Mechanisms of Resistance of Human Glioma Cells to Apo2 Ligand/TNF-Related Apoptosis-Inducing Ligand

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Key Words

Brain tumor • Caspase 8 • Death ligands • DR5 • ERK kinase

Abstract

Background: Many tumor cells are resistant to Apo2L/TRAIL-induced apoptosis in the absence of inhibitors of protein synthesis. Apo2L/TRAIL, in addition to induction of apoptosis, may therefore also activate survival pathways. **Methods:** Here we investigated whether such survival pathways mediate resistance to Apo2L.0-induced apoptosis in human glioma cells. **Results:** Apo2L.0 induced the phosphorylation of ERK1/2, but not of Akt. This effect was unaffected by caspase inhibition. Inhibitors of protein synthesis, PI3 kinase, ERK kinase, NF- κ B or casein kinase 2 sensitized for Apo2L.0-induced apoptosis to a different extent in a panel of human malignant glioma cell lines. However, none of the sensitizers overcame resistance mediated by ectopic expression of the viral caspase 8 inhibitor, crm-A. Primary glioma cultures were almost completely resistant to Apo2L.0-induced cell death even in the presence of the inhibitors. Caspase-8 was expressed in these cells whereas only weak expression of DR5 was detected. Transient expres-

sion of DR5 conferred sensitivity to Apo2L.0. **Conclusion:** These data challenge the view that specific cell lines harbour specific mechanisms of resistance to Apo2L/TRAIL. Weak expression of DR5 in primary glioma might limit the therapeutic application of Apo2L/TRAIL in human glioblastoma patients.

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Introduction

The tumor necrosis factor (TNF) family member Apo2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) has been shown to induce apoptosis in a variety of human tumor cells [1, 2], including human malignant glioma cells [3]. The toxicity of Apo2L/TRAIL for normal tissues depends on its preparation: His-tagged or FLAG-tagged preparations are toxic for normal hepatocytes [4-6] whereas Apo2L.0, a recombinant Apo2L/TRAIL preparation containing the amino acids 114-281 is not toxic for hepatocytes [6]. For Apo2L/TRAIL, five receptors are known: death receptor (DR)4/TRAIL-R1 and DR5/TRAIL-R2 transmit an apoptotic

signal *via* their death domain whereas decoy receptor (DcR)1/TRAIL-R3 and DcR2/TRAIL-R4 do not transmit an apoptotic signal [7]. Further, osteoprotegerin (OPG) is a soluble receptor for Apo2L/TRAIL with a lower affinity compared with the other receptors [8]. After binding of Apo2L/TRAIL to DR4/5, Fas-associated death domain protein (FADD) is bound to the receptors. As a consequence, caspases 8 or 10 are activated. They activate additional caspases, resulting in mitochondrial cytochrome c release and apoptotic cell death [7]. Although some tumor cell lines are susceptible to Apo2L/TRAIL-induced apoptosis, many tumor cells exhibit resistance unless sensitized to apoptosis by various strategies.

The gold standard for sensitization to death ligands such as Apo2L/TRAIL is by inhibition of RNA or protein synthesis. Interestingly, it has not been clarified for Apo2L/TRAIL-induced cell death or cell death induced by TNF or CD95 ligand (CD95L) which specific mRNAs or proteins need to be down-regulated to facilitate the induction of cell death by death ligands. Candidates include FLIP in keratinocytes [9] and in fibroblasts [10] as well as p21 in glioma cells [11].

In addition to the induction of proapoptotic caspase signaling by Apo2L/TRAIL, other signalling pathways are activated by Apo2L/TRAIL. Extracellular signal-related kinase (ERK) 1/2 is activated by Apo2L/TRAIL in HeLa cells whereas inhibition of MEK1/2 (MAP kinase kinase/ERK kinase) by PD98059 results in increased sensitivity to Apo2L/TRAIL [12]. Apo2L/TRAIL leads to phosphorylation of Akt in SK-N-MC neuroblastoma cells [13], in vascular endothelial cells [14] as well as in synovial fibroblasts [15]. Further, activation of nuclear factor (NF)- κ B by Apo2L/TRAIL has been demonstrated in different cell types [16-18]. Activation of these survival signaling pathways could be part of the cellular counterattack to block an Apo2L/TRAIL-transduced apoptotic signal. Inhibition of the MEK/ERK 1/2 and PI3 kinase/Akt pathways sensitizes HeLa cells and rheumatoid synovial cells to Apo2L/TRAIL [12, 19].

Further strategies to sensitize for Apo2L/TRAIL-induced apoptosis include the inhibition of casein kinase 2 (CK2) [20] or epidermal growth factor receptor (EGFR) [21]. CK2 is a protein kinase which phosphorylates more than 100 proteins, is important for cell survival and inhibits apoptosis [22]. CK2 inhibits the cleavage of BID by caspase 8 and promotes the NF- κ B-mediated expression of BCL-X_L, thereby preventing the activation of caspase 9 [20].

Most glioma cells are resistant to Apo2L/TRAIL in the absence of inhibitors of protein synthesis. Therefore,

we analyzed whether Apo2L/TRAIL induces survival signals and whether inhibition of survival signals sensitizes glioma cells to Apo2L/TRAIL. Inhibition of these survival signals has already been shown to sensitize different cell types to Apo2L/TRAIL [12, 19, 20]. The present data aim to analyse which of these mechanisms mediate resistance against Apo2L/TRAIL in a larger panel of glioma cells. Because of the well-appreciated shortcomings of cell lines to forecast the clinical situation, we further investigated whether short-term cultured primary glioma cells express receptors for Apo2L/TRAIL and are susceptible to Apo2L/TRAIL-induced apoptosis.

Materials and Methods

Materials

Cycloheximide (CHX), an inhibitor of protein synthesis, and emodin, an inhibitor of CK2, were obtained from Sigma (München, Germany), LY294002, an inhibitor of PI3 kinase, U0126, a MEK1/2 inhibitor as well as 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB), an inhibitor of CK2, were obtained from Calbiochem (Schwalbach, Germany). Helenalin, an inhibitor of NF- κ B [23], was obtained from Biomol (Hamburg, Germany). Recombinant Apo2L.0 (amino acids 114-281) was a gift from A. Ashkenazi (Genentech, CA) [24] and stored at -80°C until use. The concentrations and exposure times of the various inhibitors were titrated to achieve a maximum effect of sensitization in the absence of relevant cytotoxicity of the inhibitor itself and correspond to those previously employed for CHX [25], LY294002 and U0126 [26]. The caspase inhibitor zVAD-fmk was from Bachem (Weil am Rhein, Germany). CCNU was from obtained from Medac (Hamburg, Germany).

Cell culture

The established glioma cell lines have been characterized previously [27]. The generation of primary glioma cell cultures has also been described [28]. They were used at passage numbers below 15. The glioma cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 ng/ml streptomycin (Cambrex, Verviers, Belgium). For crystal violet staining, glioma cell lines were seeded at 20000 cells per well in 96-well plates in DMEM/10% FCS. After 24 h, medium was removed, cells were treated in serum-free medium as indicated for 20 h, and viable cells were stained with crystal violet.

Immunoblot analysis

Cells were treated in medium containing 10% FCS. Thereafter, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested into PBS with phenylmethylsulfonylfluoride (PMSF). The cells were centrifuged, lysed in lysis buffer containing phosphatase inhibitor cocktails 1 and 2 (Sigma), and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For detection of phos-

phorylated proteins, tris-buffered saline (TBS) was used instead of PBS. Membranes were probed overnight with the following antibodies: anti-Phospho-ERK1/2 (No. 9106), anti-Phospho-Akt (No. 92715), anti-Akt (No. 9272) from Cell Signaling (Frankfurt, Germany), anti-ERK1/2 (sc 94), anti-Actin (sc1616) from Santa Cruz (Heidelberg, Germany), anti-Crm-A (No. 556427) from Pharmingen (Heidelberg, Germany), anti-Caspase-8 (ALX-804-429-C100) from Alexis (Grünberg, Germany). The secondary antibodies horse radish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) G, anti-goat IgG and anti-rabbit IgG were from Santa Cruz. Enhanced chemoluminescence (ECL) from Amersham (Braunschweig, Germany) was used for detection.

Flow cytometry

For the detection of receptors for Apo2L/TRAIL, cells were detached with accutase (PAA, Pasching, Germany), washed in flow cytometry buffer (PBS/1% bovine serum albumine/0.01% sodium azide) and blocked for 30 min in flow cytometry buffer supplemented with 10% rabbit serum. Thereafter, cells were incubated for 1 h with IgG₁ isotype antibody (MAB002), mouse anti-DR4 antibody (MAB347), mouse anti-DR5 antibody (MAB6311), mouse anti-DcR1 antibody (MAB6302) or mouse anti-DcR2 antibody (MAB633), purchased from R&D (Wiesbaden, Germany), at 10 µg/ml in flow cytometry buffer, washed, and incubated at 0.05 mmol/l with biotin-conjugated rabbit anti-mouse F(ab)₂ antibody E0413 obtained from DAKO (Hamburg, Germany) for 30 min. After incubating with streptavidin-APC (B&D, Heidelberg, Germany) at 0.1 µg/ml and washing, the cells were analysed in a DAKO CyAn flow cytometer using Summit 4.2. The specific fluorescence index (SFI) was calculated as the ratio of median fluorescence of specific versus isotype antibody.

Luciferase assay

The cells were seeded at 10,000 cells per well in 96-well plates. After 24 h, the cells were transfected with 150 ng NF-κB luciferase reporter plasmid (219077, Strategene, La Jolla, CA) and 20 ng RL-CMV renilla plasmid per well by Eugene 6 (Roche, Mannheim, Germany) [29, 30]. Twenty-four h after transfection, medium was removed, and the cells were treated with Apo2L.0 (10 ng/ml) or TNF (10 ng/ml) in DMEM for 6 h. The cells were washed with PBS, lysed with lysis buffer (Promega, Madison, WI), and frozen at -20°C. For analysis, the lysates were transferred to a LumiNunc™ Plate (Nunc, Roskilde, Denmark). Luciferase assay substrate (pH 8.0) and renilla assay substrate (pH 5.1) were added, and luminescence was measured in a LuminatPlus (EG&G, Pforzheim, Germany). The luciferase values were corrected for renilla activity.

Transient transfection

For cell death analysis after transient transfection, a membrane-bound enhanced green fluorescence protein (EGFP), EGFP-F, was transfected together with control vector (prK) or prK-DR5, provided by A. Ashkenazi (South San Francisco, CA) [31]. Because EGFP is lost from the cells during cell death, the membrane-bound EGFP-F was used in these experiments to control for transfection efficacy [32].

Primary glioma cells were seeded in 6-well plates and, after 24 h, transiently transfected with 0.2 µg pEGFP-F, and 0.8 µg prK or 0.8 µg prK-DR5 for 24 h using Eugene6 (Roche). After 12 h, cells were incubated with CHX (10 µg/ml) without or with Apo2L.0 (1 µg/ml) in serum-free medium. After 8 h, the cells were incubated with propidium iodide (PI) (50 µg/ml) for 10 min in flow cytometry buffer (PBS/0.2% bovine serum albumine), and analysed in a DAKO CyAn flow cytometer using Summit 4.2. Survival of transfected cells was calculated as the percentage of PI-negative cells of all EGFP-positive cells. A t-test for paired samples was performed to test for significance. To confirm DR5 expression, cells were transfected with EGFP-F and prK or prK-DR5 in the presence of zVAD-fmk, and analysed by flow cytometry.

Immunofluorescence analysis

The cells were seeded on poly-L-Lysine coated glass slides in 24-well plates. After 24 h, the medium was removed, and the cells were incubated with serum-free medium for 24 h. Thereafter, the cells were left untreated or treated with Apo2L.0 (10 ng/ml) or TNF (10 ng/ml) for 1 h or 3 h. The cells were fixed for 10 min. in 3.7% paraformaldehyde/PBS, washed with PBS and permeabilized in Triton-X 0.05%/PBS for 5 min., and washed again with PBS. Thereafter, the cells were blocked for 1 h in PBS/10% goat serum, and incubated with isotype antibody or anti-NF-κB-p65 antibody (sc-8008, Santa Cruz). After washing three times with PBS, the cells were incubated with Alexa-Fluor-488-goat-anti-mouse-IgG-antibody (Molecular Probes/Invitrogen, Karlsruhe) for 20 min., washed three times with PBS, and mounted with Vectashield containing DAPI (Vectorlabs, Axxora, Gruenberg), and analysed on a Zeiss Axiovert 100 M confocal laser microscope.

Statistics

Correlation analysis of susceptibility to Apo2L.0 and expression of DR4 and DR5 was performed using Pearson product-moment correlation of survival fractions after treatment and SFI values of receptor expression.

Results

Modulation of survival signalling pathways by Apo2L.0

To determine whether the resistance of some glioma cell lines to Apo2L.0 might be mediated by the activation of survival signalling pathways by Apo2L.0, we investigated the phosphorylation of Akt, ERK1/2 and IκBα after exposure to Apo2L.0. Apo2L.0 did not induce the phosphorylation of Akt in either of three examined cell lines. In contrast, there was a reduction of phosphorylated Akt after 3 h of treatment in LN-18 cells and LNT-229 cells. ERK1/2 was rapidly phosphorylated after treatment with Apo2L.0 in the three cell lines (Fig. 1A). There was no clear modulation of phosphorylation of IκBα by

Fig. 1. Modulation of signalling pathways by Apo2L.0. A. LN-18, U87MG or LNT-229 cells were treated with Apo2L.0 (10 ng/ml) for the indicated periods of time, and the phosphorylation of ERK1/2 and Akt was examined by immunoblot analysis. Total ERK1/2 and Akt were determined in parallel. B. LNT-229 cells were transfected with NF- κ B luciferase reporter and renilla luciferase plasmids and treated for 6 h with Apo2L.0 (10 ng/ml) or TNF (10 ng/ml). Luciferase activities were measured and corrected for renilla luciferase activity and are shown as relative light units (RLU) (* $p < 0.05$ compared to untreated cells). C. LN-18 puro or crm-A cells were treated for 10 min with Apo2L.0. The levels of phosphorylated and total ERK1/2 and crm-A were analysed by immunoblot. Actin was included as a control.

Apo2L.0 (data not shown). Accordingly, Apo2L.0, in contrast to TNF, failed to activate NF- κ B in a luciferase assay (Fig. 1B), and to induce nuclear p65 translocation as analysed by immunofluorescence (not shown).

To investigate whether the phosphorylation of ERK1/2 was mediated by caspases, we took advantage of LN-18 cells stably expressing the viral caspase inhibitor crm-A. Apo2L.0 induced the phosphorylation of ERK1/2 to a similar extent in control-transfected cells and in crm-A transfected cells (Fig. 1C). In contrast, crm-A transfected cells were protected against Apo2L.0-induced cell death (Fig. 2B). Similarly, treatment of cells

with the caspase inhibitor zVAD-fmk did not inhibit the phosphorylation of ERK1/2 by Apo2L.0 (not shown), indicating that the phosphorylation of ERK1/2 does not depend on caspase activation.

Pharmacological modulation of survival pathways putatively involved in Apo2L.0-induced cell death

We next tested whether inhibitors of Akt, ERK1/2, NF- κ B or CK2 modulate Apo2L.0-induced apoptosis. As a control, CHX was included in these experiments. Three

Fig. 2. Pharmacological modulation of Apo2L.0-induced cell death. A. The glioma cells were exposed to Apo2L.0 (1 μ g/ml) for 16 h in the absence or presence of CHX (10 μ g/ml), LY294002 (10 μ M), U0126 (20 μ M), helenalin (5 μ M) or DRB (25 μ M). Survival was assessed by crystal violet staining (mean and SD) and is expressed as survival relative to survival with inhibitor alone (* $p < 0.05$ compared to survival of cells treated with Apo2L.0 in the absence of inhibitors). B. LN-18 puro or crm-A cells were treated as described and survival was analysed. Note that in the absence of inhibitors, LN-18 puro control transfected cells were more resistant to Apo2L.0 than untransfected parental LN-18 cells, the reason for these effects is unclear.

Fig. 3. Expression of receptors for Apo2L/TRAIL and apoptosis induced by Apo2L.0 in primary glioma cultures. A. Expression of Apo2L/TRAIL receptors in Tu-113 cells was analysed by flow cytometry. Light, isotype antibody, solid line, specific antibody. B. (left panel) Expression of receptors for Apo2L/TRAIL was analysed by flow cytometry analysis and is shown as SFI. (right panel) Glioma cells were treated with Apo2L.0 (1 µg/ml) in the absence or presence of CHX, LY294002, U0126, Helenalin or DRB. Survival was assessed by crystal violet staining and is expressed as survival relative to survival with inhibitor without Apo2L.0.

of 12 cell lines, LN-18, T98G and A172, were sensitive to Apo2L.0 in the absence of sensitizing agents, and these cell lines were further sensitized by CHX. At the concentration of Apo2L.0 shown in Fig. 2, the sensitivity of

LN-18 to Apo2L.0 was high, therefore, an additional sensitizing effect of the inhibitors was not evaluable; the effects of the inhibitors in LN-18 cells described below were only obvious at lower concentrations of Apo2L.0.

Fig. 4. Expression of caspase 8 and ERK1/2 and induction of expression of DR5 in primary glioma cells. **A.** Primary glioma cells were treated with Apo2L.0 (10 ng/ml) for 24 h, and caspase 8 expression was analysed by immunoblot analysis. As a control, LNT-229 cells were treated for 6 h with CD95L (100 U/ml) + CHX. **B.** Primary glioma cells were treated with Apo2L.0 (10 ng/ml) for 10 min, and the phosphorylation of ERK1/2 was analysed by immunoblot. Total ERK1/2 served as a reference. **C.** Primary glioma cells Tu-140 were transiently transfected with EGFP-F and control vector (prK) (left) or DR5 (right) in the presence of zVAD-fmk, and expression of DR5 of the EGFP-positive cells was analysed by flow cytometry, broken line, isotype antibody, solid line, specific antibody (left panel). Primary glioma cells Tu-140 were transiently transfected with EGFP-F and prK or DR5, treated with CHX (10 µg/ml) without (control) or with Apo2L.0 (1 µg/ml) for 16 h, and cell death was analysed by PI staining. Surviving transfected cells are EGFP-positive and PI-negative. The percent of surviving transfected cells relative to the total transfected cells after control transfection (prK) or after DR5 transfection (DR5), after treatment with CHX (Co.) or Apo2L.0 + CHX (Apo2L.0), was analysed (Mean and SD of three independent experiments. * $p < 0.05$ paired t-test, difference between mean survival of DR5-transfected cells treated with CHX and Apo2L.0 + CHX) (right panel). **D.** Primary glioma cells were treated for 24 h without or with CCNU (50 µM or 250 µM), in the absence (white bars) or presence of Apo2L.0 (1 µg/ml), and survival was analysed by crystal violet staining.

Five cell lines were resistant to Apo2L.0 alone but became sensitive when they were coexposed to CHX: U87MG, LN-428, D247MG, U251MG, U373MG. In contrast, LN-319, LNT-229, U138MG and LN-308 remained resistant in the presence of CHX. LY294002, an inhibitor

of PI3 kinase, sensitized T98G, U87MG, D247MG and A172, but not the other cell lines, to Apo2L.0-induced cell death. Apo2L.0-induced cell death was increased by the MEK1/2 inhibitor U0126 in LN-18, T98G and A172 cell lines. Helenalin, which inhibits the activity of the p65

	Apo2L.0+ CHX	Apo2L.0+ LY294002	Apo2L.0+ U0126	Apo2L.0+ Helenalin	Apo2L.0+ DRB	DR4	DR5
Apo2L.0	0.54	0.88*	0.89*	0.49	0.68*	-.52	-.58*
Apo2L.0 +CHX		0.68*	0.68*	0.67*	0.91*	-.05	-.70*
Apo2L.0 +LY294002	0.68*		0.92*	0.45	0.75*	-.31	-.64*
Apo2L.0 +U0126	0.68*	0.92*		0.44	0.76*	-.36	-.66*
Apo2L.0 +Helenalin	0.67*	0.45	0.44		0.78*	-.10	-.58*
Apo2L.0 +DRB	0.91*	0.75*	0.76*	0.78*		-.05	-.68*

Table 1. Correlation matrix. Correlation analysis of sensitivity to Apo2L.0. (left) The glioma cell lines were treated with Apo2L.0 (1 µg/ml) without or with inhibitor. Percentages of survival were determined and analysed by correlation analysis. (right) The previously determined expression of DR4 and DR5, analysed by flow cytometry, [33] was correlated with sensitivity to Apo2L.0 without or with inhibitor. The values were analysed by correlation analysis. R-values are shown. Note that in the right part of the table, significant negative r-values indicate that stronger expression is associated with lower survival and therefore higher sensitivity to Apo2L.0. (*, $p < 0.05$, Pearson product-moment correlation).

subunit of NF- κ B, sensitized LN-18, U87MG, LN-428, D247MG, LN-319, LNT-229, A172, U251MG and U373MG cell lines. The CK2 inhibitor, DRB, increased Apo2L.0-induced cell death in LN-18, U87MG, LN-428, D247MG, T98G, LNT-229, A172, U251MG and U373MG cell lines (Fig. 2A). Similar results were found with emodin, another inhibitor of CK2 (data not shown).

None of the cell death-sensitizing agents overcame resistance mediated by ectopic expression of the viral caspase 8 inhibitor, crm-A, in LN-18 (Fig. 2B) or LNT-229 cells (data not shown), indicating that caspase 8 cannot be bypassed to induce cell death. To assess that the inhibitors achieved the desired biological effect, inhibition of protein synthesis by CHX had been verified by 3 H leucine incorporation [25], LY294002 inhibited the phosphorylation of Akt, U0126 inhibited the phosphorylation of ERK1/2, and inhibition of NF- κ B by helenalin was shown by prevention of NF- κ B reporter activity induced by TNF (not shown). At the concentrations used here, inhibitors of protein synthesis, NF- κ B and CK2 were stronger sensitizers than inhibitors of PI3 kinase and MEK. There was a strong correlation between Apo2L.0 sensitivity in the absence or presence of the inhibitors. More importantly, across the spectrum of inhibitors (Table 1, left), there was also a strong correlation, suggesting that glioma cells can either be

sensitized or not, but that there are no cell-line specific pathways of resistance that can be selectively targeted. The expression of DR5 at the cell surface determined by flow cytometry was a strong predictor of sensitivity to Apo2L.0, without or with inhibitors, as previously observed for His-tagged Apo2L/TRAIL [33] (Table 1, right).

Apo2L.0 sensitivity of primary glioma cells

The heterogenous Apo2L.0 sensitivity profile of 12 glioma cell lines and the promising preclinical data with Apo2L.0 [34] prompted us to analyze the *in vitro* Apo2L.0 sensitivity of primary glioma cells. We first determined the expression of Apo2L/TRAIL receptors in primary glioma cells. DR4 and DR5 were expressed in all of five primary glioma cell cultures analysed, whereas DcR1 was not detected (as defined by a specific fluorescence index (SFI) < 1.1) in 2, and DcR2 was not detected in 1 primary glioma cell culture (Fig. 3A for Tu-113 and B, left panel). To analyse whether these primary glioma cells are sensitive to Apo2L.0, the cells were treated with Apo2L.0 alone or coexposed to Apo2L.0 and the survival pathways inhibitors. All primary glioma cell cultures were resistant to Apo2L.0. Only two of these primary glioma cells were moderately sensitized to Apo2L.0 by CHX or helenalin. Thus, although primary glioma cells express death signal-transmitting receptors,

they are almost completely resistant to Apo2L.0.

Caspase 8 expression and Apo2L.0-induced phosphorylation of ERK1/2 in primary glioma cells

The data obtained with the crmA-expressing sublines had suggested a pivotal role for caspase 8 in mediating cell death. Therefore, the expression of caspase 8 in primary glioma cells was also analysed. Fig. 4A shows that caspase 8 was expressed in primary glioma cells, indicating that reduced expression of caspase 8 is not the cause of resistance to Apo2L.0. Since we had shown that phosphorylation of ERK1/2 is independent of caspase 8 activation, we were interested in the effect induced by Apo2L.0 in the primary glioma cells. Whereas Tu-159 shows an increased phosphorylation of ERK1/2, no effect was detectable in the other primary glioma cells (Fig. 4B). These observations provide further evidence for an upstream inhibition of Apo2L/TRAIL signalling in primary glioma cells.

Expression of DR5 increases sensitivity to Apo2L.0 in primary glioma cells

The expression of DR5 was stronger in the two cell cultures, Tu-107 and Tu-113 and both demonstrated some Apo2L.0 sensitivity. Expression of DR5 was low in the three resistant cell cultures (Fig. 3B). We therefore hypothesized that low expression of DR5 could be responsible for the resistance to Apo2L.0. Therefore, glioma cell lines Tu-132 and Tu-140 were transiently transfected with p-EGFP-F and DR5 and subsequently treated with Apo2L.0 and CHX. Expression of DR5 was confirmed by flow cytometry (Fig. 4C, left panel). The expression of DR5 alone did not significantly affect the viability of Tu-140 cells ($p=0.18$). Further, whereas Apo2L.0 + CHX had no effect on the survival of control-transfected cells, there was induction of cell death by Apo2L.0 in the presence of CHX in DR5 transfected Tu-140 cells (Fig. 4C, right panel). In contrast, no significant Apo2L.0-sensitizing effect of DR5 expression was detected in Tu-132 cells (not shown). These data suggest that reduced expression of DR5 might contribute to the resistance to Apo2L.0 in some primary glioma cell lines but further stress the heterogeneity among malignant glioma cell cultures. Because tumor cell treatment with cytotoxic agents increased susceptibility against Apo2L/TRAIL [33], we investigated whether treatment with CCNU increases sensitivity of primary glioma cells against Apo2L/TRAIL. As shown in Fig. 4D, treatment with CCNU did not sensitize these cells against apoptosis induced by

Apo2L/TRAIL. Furthermore, treatment with CCNU for 3 h resulted only in a weak induction of DR5 in Tu-132 cells (SFI of control-treated cells, 1.28; SFI of cells treated with CCNU 250 μ M, 2.46). Expression of DR5 was not increased in Tu-140 cells (not shown).

Discussion

Apo2L/TRAIL is a TNF family member considered a candidate for tumor therapy because of differential cytotoxicity for tumor as opposed to untransformed cells [1, 2]. Unfortunately, although some tumor cell lines are susceptible to Apo2L/TRAIL-induced apoptosis, a number of tumor cell lines are resistant and of these only a fraction is sensitized by cotreatment with RNA or protein synthesis inhibitors. In addition to the induction of apoptosis, Apo2L/TRAIL induces putative survival pathways. Therefore, we were interested in determining whether these signalling pathways are induced by Apo2L/TRAIL in glioma cells and contribute to their resistance. Importantly, we focused on the Apo2L.0 preparation that is not toxic for hepatocytes and therefore possibly relevant for clinical applications [6]. We find that Apo2L.0 induces the phosphorylation of ERK1/2, but not Akt, in glioma cells (Fig. 1). Among non-neoplastic cells, both ERK1/2 and Akt phosphorylation was observed in response to Apo2L/TRAIL in vascular smooth muscle cells [14] and synovial fibroblasts [15]. Further, Apo2L/TRAIL has been shown to induce I κ B kinase and NF- κ B activity in 293 cells, MCF-7 cells and fibroblasts [16, 35, 36]. Therefore, we analysed whether Apo2L.0 modulates the phosphorylation of I κ B α , which when phosphorylated allows the activation of NF- κ B. However, no such effect was seen in human glioma cells. Further, Apo2L.0 did not induce NF- κ B activity in a luciferase assay, and the p65 subunit of NF- κ B was not translocated to the nucleus in response to Apo2L.0 (not shown). His-tagged Apo2L/TRAIL in contrast led to consistent phosphorylation of I κ B α , but did also not translocate p65 in glioma cells [37]. Similarly, NF- κ B was not induced by Apo2L/TRAIL in vascular smooth muscle cells and synovial fibroblasts [14, 15]. Taken together, the activation of NF- κ B by Apo2L/TRAIL may depend on the ligand preparation, the cell type and culture conditions.

Because apoptosis induction by Apo2L.0 is caspase-dependent, we investigated whether the activation of ERK1/2 by Apo2L.0 depends on caspase activation, too. However, the phosphorylation of ERK1/2 was not prevented by ectopic expression of the caspase-1 and -8

inhibitor crm-A (Fig. 1C) or by the caspase inhibitor zVAD-fmk, suggesting that the activation of ERK1/2 by Apo2L.0 is independent of caspase activation. Similar, ERK1/2 phosphorylation by TNF was independent of caspase activation [38, 39].

Further, we investigated whether the inhibition of signalling pathways modulates Apo2L.0-induced apoptosis. Both the inhibition of PI3 kinase signalling and of MEK signalling slightly increased the sensitivity of some glioma cell lines to Apo2L.0. Similarly, inhibition of Akt has been shown to increase sensitivity of glioma cell lines to Apo2L/TRAIL [40]. Similar to the inhibition of protein synthesis, inhibition of NF- κ B and CK2 strongly increased sensitivity to Apo2L.0. The molecular mechanisms which mediate the sensitizing effect of inhibitors of protein synthesis, await elucidation. The correlations between the sensitizing effects of these inhibitors among a large panel of cell lines indicate these mechanisms to be similarly modulated by these inhibitors. Accordingly, it will be difficult to use distinct tumor-specific strategies to overcome resistance to Apo2L.0 in the clinic.

Among the primary glioma cell cultures, 3 of 5 primary cell cultures were resistant to Apo2L.0 even when treated with the inhibitors. Similarly, limited sensitivity to His-tagged Apo2L has been described in primary glioma cells [41], and resistance to Apo2L/TRAIL in primary glioma cell cultures has recently been shown using leucine zipper Apo2L/TRAIL [42]. Since low expression of caspase 8 has been shown to mediate the resistance to Apo2L/TRAIL in neuroblastoma cells [43] and since the expression of caspase 8 has been reported to be low in gliomas [42, 44], we examined the expression of caspase 8 in primary glioma cell cultures. Because caspase 8 was expressed in these cells, resistance in our cell cultures was probably not caused by reduced expression of caspase 8. Expression of DR5 in glioma cells has been shown previously [33, 45, 46]. The expression of DR5 was low in the 3 cell lines, which were resistant to Apo2L.0. Expression of DR5 sensitized Tu-140 cells for Apo2L.0-induced cell death, indicating that low expression of DR5 is a cause for resistance to Apo2L.0. DR5 has been shown to be the relevant receptor for induction of apoptosis by Apo2L/TRAIL, as analysed using receptor selective Apo2L-ligand proteins [47]. Similarly, a correlation between DR5 expression and susceptibility to Apo2L/TRAIL has been shown in glioma cells ([33];

Tab. 1). It is unclear why the expression of DR5 in primary glioma cells is lower than in long-term glioma cell lines. Further, there was only weak induction of DR5 by CCNU in Tu-132 cells, and consequently these cells were not made sensitive against Apo2L/TRAIL by CCNU (Fig. 4D).

Expression of DR5 is increased by p53 [48] and c-Myc [49]. As cell culture conditions might increase the activity of c-Myc, as shown in immortalized fibroblasts [50], expression of DR5 in glioma cell lines might be increased by such mechanisms. Because no significant effect of DR5 expression on cell death induction by Apo2L.0 was detectable in Tu-132 cells, other mechanisms of resistance to Apo2L.0 operate in primary glioma cells, too. Altogether these data indicate that the possible clinical effect of Apo2L.0 might be limited by the low expression of DR5 in glioma cells and that the sensitivity of glioma cells to Apo2L.0 might be overestimated by experiments with long-term cell lines. Moreover, the correlation analysis summarized in Table 1 suggests that multiple pathways of glioma cell sensitisation to Apo2L/TRAIL may converge on a common cellular target.

Abbreviations

Apo2L/TRAIL (Apo2 ligand/TNF-related apoptosis-inducing ligand); CD95L (CD95 ligand); CHX (cycloheximide); CK2 (casein kinase 2); DcR1 (decoy receptor 1); DcR2 (decoy receptor 2); DR4 (death receptor 4); DR5 (death receptor 5); Co. (control); EGFP (enhanced green fluorescent protein); EGFR (epidermal growth factor receptor); ERK (extracellular signal-related kinase); FADD (Fas-associated death domain protein); FCS (fetal calf serum); FLIP (FLICE-inhibitory protein); MEK1/2 (MAP kinase kinase/ERK kinase); NF- κ B (nuclear factor κ B); PI (propidium iodide); PI3 kinase (phosphatidylinositol 3-kinase); TNF (tumor necrosis factor); SFI (specific fluorescence index).

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