resistance of LN-308 cells to CD95-mediated apoptosis. Accordingly, U87MG and LNT-229, but not LN-308, cells were highly sensitive to APO010. At equivalent concentrations, APO010 was more effective than sCD95L or CH11 in inducing cell death in both cell lines (Fig. 1A–C). Accordingly, APO010 induced DEVD-amc–cleaving
caspase activity in U87MG and LNT-229 cells (Fig. 1D and E) but not in LN-308 cells (data not shown). Cell death induction involved the cleavage of caspases 8 and 3 and of PARP, and caspase and PARP processing were blocked by the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 1F). The induction of cell death by APO010 critically depended on the activation of caspase 8, since LNT-229 cells overexpressing crmA, which selectively blocks caspase 8, were resistant (data not shown).

Cell death induction by APO010 was further characterized by flow cytometry using annexin V labeling for the assessment of apoptosis and PI staining for the detection of cell death. APO010 critically depended on the activation of caspase 8, since LNT-229 cells overexpressing crmA, which selectively blocks caspase 8, were resistant (data not shown).

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Further, we tested primary glioma cell cultures from 9 randomly selected glioblastoma patients. These ex vivo propagated cells were treated between passages 4 and 9 with either APO010, sCD95L, or CH11. Again, APO010 was more effective in inducing apoptosis than sCD95L or CH11 as assessed by annexin V/PI labeling and flow cytometry (Table 1). APO010 killed in a concentration-dependent manner more than 50% of the cells at a concentration of 1000 ng/mL in 7 tumors, compared with 3 tumors for sCD95L and none of the tumors for CH11 at equivalent concentrations. Only 2 (TU 132 and TU 159) of these 9 tested tumors showed relative resistance to APO010-mediated cell death. TU 132 does not express CD95 on the cell surface (SFI 1.0) as assessed by flow

Fig. 1. APO010 induces DEVD-amc–cleaving caspase activity and cell death in human glioma cell lines. (A–C) U87MG, LNT-229, or LN-308 cells were exposed to increasing concentrations of APO010, sCD95L, or CH11 for 20 hours in triplicates. Cell density was assessed by crystal violet staining. Data are expressed as mean and standard deviation (n = 3); 1 representative out of 3 independent experiments with similar results is shown. (D and E) U87MG or LNT-229 cells were treated as in (A) and (B) in triplicates and assessed at 3 (U87MG) or 6 hours (LNT-229) for DEVD-cleaving caspase activity. Data are expressed in relation to untreated controls as mean and standard deviation (n = 3); 1 representative out of the 3 independent experiments with similar results is shown. The level of statistical significance for a superior effect of APO010 over sCD95L and CH11 was assessed in (A)–(E) by one-way ANOVA and Bonferroni post hoc testing (*P < .05; **P < .01; ***P < .001; ++P < .01 for APO010 and sCD95L vs CH11). (F) LNT-229 cells were treated as indicated for 20 hours. Cellular lysates were examined for cleavage of caspases 8 and 3 and for PARP and GAPDH by immunoblot.
cytometry and TU 159 only at a low level (SFI 1.4; Table 1). Most of the sensitive tumors show higher SFI values for CD95 expression. However, the SFI of the sensitive TU 447 (SFI 1.4) corresponded to that of TU 159, indicating the regulation of sensitivity at the level of downstream signal transduction too.

Cytotoxicity of APO010 Critically Depends on the Expression of CD95

To assess the specificity of the cytotoxic activity of APO010, CD95 expression was silenced using siRNA in LNT-229 and U87MG glioma cells. Successful gene silencing was verified by flow cytometry 72 hours following transfection (Fig. 3A). Time-course experiments confirmed a stable knockdown of CD95 between 72 and 96 hours following transfection (data not shown). The knockdown of CD95 led to a significant reduction in sensitivity of both cell lines toward APO010-mediated cytotoxicity (Fig. 3B). To sensitize LN-308 cells toward APO010, these cells were genetically modified to overexpress CD95 and subcloned. The clones LN308.CD95 K2 and K3 showed stable overexpression of CD95 as assessed by flow cytometry (SFIs 7.0 and 14.4) and gained sensitivity toward APO010 (Fig. 3C).

APO010 Synergizes with TMZ in Inducing Glioma Cell Death

The next set of experiments was performed to evaluate additive or synergistic effects of APO010 and TMZ, the most commonly used drug for the treatment of glioblastoma. According to the fractional product method,24 the combination of APO010 and TMZ had more than additive, that is, synergistic, cytotoxic effects (Fig. 4A). Resistance to TMZ alone is conferred by the DNA repair enzyme MGMT.18 To assess whether the combination of APO010 and TMZ bypassed the protective function of MGMT, we cotreated MGMT-transfected cells with APO010 and TMZ. No synergy was observed under these conditions (Fig. 4B), suggesting that the MGMT-sensitive effects of TMZ mediate synergy. Accordingly, when higher, equieffective concentrations for monotherapy with TMZ were employed in the MGMT-transfected cells, synergy was restored (data not shown). Moreover, preexposure of the MGMT-transfected cells to the MGMT inhibitor O6BG and subsequent coexposure to lower concentrations of TMZ and APO010 also resulted in synergistic induction of cell loss (Fig. 4C). These data corroborated that no specific, MGMT-independent cascade triggered by TMZ facilitates APO010-induced apoptosis. Importantly, LN-308 cells, which are resistant to death receptor–mediated apoptosis, were sensitized by TMZ to APO010-mediated cell death (Fig. 4D). However, since LN-308 cells are also relatively resistant to TMZ-mediated cell death,18 a higher concentration of TMZ compared with LNT-229 cells was necessary to achieve this sensitization. Moreover, the synergy of TMZ and APO010 led to enhanced DEVD-amc–cleaving caspase activity in LNT-229 cells (Fig. 4E).
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APO010 Induces Glioma Cell Death In Vivo and Prolongs Survival

Orthotopic human U87MG xenografts were established in nude mice as outlined in the “Materials and Methods” section and were confirmed to be associated with intracranial tumor growth as assessed by histology. APO010 was administered by stereotactic injection on days 7 and 14 after tumor cell implantation. The dose of 40 ng per single injection was determined from tolerability studies in preceding applications in non-tumor-bearing mice. APO010 conferred a significant survival benefit. At 40 days after tumor cell implantation, the survival rate was 80% as opposed to 0% in control-treated animals ($P < .01$; Student 2-tailed $t$-test). The median survival increased from 37 to 52 days (Fig. 5A). The subsequent death of the mice in the APO010 treatment group was related to neurological symptoms following recurrence of tumor growth as assessed macroscopically.

Systemic treatment was performed by i.p. injections of APO010 with the MTD of 0.015 mg/kg body weight 3 times per week following tumor inoculation until the mice had to be sacrificed due to the development of neurological symptoms. The survival of the mice in the APO010 treatment and PBS control groups did not differ significantly ($P = n.s.$; Student 2-tailed $t$-test; Fig. 5B).

Histological studies were performed on day 15 after tumor inoculation in i.c.-treated mice. Hematoxylin-eosin staining revealed minimal tumor burden in the APO010 group compared with controls (Fig. 5Ca and b). Apoptotic tumor cells were detected by TUNEL staining (Fig. 5Cc and d) and immunohistochemistry for active caspase 3 (Fig. 5Ce and f) only in the APO010 group but not the control group (Fig. 5C middle and lower panels).

The failure of systemic application to limit tumor growth suggested insufficient APO010 delivery to the target. Accordingly, to assess intratumoral APO010, tumors were implanted and 6 mice per group were treated by a single i.p. or i.c. administration of APO010 or PBS as a control. Three mice per group were sacrificed 4 hours and 3 mice 24 hours following treatment. Tumor lysates were assessed for APO010 by immunoblot. APO010 was detected in only the tumors from mice injected i.c. with APO010, but not in the APO010 i.p. or the PBS control groups (Fig. 5D). At 24 hours (Fig. 5D, lower panel), note a weak band in lane 3 and only background signal in lane 5. Equal protein loading was ascertained by Ponceau S staining (data not shown).

Discussion

This article reports on a further development in the field of death receptor targeting for malignant glioma. We found that APO010, a novel hexameric CD95 agonist, is superior to either soluble CD95L or the agonistic CD95 antibody CH11 in killing CD95-expressing U87MG and LNT-229 glioma cells (Figs 1 and 2).
Superior activity of APO010 over other CD95 agonists has previously been observed in leukemia and SKOV-3 ovarian cancer cells. The induction of cell death in glioma cells was specific in that it was receptor mediated. In LN-308 cells, which express very little CD95, a significant induction of cell death was observed for only the highest doses of APO010 (Figs 1C and 2C), and LNT-229 cells overexpressing crmA were resistant to APO010 (data not shown). Moreover, in contrast to human Apo2L/TRAIL, which failed to kill freshly isolated glioma cells, activity of APO010 was also observed in various primary glioma cell cultures (Table 1). The CD95 gene silencing in LNT-229 or U87MG cells abrogated, and the overexpression of CD95 in LN-308 cells conferred sensitivity of these cells (Fig. 3). Therefore, 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. However, the susceptibility of the cell lines is likely modulated additionally by downstream intracellular targets, such as caspases and inhibitors of apoptosis proteins (IAPs). Compared with LNT-229 cells, U87MG cells express similar levels of X-linked IAP but much higher levels of caspase 9, suggesting that these cells amplify the mitochondrial cell death pathway more efficiently. Such observations explain that cell lines expressing similar amounts of CD95 may show different sensitivities.

Additive or synergistic effects of death ligands and chemotherapy have been studied extensively in various cancer models, including malignant gliomas. APO010 exhibited enhanced activity when combined with platinum in ovarian cancer cells or imatinib in gastrointestinal stromal tumors. In contrast, no synergistic effect of APO010 was demonstrated in vivo when combined with irradiation. In view of a possible clinical application, we combined APO010 with TMZ, the current standard of care chemotherapy for
There was compelling evidence for synergy as assessed by the fractional product method, and this synergy required the classical MGMT-sensitive cell death pathway triggered by TMZ (Fig. 4).

Sensitization of glioma cells toward Apo2L/TRAIL-mediated apoptosis by lomustine, another alkylating chemotherapy agent, involved the mitochondrial pathway and enhanced cytochrome c release. Another report demonstrated an upregulation of CD95 following treatment of U87MG cells with the O6-methylating agent N-methyl-N-nitro-N-nitrosoguanide by immunoblot in membrane extracts. In this study, we did not observe an upregulation of CD95 on the cell surface by flow cytometry under conditions of synergistic killing in LN-308 cells (data not shown).

Local injection of APO010 conferred a significant survival benefit to glioma-bearing mice in the absence of relevant toxicity (Fig. 5). APO010 was active in vivo only when administered locally but not when given i.p. Assessment of tumors ex vivo revealed APO010 in only the i.c.-treated group but not following i.p. injection (Fig. 5D). This suggests that upon systemic treatment with the MTD, too little, if any, APO010 reaches the tumor. In general, the toxicity of the human APO010 in mice in vivo has been favorable compared with the mouse Jo2 antibody. When applied in BALB/c mice, 100 μg of Jo2 killed most of the mice and 10 μg killed half of the mice when given i.p. In contrast, APO010 has been administered systemically or i.p. or intrasleionally in the brain in this study and controlled tumor growth in the absence of unacceptable toxicity as assessed by the clinical observation of the mice and immunohistochemistry. The MTD for APO010 was 0.015 mg/kg body weight for systemic treatment and 40 ng per single injection for local intracerebral treatment as determined here. Kamei et al. used the anti-CD95 antibody, clone CH11, in a mouse model of melanoma.
and injected 5 μg of the antibody directly in the tumors grown s.c. in nude mice. This was tolerated but did not affect tumor growth. The injection of up to 10 μg of CH11 into s.c. growing tumors derived from SNB19 or SNB79 glioblastoma cell lines was tolerated too but did not alter tumor growth either. In the same study, mice were also injected with up to 10 μg of CH11 i.v. An MTD was not formally determined in either study.

The MTDs of APO010 (40 ng) and CH11 (10 μg) for local treatment are difficult to compare, since APO010 was used in an orthotopic model and injected i.c., whereas CH11 was tested only in s.c. inoculated tumors. No study available has directly compared APO010 with an anti-CD95 antibody or a soluble Fas ligand in vivo. However, in the present study, APO010 exhibited antiglioma activity when injected directly in the tumor, whereas CH11 failed to exhibit activity when injected s.c. in mouse models of melanoma or glioblastoma as indicated above.

APO010 is now being explored in a phase I dose-escalation study in up to 35 patients with untreatable advanced or refractory solid tumors in order to establish the safety and tolerability and MTD in humans (ClinicalTrials.gov identification NCT00437736). In addition, pharmacokinetics, immunogenicity, and preliminary signs of anticancer activity are being evaluated.

Conflict of interest statement. None declared.

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