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REVIEW ARTICLE

CD95 Ligand: Lethal Weapon Against Malignant Glioma?

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CD95 (Fas/APO-1) and its ligand (CD95L) belong to a growing cytokine and cytokine receptor family that includes nerve growth factor (NGF) and tumor necrosis factor (TNF) and their corresponding receptors. CD95 expression increases during malignant progression from low-grade to anaplastic astrocytoma and is most prominent in perinecrotic areas of glioblastoma. There is, however, no evidence that CD95 expression in malignant gliomas is triggered by hypoxia or ischemia. Agonistic antibodies to CD95, or the natural ligand, CD95L, induce apoptosis in human malignant glioma cells *in vitro*. Glioma cell sensitivity to CD95-mediated apoptosis is regulated by CD95 expression at the cell surface and by the levels of intracellular apoptosis-regulatory proteins, including bcl-2 family members. Several cytotoxic drugs synergize with CD95L to kill glioma cells. For as yet unknown reasons, glioma cells may co-express CD95 and CD95L *in vitro* without undergoing suicide or fratricide. Yet, they kill T cells *via* CD95/CD95L interactions and are sensitive to exogenously added CD95L. Since CD95L is expressed in gliomas *in vivo*, too, forced induction of CD95 expression might promote therapeutic apoptosis in these tumors. That glioma cells differ from non-transformed T cells in their sensitivity to CD95 antibodies or recombinant ligand, may allow the development of selective CD95 agonists with high antitumor activity that spare normal brain tissue. A family of death ligand/receptor pairs related to CD95L/CD95, including APO2L (TRAIL) and its multiple receptors is beginning to emerge. Although several issues regarding glioma cell sensitivity to CD95L/CD95-mediated apoptosis await elucidation, CD95 is a promising target for the treatment of malignant glioma.

Introduction

CD95 (Fas/APO-1) is a member of a growing family of cytokine receptors that includes the receptors for TNF-α and NGF. The natural CD95 ligand (CD95L), a cytotoxic cytokine homologous to TNF, induces apoptotic cell death in susceptible target cells. Natural mouse mutants with defects in the CD95 gene (*lpr*) or CD95L gene (*gld*) develop lymphadenopathy and exhibit features of autoimmune disease, suggesting that the CD95/CD95L system plays a role in the peripheral deletion of expanded immune effector cell populations. In fact, besides the perforin pathway, CD95/CD95L interactions are the second principal pathway of T cell-mediated cytotoxicity (17).

Key steps in the CD95-dependent subcellular killing cascade have been delineated in recent years (Fig. 1) (13,26). The CD95L needs to trimerize for signal transduction and in turn promotes aggregation of the cytoplasmic death domains of CD95. This results in the recruitment of the Fas-associating protein with death domain (FADD) and FLICE (FADD-like ICE, interleukin 1-convertase, caspase 8) to the death-inducing signalling complex (DISC). Thus, activation of a caspase is a rather early event in CD95-mediated apoptosis. Caspase-3 (CPP32) appears to be subsequently activated in all cell types undergoing CD95-mediated apoptosis. The proximate cause of death after CD95 ligation is unknown. Although several potential substrates for caspase 3 have been identified, including poly-(ADP-ribose) polymerase (PARP), retinoblastoma protein, actin, lamin fodrin, and even the bcl-2 protein (3), it has not been clarified which specific substrates need to be cleaved for death to occur. DNA fragmentation is part of

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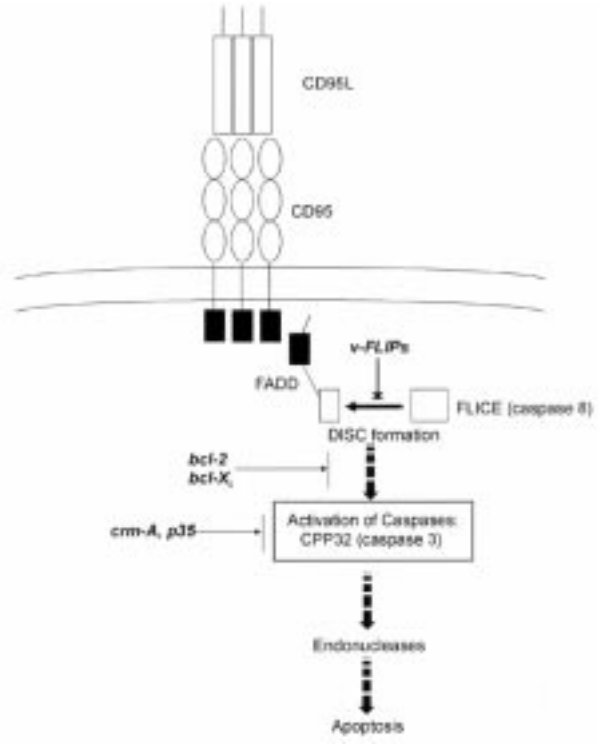

Figure 1. CD95-dependent signal transduction (for details, see text).

the terminal events in CD95-mediated apoptosis since inhibition of CAD, a novel caspase-activated DNase, prevents DNA fragmentation but not cell death (5,34). The point of commitment to death is therefore upstream of DNA fragmentation. The signalling cascade leading to cell death can be inhibited at the level of DISC formation by viral FLICE inhibitory proteins (FLIP), upstream of caspase 3 activation by bcl-2 or bcl-X, and at the level of caspase 3 activation by viral proteins such as crm-A or p35 (Fig. 1).

CD95: a novel therapeutic target in malignant glioma

We became interested in targeting CD95 in malignant glioma when we noticed that human malignant glioma cells were susceptible to CD95 antibody-induced apoptosis *in vitro* and that CD95 was not expressed in normal brain tissue except for endothelial cells (52). At least *in vitro*, endothelial cells resist CD95 antibody-induced apoptosis (28), suggesting that endothelial damage would not be a major side effect of CD95 targeting in the brain *in vivo*. Further, freshly isolated *ex vivo* glioma cells were also susceptible to CD95-mediated apoptosis (53). Like chemotherapy-induced apoptosis (56), CD95-mediated apoptosis of glioma cells is attenuated by dexamethasone (52). This may be of clinical relevance in light of the frequent use of corticosteroids for the control of cerebral edema in affected patients. Alternative drugs to control cerebral edema that lack the cytoprotective properties of steroids would be welcome. In that regard, we have obtained preliminary evidence that boswellic acids, phytotherapeutic inhibitors of the lipoxygenase pathway (33), show anti-edema activity in human glioma patients *in vivo* (Weller and Schabet, unpublished) but do not attenuate CD95L- or drug-induced apoptosis of glioma cells *in vitro* (Glaser et al. submitted).

Two problems became apparent early on. First, some glioma cell lines appeared to resist CD95 antibody-induced apoptosis because of little CD95 expression at the cell surface. Second, most glioma cell lines required inhibition of mRNA or protein synthesis to undergo apoptosis in response to CD95 antibodies, suggesting that glioma cells express cytoprotective proteins which interfere with the killing cascade. Glioma cells express these proteins either constitutively or are induced to do so when CD95 ligation is triggered. Therefore, enhancing CD95 expression at the cell surface and overcoming subcellular resistance became important issues for a possible therapeutic exploitation of the CD95/CD95L system in malignant glioma.

The role of CD95 expression at the cell surface

The first comparative study of various glioma cell lines for CD95 expression at the cell surface and susceptibility of CD95-mediated apoptosis indicated that there was a certain level of CD95 expression necessary to permit transduction of a CD95-dependent apoptotic signal (52). Further, pre-exposure of glioma cells to the cytokines TNF-α and interferon-γ (IFN-γ), which increase CD95 expression at the cell surface, enhanced their sensitivity to agonistic CD95 antibodies. Sensitizing effects of TNF-α to CD95-mediated apoptosis have been confirmed in direct TNF-α gene transfer studies (15). Recent work suggests that the sensitivity of human malignant glioma cells to CD95 antibodies can also be enhanced using CD95 antibody-loaded liposomes (23).

The next step was to assess whether gene transfer-mediated augmentation of CD95 expression in CD95-negative glioma cells would suffice to reconstitute susceptibility to the induction of apoptosis (55). Up to a certain level of ectopic CD95 expression, we observed a corresponding increase in sensitivity to CD95 antibody-induced apoptosis when different CD95-transfected clones derived from the same cell line were compared. Beyond that level, this correlation was no more

detectable, that is, factors other than merely ectopic CD95 expression determined sensitivity to apoptosis. We thus identified a critical threshold of CD95 expression that appeared to be required to transmit the pro-apoptotic signal. This threshold differed significantly among the cell lines, suggesting a complex regulation of CD95-dependent signal transduction. Interestingly, above that threshold of enforced CD95 expression, the level of CD95 expression did not correlate with susceptibility to apoptosis when different CD95-transfected clones from the same glioma cell line were compared. These data confirmed that subcellular factors acquire a major role in modulating apoptosis once the critical level of CD95 expression sufficient for signal transduction is passed. Collectively, these observations also clearly indicate that augmenting CD95 expression, by whatever approach, alone will not be sufficient to promote apoptosis in glioma cells but that the intracellular signalling cascade needs to be considered as well.

Synergy with chemotherapy

The potentiation of CD95 antibody-induced apoptosis by inhibitors of RNA or protein synthesis such as actinomycin D or cycloheximide (52) led us to screen commonly used cancer chemotherapy drugs, including adriamycin, BCNU, vincristin, etoposide, teniposide, 5-fluoruracil and cytarabin, for possible synergy with CD95L-induced apoptosis. While none of these drugs augmented CD95L-induced apoptosis to the same degree as inhibitors of RNA and protein synthesis in acute cytotoxicity assays, all drugs synergized with CD95L in inhibiting clonogenic glioma cell survival (30). These data suggested that systemic chemotherapy might be a useful adjunct to therapeutic CD95 targeting in malignant glioma. Topoisomerase I inhibitors were subsequently found to be rather exceptional in augmenting CD95-mediated apoptosis even in acute toxicity assays (58) but this effect was restricted to high concentrations of these agents and could be linked to drug-induced inhibition of RNA synthesis (Winter and Weller, submitted). Taxol was identified as another candidate drug for CD95L-based immunochemotherapy of malignant glioma (31). Here, inactivation of the endogenous bcl-2 protein by phosphorylation appeared to underlie the facilitation of CD95-mediated apoptosis, given that bcl-2 gene transfer had previously been shown to induce resistance to CD95-mediated apoptosis (54). This protection of glioma cells from apoptosis mediated by bcl-2 operates upstream of caspase 3 activation (Wagenknecht et al. submitted) (Fig. 1). The apparent resistance to apoptotic stimuli provided by pro-

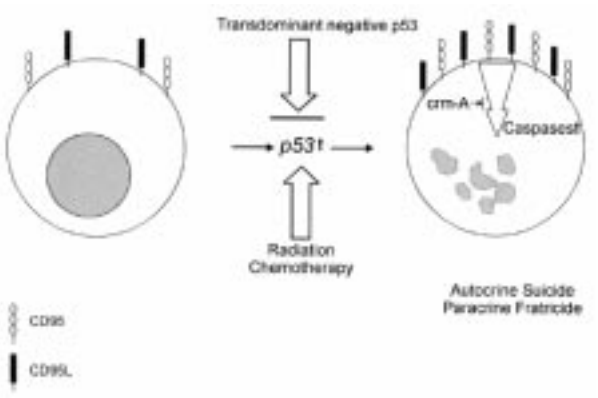

Figure 2. Drug-induced tumor cell apoptosis: a role for CD95L/CD95 interactions?
According to studies in nonglial cells, genotoxic stress induced by irradiation or chemotherapy results in enhanced expression of CD95 and CD95L at the cell surface, possibly in a p53-dependent pathway, triggers CD95/CD95L interaction-dependent caspase activation, and leads to autocrine suicide or paracrine fratricide. Cytotoxicity might be blocked by transdominant negative p53 at the level of induction of CD95/CD95L expression or by crm-A at the level of caspase activation (for details, see text).

teins such as bcl-2 has led some authors to try to bypass this cytoprotective mechanism of gliomas by activating a caspase directly by gene transfer methods (63). The feasibility of that approach needs to be confirmed in human patients. Given the central role of caspases in mediating neuronal cell death (e.g., 35,36), targeting caspases in the brain directly seems to carry a high risk of side effects.

The CD95/CD95L system does not mediate drug toxicity in human malignant glioma cells

Recent studies have proposed a role for the CD95/CD95L system in drug-induced tumor cell cytotoxicity. Thus, drug-induced apoptosis of lymphoma cells was linked to drug-induced CD95L expression and autocrine suicide or paracrine fratricide (7). Similarly, drug-induced increases of CD95 and CD95L expression were held responsible for drug-induced hepatoma cell death (16) (Fig. 2). This process appeared to be p53-dependent. We have not been able to delineate a similar role of CD95/CD95L interactions in the drug toxicity of glioma cells. Thus, gene transfer-induced changes of CD95 expression do not alter drug sensitivity of glioma cell lines (60). Further, expression of transdominant negative p53 in p53 wild-type LN-229 glioma cells abrogates drug-induced increases in CD95 expression but does not inhibit drug toxicity (60). Eventually,

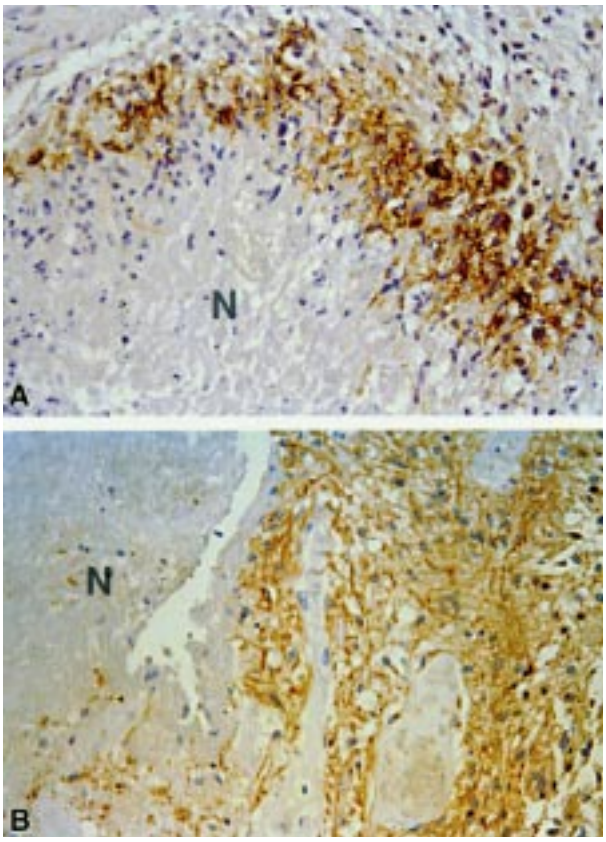

Figure 3. CD95 and CD95L expression in human malignant glioma.
Immunochemical detection was performed as described (9,41) and identified CD95 and CD95L expression by peroxidase staining (brown). Note that CD95 is expressed most abundantly in perinecrotic areas (A) whereas CD95L is expressed in a diffuse pattern (B) (x 162, N = necrosis).

	WHO grade	CD95	Ref.	CD95L	Ref.
Pilocytic astrocytoma	I	0/4, RT-PCR	40		
Low-grade astrocytoma	II	1/9, RT-PCR 3/3, RT-PCR 1/9, IHC 1/3, IHC	40 9 41 9	3/3, RT-PCR 1/3, IHC	9 9
"Benign" astrocytoma	I and II			4/4, IHC	32
Anaplastic astrocytoma	III	6/12, RT-PCR 2/11, IHC	40 41		
Glioblastoma	IV	9/9, RT-PCR 9/9, RT-PCR 13/15, IHC 7/9, IHC	40 9 41 9	9/9, RT-PCR 9/9, IHC	9 9
Malignant astrocytoma	III and IV			6/7, IHC	32
Primary (de novo) glioblastoma	IV	18/18, IHC	44	22/22, IHC	A*
Secondary glioblastoma	IV	4/19, IHC	44	6/6, IHC	A*

A*, Ohgaki et al. unpublished results, RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry.

Table 1. CD95 and CD95L expression in human astrocytomas in vivo.

forced expression of the viral caspase inhibitor, crm-A, blocks CD95L-mediated glioma cell death but does not modulate drug toxicity, excluding the role of CD95/CD95L interactions in the drug toxicity of glioma cells (Roller et al. submitted). Using a similar approach with crm-A, Villunger et al. (45) have recently questioned the data on the role of CD95/CD95L interactions in the drug toxicity of lymphoma cells, too.

CD95 expression in human malignant gliomas *in vivo*

Using immunohistochemistry of glioma sections and immunoblot analysis of fresh glioma tissue protein lysates, we were able to detect CD95 expression in malignant gliomas *in vivo* in a preliminary study (52). CD95 expression in normal brain is restricted to some endothelial cells. Systematic analyses of grade I-IV astrocytomas revealed that CD95 mRNA expression correlated with malignant progression of these tumors and that CD95 mRNA was detected in all glioblastomas (40) (Table 1). Large areas of ischemic necroses are observed in virtually all primary (*de novo*) glioblastomas but are significantly less frequent in secondary glioblastomas which develop through progression from low-grade or anaplastic astrocytoma. At the protein level, CD95 expression is predominantly observed in glioma cells surrounding large areas of necrosis (Fig. 3) (41,44) and thus significantly more frequent in primary (100%) than in secondary glioblastoma (21%). This suggests that these clinically and genetically defined subtypes of glioblastoma also differ in the extent and mechanism of necrogenesis and in their response to CD95/CD95L-based immunotherapy.

Although very low levels of CD95 expression can be sufficient to transmit a pro-apoptotic signal, provided an appropriate stimulus is presented, these data have questioned the feasibility of targeting the CD95 system in malignant glioma *in vivo*. To overcome low level CD95 expression as a mechanism underlying resistance to apoptosis, one might envisage various strategies, such as promoting CD95 expression by pretreatment with cytokines such as TNF-α or IFN-γ which can be administered to glioma patients without serious side effects (6,62) and strongly enhance CD95 expression in glioma cells *in vitro* (52). However, since glioma cells may coexpress CD95 and CD95L at the cell surface without undergoing apoptosis (see below), enhanced CD95

expression may be a precondition for an exogenous CD95 agonist to be active against glioma cells but will probably not promote apoptosis in the absence of externally triggered CD95 ligation. Further, it is unclear whether the proinflammatory cytokines that induce CD95 expression *in vitro* (52) will also do so in the microenvironment of the glioma *in vivo* that is characterized by the presence of multiple immunomodulatory and also potent immunosuppressive factors such as TGF-β or interleukin-10. In that regard, it is noteworthy that intracerebral injections of IFN-γ have been shown to induce MHC antigen expression in the brain several years ago (61). However, intratumoral injections of IFN-γ in the rat 9L gliosarcoma model induced MHC class I and II expression in nonneoplastic cells such as mononuclear phagocytes and endothelial or ependymal cells but not in the tumor cells (59). These observations raise the possibility that exogenous cytokines might enhance CD95 expression in untransformed brain cells more efficiently than in glioma cells *in vivo*, an undesirable effect from a therapeutic point of view.

In contrast to vascular endothelial growth factor (VEGF), CD95 expression does not appear to be induced by hypoxia (44). Although there is evidence that the presence of wild type p53 is necessary for expression of CD95 (24,42), analysis of CD95 mRNA levels in a glioblastoma cell line containing a *p53* mutation and an inducible wild-type *p53* gene showed little difference under induced and non-induced conditions, suggesting that up-regulation of CD95 expression is not directly linked to the *p53* gene status in glioblastoma (44). Yet, ectopic expression of the murine temperature-sensitive p53 val^{135} mutant enhanced CD95 expression uniformly when induced to assume wild-type conformation (49, Pohl et al. submitted).

In vitro studies predict that the levels of antiapoptotic bcl-2 family proteins will play an important role in determining the response of malignant gliomas to CD95 targeting (54). Interestingly, the correlation of bcl-2 family protein expression with the progression of astrocytomas is not as clear as for CD95 (Table 1). While we noted higher bcl-2 expression in anaplastic astrocytomas and glioblastomas compared with grade I and II astrocytomas (54), other authors reported the opposite in that low-grade gliomas were more likely to express bcl-2 than high-grade gliomas (4,12,18) or no relation to the malignancy grade (19). Further, bcl-2 expression may or may not be associated with p53 wild-type status (1,19). Since human malignant gliomas do not only express bcl-2 but also other antiapoptotic and proapoptotic members of the bcl-2 protein family (11,29), the net effect of bcl-2 family protein expression on CD95L-induced apoptosis in a specific tumor will be difficult to predict.

Co-expression of CD95 and CD95L in malignant gliomas

Among the rationales of activating CD95, a natural target of immune effector cells, was the state of immunosuppression observed in malignant glioma patients that might interfere with strategies of active cellular immunotherapy (50). We viewed transforming growth factor-β (TGF-β) as a principal soluble mediator of T cell suppression and had hypothesized that glioma cells induced TGF-β-mediated apoptosis of invading immune cells (51). However, more recently, we and others (9,32,57) have shown that human malignant glioma cells express not only CD95 but also CD95L on their cell surface and that CD95L expressed on the surface of glioma cell lines is functionally active in that it kills sensitive target cells of the immune system. Interestingly, similar data on CD95L expression and immune surveillance have been published for various other nonglial tumors, including carcinoma of colon, lung and liver, melanoma and plasmacytoma (10,20,21,39,46,48). Unexpectedly, however, ectopic expression of CD95L on various tumor cell lines induced tumor regression *in vivo*, an effect that was attributed to neutrophil activation and CD8 T cell-mediated immunity (37).

Why glioma cells co-expressing CD95 and CD95L fail to undergo suicide or fratricide, is at present unknown (Fig. 4A). Topographic restriction of CD95 and CD95L expression at the cell surface may prevent suicide, but not fratricide. In most glioma cell lines, CD95-mediated apoptosis is greatly facilitated when RNA or protein synthesis are blocked (52), suggesting the expression of labile, anti-apoptotic proteins in the glioma cells which inhibit both suicide and fratricide. Yet, the cell line most sensitive to CD95L, LN-18, does not seem to commit suicide or fratricide even when grown to tight confluency (Weller, unpublished observation). Future studies will have to examine the possible modulation of suicide, fratricide, and bystander cell death by shedding of the CD95L (43).

Since CD95L appears to be uniformly expressed throughout the tumor tissue in malignant gliomas *in vivo* (9) (Fig. 3), induced expression of CD95 might suffice to promote glioma cell suicide or fratricide. However, this idea does not gain support from studies which show that agents known to promote CD95 expression in malignant glioma cells *in vitro*, e.g. cytokines such as IFN-γ or TNF-α (52) or cytotoxic drugs such as VM26

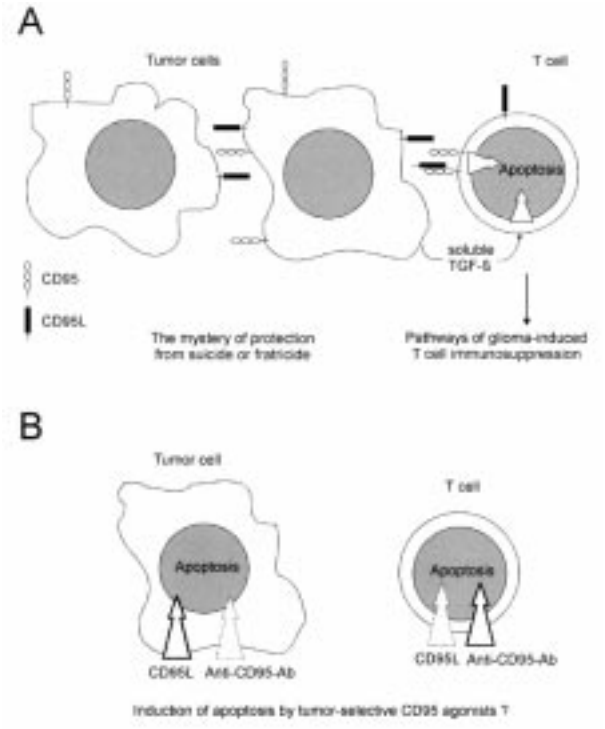

Figure 4. Coexpression of CD95L and CD95 in glioma cells: implications for immune surveillance and immunotherapy. **A.** Human glioma cells coexpress CD95 and CD95L and kill T cells in a CD95/CD95L-dependent manner (right) but fail to undergo suicide or fratricide (left) (57). **B.** Soluble murine CD95L kills glioma cells much better than untransformed T cells whereas the opposite is true for the CH-11 CD95 antibody (65).

or BCNU (60, Pohl and Weller, unpublished), are not very active against malignant glioma *in vivo*.

Soluble CD95 and CD95L: implications for CD95 targeting *in vivo*

Similar to the TNF receptor, soluble CD95 has been detected in biological fluids and has been suggested to inhibit CD95-mediated apoptosis (2). Soluble CD95 may modulate the immune response in human cancers (14) but has been examined more extensively in autoimmune disorders such as systemic lupus erythematosus (2) and multiple sclerosis. In the latter, there is some correlation between disease activity and levels of serum CD95 (66), and the levels of CD95 detected in multiple sclerosis sera are biologically active in that they interfere with CD95L-induced apoptosis of susceptible target cells (67). In contrast, we detected only low levels of the CD95 mRNA that encodes the transmembrane domain-lacking CD95 ΔTM variant and no soluble CD95 protein released into the cell culture medium in several human malignant glioma cell lines (55). Low levels of CD95 ΔTM mRNA have also been detected in human malignant gliomas *in vivo* (9,40). Taken together, these data suggest that soluble CD95 would not significantly interfere with CD95 targeting in malignant glioma.

Toxicity and Targeting

The general interest in CD95-mediated apoptosis as a novel approach to systemic, notably hematological and lymphoid, neoplasias decreased when Ogasawara et al. (22) reported that agonistic CD95 antibodies induced fatal liver toxicity in mice within a few hours. Yet, intraperitoneally administered recombinant CD95L prolonged survival in mice with intraperitoneal lymphoma without major toxicity (27). Further, local intra-articular injection of CD95 antibodies has been successfully used for disease control in a transgenic mouse model of arthritis (8). Similar results have been obtained in collagen-induced experimental arthritis using an adenoviral vector encoding CD95L (64). The development of CD95 agonists that specifically target neoplastic as opposed to untransformed cells would seem to be another way to prepare for a possible therapeutic strategy for human malignant glioma. In this regard, we were able to show inverse properties to kill either untransformed antigen-specific T cells or human malignant glioma cells of CD95 antibodies on the one hand and soluble murine CD95L on the other (65). Although the molecular basis of this differential sensitivity of T cells and glioma cells to different triggers of CD95 ligation is poorly understood, these observations give rise to the hope that tumor-selective induction of CD95-dependent apoptosis might be achieved by designing specific agonists for tumor cell targeting (Fig. 4B). Of note, efficacy of CD95 targeting in a glioma model in experimental animals has yet to be demonstrated. In our hands, the major obstacle has been to obtain purified *bioactive* CD95L in sufficient quantities for such a study (unpublished). Current efforts focus on an adenoviral CD95L-encoding vector. Potential systemic toxicity from locoregionary therapy could probably be prevented by systemic application of CD95L scavengers, e.g., soluble CD95 or soluble CD95 fragments.

The emerging family of death ligands and their receptors

Recent evidence suggests that there is a family of death ligands and receptors homologous to CD95L and CD95. Apo-2L (TRAIL) is a novel member of that family with interacts with various receptors, some of which

induce whereas others block apoptosis (25,38). Studies to delineate a possible suitability of the Apo2L (TRAIL) system for glioma cell targeting are currently in progress. In conclusion, the CD95/CD95L system and related pairs of ligands and receptors efficiently transmit an apoptotic signal that is unaffected by the molecular alterations that confer resistance of glioma cells to multiple other proapoptotic stimuli. However, significant improvements in targeting the apoptotic signal specifically to tumor cells in the brain are needed before the first clinical trials can be undertaken.

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