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Review

Chemotherapy and immunotherapy of malignant glioma: molecular mechanisms and clinical perspectives

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Abstract. Despite the considerable progress in modern tumor therapy, the prognosis for patients with glioblastoma, the most frequent malignant brain tumor, has not been substantially improved. Although cytoreductive surgery and radiotherapy are the mainstays of treatment for malignant glioma at present, novel cytotoxic drugs and immunotherapeutic approaches hold great promise as effective weapons against these malignancies. Thus, great efforts are being made to enhance antitumoral efficacy by combining various cytotoxic agents, by novel routes of drug administration, or by combining anticancer drugs and immune modulators. Immunotherapeutic approaches include cytotoxic cytokines, targeted antibodies, and vaccination strategies. However, the success of most of these experimental therapies is prevented by the marked molecular resistance of glioma cells to diverse cytotoxic agents or by glioma-associated immunosuppression. One promising experimental strategy to target glioma is the employment of death ligands such as CD95 (Fas/Apo1) ligand or Apo2 ligand (TRAIL). Specific proapoptotic approaches may overcome many of the obvious obstacles to a satisfactory management of malignant brain tumors.

Key words. Immunotherapy; chemotherapy; malignant glioma; CD95 (Fas/Apo1); Apo2L (TRAIL); apoptosis.

Current treatments for malignant glioma

Introduction

The treatment of malignant glioma belongs to one of the more depressing fields of modern medicine. While great advances have been made in other fields of oncology, neurooncologists can offer their patients only marginal gain in quantity and quality of life. After diagnosis, the median survival time of patients with malignant glioma is less than 6 months with surgery alone. Radiotherapy can increase this period by only several months, and additional chemotherapy does not lead to a substantial improvement of this dismal prognosis. Glioblastomas are the most malignant of the astrocytic-lineage tumors. They arise either from less anaplastic astrocytoma by malignant transformation or without apparant antecedent, that is, de novo. Pathophysiologic and clinical properties of gliomas include infiltrative growth, induction of vasogenic edema, local compression of brain tissue due to elevated intracranial pressure, obstruction of cerebrospinal fluid flow, venous occlusion and hemorrhage (fig. 1). Damaged brain tissue often causes focal seizures by irritation or neurologic deficits by depression of central nervous system (CNS) functions. Importantly, malignant gliomas do not usually metastasize outside the brain. This outstanding feature may have crucial implications for some novel therapeutic strategies. From an immunologic point of view, this finding indicates that the immune system of glioma patients may fail only in the CNS,

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whereas the efficient immune-mediated killing of metastasizing glioma cells in the periphery may still take place. The peculiarity of the intracerebral immunologic environment requires a specifically tailored therapeutic approach to this neoplastic disorder. Thus, the future development of successful therapies will depend on understanding the pathophysiologic and molecular features of glioma arising in an immunologically separated compartment. More insight into this complex process of tumorigenesis will enable us to identify possible targets of therapeutic attack and perhaps to develop strategies to intervene with the natural course of these malignancies.

The first part of this review outlines the established therapy for malignant glioma and provides a comprehensive survey of innovative chemo- and immunotherapeutic strategies. Since chemotherapies and immunotherapies fail, at least in part, for the same reasons, the second section discusses the most essential impediments to successful glioma treatment. In the third part of the review, a recently discovered group of cytotoxic cytokines, the death ligands, are described as promising candidates for an experimental glioma therapy which may overcome many of the pathophysiologically relevant mechanisms of resistance.

Surgery

At present, the management of malignant glioma is based on a multimodality treatment. First, most of the tumor tissue is commonly reduced by surgical resection
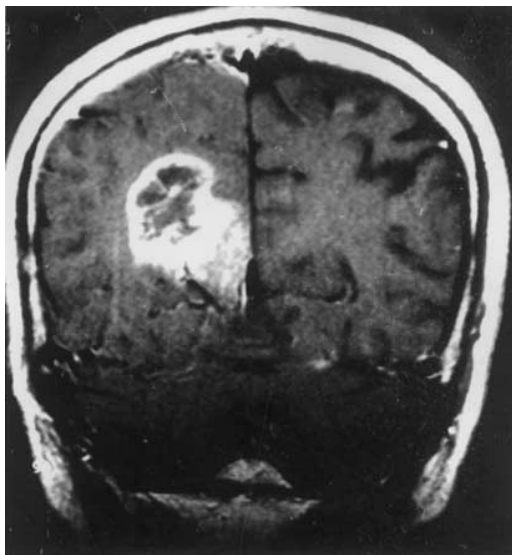

Figure 1. T1-weighted, gadolinium-enhanced magnetic resonance tomograph of a grade IV malignant glioma, demonstrating the invasive growth pattern, brain edema, and central necrosis.

in order to diminish the tumor burden and to prevent vital complications due to compression and elevated intracranial pressure. Moreover, cytoreduction is thought to improve the efficacy of the subsequent therapeutic interventions. However, gliomas often grow in neurologically important sites, thereby precluding a macroscopically complete resection. Another key problem is the marked propensity for infiltrative growth patterns such that individual cells migrate deeply into otherwise healthy CNS tissue. These cells are not macroscopically visible and can affect a considerable part of the surrounding brain. Thus, the neurosurgical resection of gliomas is virtually never total. Of note, there is no precise evidence from a controlled prospective trial that the extent of surgery has any influence on the prognosis of patients with gliomas [1].

Radiotherapy

Surgical resection is commonly followed by radiation therapy, which currently must be considered as the single most effective treatment for malignant glioma. Radiotherapy is commonly carried out as involved-field irradiation with a cumulative dose of 54–60 Gy. The most important impediment to radiotherapy is the intrinsic resistance of many gliomas to irradiation-induced cytotoxicity. Moreover, because the tolerance of brain tissue is limited to 50–60 Gy, irradiation can neither be dose escalated nor repeated. Glioma patients with long remissions often suffer from late side effects of radiotherapy, diffuse leukoencephalopathy being the most feared complication that may appear several months or years after completion of radiotherapy.

Chemotherapy

As with radiotherapy, toxicity is also a major problem of chemotherapy for malignant glioma, limiting the antitumoral efficacy and therapeutic success of cytotoxic drugs. Besides local side effects such as necrotizing leukoencephalopathy after radiochemotherapy, the most important complications include myelosuppression, e.g., caused by nitrosoureas, or peripheral neurotoxicity, especially due to vinca alkaloids. A further mainstay of the clinical managemant of brain tumors is the treatment of tumor-associated edema with steroids. The application of steroids like dexamethasone is also one of the most effective measures in the palliative treatment of glioma. However, steroid medications may interfere with the efficacy of chemo- or immunotherapy for glioma [2, 3].

Mono- versus polychemotherapy. If chemotherapy is initiated, several courses of the respective protocol are usually carried out during or after radiotherapy. Standard protocols include monotherapy with nitrosoureas,

mostly 1,3-bis-(chloroethyl)-1-nitrosourea (BCNU) or the PCV protocol [procarbazine, N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU), vincristine], which has proven highly active against anaplastic oligodendroglioma [4, 5], though much less so against malignant astrocytic tumors [6–8]. The impact of these therapies on the natural course of glioblastoma multiforme is rather limited. At present, response rates to chemotherapy range between 20% and 30% and median survival is prolonged for 2–3 months when chemotherapy is given after surgery and radiotherapy [9, 10]. Major prognostic factors for overall survival include age, general physical condition (assessed by the Karnofsky index), and histologic grade. It has been suggested that the benefit from adjuvant chemotherapy depends on these prognostic factors, that is, only young patients with good general condition and grade III astrocytoma will benefit substantially from chemotherapy, but this remains to be demonstrated in a prospective trial. Moreover, in this subgroup of patients, the percentage of long-term survivors might be increased by adjuvant chemotherapy [9, 11].

To improve the dismal prognosis for malignant glioma patients, great efforts have been made during the last decade to develop more powerful therapies. One of these approaches is the design of combination chemotherapies that include two or more cytotoxic drugs. Most of the polychemotherapies have been performed after cytoreductive surgery and radiotherapy with established anticancer drugs which have proven to be effective in other cancers, e.g., cisplatin, etoposide, cytarabine, or vincristine. Table 1 provides a survey of phase I and II trials on polychemotherapies for malignant glioma or anaplastic astrocytoma of adults since 1992. Monotherapies with substances other than nitrosoureas are also listed. Not considered are trials in pediatric oncology, local or intraarterial chemotherapies and high dose chemotherapies with stem cell transplantation. To date, none of the polychemotherapies has been shown to be superior to BCNU monotherapy or PCV polychemotherapy.

New cytotoxic drugs. Since an important cause for the failure of common anticancer drugs to eliminate glioma cells may be cell resistance to various mechanisms of drug action, the search for novel substances with alternative mechanisms of action appears mandatory. Some promising new drugs tested for their ability to induce glioma cell death are listed in table 2. Several of these substances are still in preclinical evaluation, others have been tested in clincial trials. Future investigations will have to prove their efficacy and safety in human trials. In our opinion, temozolomide may at present be one of the most promising candidate drugs. It is an alkylating agent that penetrates the blood-brain barrier well and is suitable for oral administration. Recently, in a worldwide multicentric phase II study enrolling 225 patients with glioblastoma multiforme, temozolomide was found to be superior to procarbazine when progression-free survival after 6 months and overall survival were assessed [70].

Novel routes of administration. Because insufficient drug delivery to intracerebral tumors is regarded as a serious impediment for successful chemotherapy, some innovative strategies are based on alternative routes of drug administration. To ensure efficacious drug concentrations in the tumor tissue, various antitumor substances have been administered directly into cerebral arteries, with the hope that this would be a more potent drug therapy through increased drug delivery to the tumor. However, the first studies had to be stopped due to severe local toxicity [75]. Later studies of intraarterial chemotherapy were less toxic [e.g., 76] but whether this mode of administration is superior to the common intravenous chemotherapy remains to be clarified. Another route of administration is the direct application of antitumor drugs into the CNS. Intrathecal administration is limited by drug neurotoxicity to a few agents such as methotrexate and cytarabine. Moreover, the intrathecal approach will probably not be suitable to deliver drugs to the bulk tumor tissue at relevant concentrations. More promising may be strategies based on the deposition of the cytotoxic drug in the form of biodegradable polymers into the surgical cavity after resection [77]. It has been shown that BCNU, administered via this route, is released at the tumor site constantly over a long period of time [78]. Other modes of direct application of drugs into the CNS include intratumoral injections and the use of reservoirs, pumps, or catheters. Finally, the response of malignant glioma to chemotherapy may be increased by a modified time schedule of the multimodality treatment. Neoadjuvant schedules, that is, chemotherapy before radiotherapy, may be more efficient and less toxic [28, 79, 80].

Taken together, considerable efforts have been made to improve the poor prognosis for patients with malignant glioma: mono- and polychemotherapies with common anticancer drugs, therapies based on cytotoxic agents with novel mechanisms of actions, and various innovative strategies aimed at transporting the drugs directly into the brain tissue. None of these new approaches has changed the overall outcome for patients with malignant glioma. Therefore, immunotherapy has remained a promising alternative for several years now, even though all clinical trials performed so far have been disappointing.

Table 1. Mono- and polychemotherapy trials in malignant glioma.

	AZQ (Aziridinylben-zoquinone)	Carbo-platin	Car-mustine	Cis-platin	Cyclophos-phamide	Cy-tarabine	Dacar-bazine	Etoposide	Fluoro-uracil	Folinic acid	Hydroxy-urea	Ifosfa-mide	Lo-mustine	Mercapto-purine	Mitomy-cin C	Nimustine	PCNU	Procar-bazine	Taxol	Teniposide	Thio-guanine	Thiotepa	Vin-cristine
Ameri et al. 1993 [12]																		X				X	X
Ameri et al. 1997 [13]		X						X															
Balmaceda et al. 1997 [14]								X															
Boiardi et al. 1992 [15]		X	X	X				X															
Boiardi et al. 1992 [16]				X		X	X				X		X					X					X
Brandes et al. 1993 [17]		X																		X			
Chamberlain et al. 1995 [18]				X																			
Chamberlain and Kormanik 195 [19]																			X				
Cvitkovic et al. 1993 [20]									X		X												
Dinapoli et al. 1993 [21]																	X						
Eyre et al. 1993 [22]													X										
Fetell et al. 1997 [23]																			X				
Forsyth et al. 1996 [24]																			X				
Fulton et al. 1996 [25]								X															
Galanis et al. 1998[26]					X													X					X
Grossman et al. 1997 [27]			X	X																			
Gruber et al. 1998 [28]		X																					
Halperin et al. 1993 [29]	X																						
Halperin et al. 2996 [30]			X											X	X								
Hellman et al. 1998 [31]								X										X					X
Hildebrand et al. 1998 [32]			X															X					
Ikeda et al. 1996 [33]							X									X							X

Table 1. Continued.

	AZQ (Aziridinylben-zoquinone)	Carbo-platin	Car-mustine	Cis-platin	Cyclophos-phamide	Cy-tarabine	Dacar-bazine	Etoposide	Fluoro-uracil	Folinic acid	Hydroxy-urea	Ifosfa-mide	Lo-mustine	Mercapto-purine	Mitomy-cin C	Nimustine	PCNU	Procar-bazine	Taxol	Teniposide	Thio-guanine	Thiotepa	Vin-cristine
Jeremic et al. 1992 [34]													X							X			
Jeremic et al. 1992 [34]													X					X					X
Jeremic et al. 1994 [35]			X	X														X					X
Jeremic et al. 1992 [6]		X						X															
Kiu et al. 1995 [36]			X	X																			
Kyritsis et al. 1996 [37]									X				X					X			X		
Lederman et al. 1998 [38]																			X				
Levin et al. 1992 [39]									X		X		X					X			X		
Levin et al. 1995 [8]													X					X					X
Levin et al. 1995 [40]		X																					X
Lunardi et al. 1996 [41]		X																					
Malkin et al. 1994 [42]																	X						
Malkin et al. 1994 [42]	X																						
Newton et al. 1993 [43]																		X					
Prados et al. 1996 [44]																			X				
Prados et al. 1998 [45]			X								X										X		
Rostomily et al. 1994 [46]									X		X		X					X			X		
Sanson et al. 1996 [47]		X						X				X											
Schold et al. 1993 [48]	X																						
Shinoda et al. 1997 [49]				X																			
Spence et al. 1992 [50]				X																			
Stewart et al. 1995 [51]									X	X													
Stewart et al. 1997 [52]				X											X								
Yung et al. 1992 [53]			X	X																			

Table 2. Experimental drugs for the treatment of malignant gliomas.

Name of drug	References	
	preclinical	clinical
Amonafide		[54]
Bromodeoxyuridine		[55]
Crisnatol mesylate		[56]
Dibromodulcitol		[57]
Eflornithine		[58]
Estramustine		[59]
Fotemustine		[60]
Gemcitabine	[61]	
Idarubicin	[62]	
Menogaril		[63]
Merbarone		[64]
Phenylacetate		[65]
Polyinosinic-polycytidylic acid		[66]
Retinoic acid		[67]
Tamoxifen		[68, 69]
Temozolomide		[70, 71]
Topotecan		[72]
Tresulfan	[73]	
Vinorelbine	[74]	

Immunotherapy

There are several reasons why malignant glioma could be a suitable target for immunotherapeutic approaches. First, the localized, non-metastasizing growth of glioma in the immunologically separated environment of the CNS may be a good precondition for local immunotherapy. For example, it appears feasible to administer activated immune effector cells locally without causing a severe systemic immune reaction which could damage

Table 3. Categories of immunotherapy for malignant glioma.

Antibody-targeted immunotherapy
  • Monoclonal/polyclonal
  • Conjugates (isotopes, toxins, cytotoxic drugs)

Cytokine-based immunotherapy
  • Interferons
  • Interleukins
  • Antagonism of transforming growth factor-β (TGF-β)

Immunotherapy based on death ligand/death receptor interactions
  • CD95L-induced cytotoxicity
  • Apo2L-induced cytotoxicity

Cellular immunotherapy
  • Natural killer cells
  • Lymphokine-activated killer cells
  • Tumor-infiltrating lymphocytes
  • Antigen-specific lymphocytes

Vaccination-based immunotherapy
  • Autologous tumor-cell immunization

Immunogene therapy
  • Inhibition of immunosuppressive factors, e.g., TGF-β by antisense TGF-β or decorin
  • Gene transfer of interferons, interleukins or tumor necrosis factor-α
  • Gene transfer of death receptors or death ligands

peripheral organs. Second, a typical feature of malignant glioma is the local as well as systemic immunosuppression induced by several immunodepressive mechanisms. Thus, glioma patients may exhibit lymphopenia, impaired antibody production, cutaneous anergy, reduced lymphocyte protein synthesis, and diminished lymphocyte responsiveness. Therefore, a therapeutic approach counteracting glioma-induced immunosuppression could be an effective tool to restore the physiological ability of cytotoxic immune cells to attack malignant cells. Table 3 provides a survey of the most promising immunotherapies thought to be suitable for malignant glioma.

Antibody-targeted immunotherapy. A more direct approach to kill glioma cells is employed by antibody-based immunotherapy [81]. Various toxins or radioactive isotopes can be specifically targeted to glioma cells by conjugation to antibodies. However, this strategy depends on the recognition of specific antigens on tumor cells by the antibodies. As with vaccination therapies, the main obstacle to antibody-targeted immunotherapy is the lack of identified specific tumor antigens on human malignant glioma cells. Nevertheless, antibody conjugates have been used to treat glioma by employing antigens which are not exclusively but preferentially expressed on malignant brain tumors, including transferrin- or epidermal growth factor (EGF)-receptor-specific antibodies [82, 83]. An even more refined strategy is to create bispecific antibodies recognizing both a tumor epitope and antigens on cytotoxic T cells, thereby mediating a deadly cell contact resulting in the cytolysis of the tumor cell [84, 85].

Cytokine-based immunotherapy. The designation 'tumor necrosis factor' discloses that great expectations were once placed on the direct tumoricidal effects of cytokines on tumor cells. However, it soon became clear that the prevailing actions of cytokines consist in the complex modulation of immunologic mechanisms rather than in direct induction of target cell death. Provided that these immune modifiers are prudently employed for therapeutic purposes, they could turn out to be powerful tools to fight various neoplastic disorders.

1) *Interleukin-2 (IL-2).* This proinflammatory cytokine has mainly been used in combination with lymphokine-activated killer (LAK) cells because of its immunoactivating properties [86]. The potential use of IL-2 as a single agent to treat glioma is complicated by two problems. On the one hand, local application of IL-2 into the CNS results in strong inflammatory reactions at the injection site, impeding a possible therapeutic administration [87, 88]. On the other hand, glioma-infiltrating T cells frequently appear to express low levels of IL-2 receptor or a defective form of the receptor [89]. However, it has also been demonstrated that gene transfer for IL-2 into malignant tumor cells can lead to an enhanced specific immune response and the prevention or even regression

of tumors [90]. Unfortunately, this approach was not successful with malignant glioma [91–93]. IL-2 could nevertheless gain further attention because of its ability to partially overcome transforming growth factor (TGF)-β-induced immunosuppression associated with malignant glioma [94, 95].

2) *Interferons (IFNs)*. IFN-γ, produced by T cells, is of crucial importance for the interaction of T cells with antigen-presenting cells such as macrophages. IFN-γ enhances the expression of MHC class I and II antigens on immune cells, thereby enabling a more efficient antigen recognition by CD4 + and CD8 + T cells [96]. Further improvement of antigen recognition could be achieved by the IFN-γ-induced upregulation of MHC I molecules on glioma cells. This mechanism may antagonize the TGF-β-mediated downregulation of MHC expression [97, 98]. The importance of these mechanisms in the immunologic environment of the CNS has not been elucidated [99]. IFN-γ exerts growth inhibitory effects on glioma cells and the inhibition of proliferation correlates with elevated levels of the cyclin-dependent kinase inhibitor p21 (waf1/cip1) [100]. There are several clinical studies on the effects of IFN-γ on patients with malignant glioma. IFN-γ, which is clinically well tolerated, has been administered locally-intratumorally [101, 102] as well as systemically [102–104]. Moreover, IFN-γ potentiates LAK-induced glioma cell killing [105–107]. However, none of the clinical trials with IFN-γ for glioma patients was clearly successful. On the other hand, that IFN-γ induces the expression of the proapoptotic receptor CD95 [108] and that IFN-α sensitizes glioma cells to the cytotoxic actions of the CD95 ligand [109] could become meaningful for a death receptor/ligand-based immunotherapy of malignant glioma (see below).

3) *Tumor necrosis factor (TNF)-α*. The pleiotropic cytokine TNF-α induces the death of only a few tumor cell types, whereas its main function appears to be the regulation of a broad range of immune reactions. TNF-α stimulates the growth of T cells, enhances the cytotoxicity of monocytes, granulocytes and natural killer (NK) cells, induces the secretion of other cytokines such as interleukin (IL)-1, IL-6, colony-stimulating factor (CSF) or platelet-derived growth factor (PDGF) by immune cells in vitro, induces the expression of adhesion molecules such as ICAM-1 and enhances the expression of IL-1β. Moreover, MHC class I and II molecules on immune cells are upregulated by TNF-α [96]. However, cultured glioma cells are usually resistant to TNF-α [108, 110], and some authors even report growth-stimulating effects on glioma cells by TNF-α [111]. Not surprisingly, clinical trials studying the therapeutic efficacy of TNF-α on glioma have been rather disappointing [112]. Other studies tried to achieve an enhancing effect on the activity of LAK cells by TNF-α gene transfer [113]. Regarding a death-receptor-based immunotherapy, it may be impor-

tant to take into consideration TNF-α-induced expression of CD95 on glioma cells [108].

4) *IL-4*. Although IL-4 is not cytotoxic to glioma cells in vitro [108, 114], it has been employed against malignant glioma cells because it is able to induce a strong local immune response if administered intracerebrally [115, 116]. In the study of Yu et al. [116], glioma growth could be retarded after simultaneous intracranial application of glioma cells and IL-4-transfected plasmocytoma cells. Interestingly, in this mouse model, a T-cell-independent prominent activation of eosinophils occurred which was presumably responsible for the antiglioma effect. To our knowledge, no clinical trials on IL-4 administration to glioma patients have been published.

Death ligands. Death ligands are cytotoxic cytokines that induce apoptotic cell death of target cells after they have bound to their cognate receptors. Besides TNF-α, important members of this protein family include CD95 ligand and Apo2 ligand. Many malignant tumor cell lines are sensitive to these ligands. Great hope is placed in the ability of death ligands to induce specifically apoptotic cell death of tumor cells without damaging healthy tissue. This promising approach is discussed in detail below.

Cellular immunotherapy. The killing of tumor cells belongs to the genuine functions of immune effector cells. These cells control virtually all regions of the organism, circulating from blood vessels to peripheral tissues and back. Despite the immune privilege of the brain (see below), lymphocytes also circulate into the brain and from the brain back into the peripheral blood. Thus, there are various ways to employ immune cells as a tool against glioma cells.

1) *NK cells*. NK cells are able to kill their target cells in a MHC-independent manner. They recognize foreign antigens on cell surfaces and initiate the death of the respective target cell by perforin- or CD95-mediated death signalling. However, it has been shown that glioma cells are rather resistant to NK cells in vitro [117]. On the other hand, glioma cells can be sensitized to NK cell cytotoxicity by treatment with TNF-α [105]. To our knowledge, no clinical trial using NK cells for the treatment of glioma patients has been published.

2) *LAK cells*. LAK cells are cytotoxic effector cells that presumably stem from peripheral mononuclear cells. They are purified from the peripheral blood of patients and cultured for several days in IL-2-containing medium. Following IL-2-induced activation, they are readministered intravenously. For some time the patient repeatedly receives IL-2 to maintain the activated state of the LAK cells. Presumably, the LAK cells can also kill their target cells MHC independently. Interestingly, synergistic effects of LAK cells and anticancer drugs have been reported [118]. Many clinical studies using LAK cells to treat patients with malignant glioma have been published [88, 119–126]. Their results have not been very encouraging.

3) *Tumor-infiltrating lymphocytes (TILs)*. TILs are isolated from surgically removed tumor tissue and cultured in vitro in the presence of IL-2. Subsequently, the cells, which are supposed to include cytotoxic T cells, are reinfused into the patient. In theory, these cells should be specific for glioma antigens because of a clonal expansion of antigen-specific immune cells. However, it is obvious that the putative antigen-specific lymphocytes may constitute only a part of all tumor-infiltrating immune cells. This could turn out to be a major obstacle to the desired employment of antigen-specific lymphocytes. Moreover, to date, the feasibility of this approach has only been shown in animal models [127].

4) *Tumor-specific cytotoxic T cells*. These would be T cells recognizing a specific tumor antigen which is not expressed on normal cells. Such T cells could be stimulated either with autologous tumor cells or with purified tumor antigen in vitro. In contrast to malignant melanoma, to date no glioma-specific antigen has been identified. In principle, tumor-specific T cells could be generated not only in vitro, but also directly in the organism. These approaches would employ a common vaccination strategy. Of note, tumor-specific T cells have not yet assumed clinical significance for any neoplasm.

Vaccination strategies. Vaccination strategies differ from other immunotherapies in that they do not aim at strengthening the immune response directly. Instead, they are targeted to support the recognition of tumor antigens and thereby elicit a more effective immune attack [128]. In general, vaccination strategies are most suitable for neoplastic disorders where tumor-specific antigens are already identified, e.g. for malignant melanoma. The vaccination itself is then carried out with antigen peptides, recombinant antigen-like proteins, naked DNA or dendritic cells which are pulsed with tumor peptides or tumor cell lysates. Because of the lack of glioma-specific antigens, these common vaccination strategies cannot at present be applied to patients with malignant glioma. However, alternative approaches have been developed. For example, patients can be vaccinated with attenuated autologous glioma cells, resulting in an immune response to unknown putative glioma antigens. This immune reaction can be further enhanced by rendering the glioma cells more immunogenic before administration. MHC class I molecules on glioma cells can be upregulated by irradiation, leading to enhanced immunogenicity and to a better antigen recognition [129]. Increased immunogenicity can also be achieved by gene transfer of MHC class II into glioma cells [130]. Moreover, transfection of glioma cells with various cytokine genes has proven effective in activating cytotoxic immune cells [131]. Gene transfer of granulocyte-macrophage-colony-stimulating factor (GM-CSF) into malignant glioma cells resulted in long-lasting tumor rejection in an animal model [132]. It has been suggested that dendritic cells are involved in such strong immune reactions [133]. However, despite these sophisticated strategies, to date vaccination for malignant glioma has been found to be an insufficient therapeutic tool in human patients [134, 135].

A major obstacle to the above outlined cellular immunotherapies and vaccination strategies is their susceptibility to inhibition by the glioma-mediated immune suppression which is mainly based on the secretion of TGF-β and other cytokines. Although IL-2 can partially overcome the TGF-β-mediated suppression of T cells, e.g., through gene transfer of IL-2 into immune cells [136], even more powerful measures may be necessary to counteract the paralysis of the immune system by gliomas. A promising approach is to inhibit TGF-β activity by decorin gene transfer (see below) [137].

Immunochemotherapy

Preclinical studies. The immunotherapeutical studies discussed above offer diverse interesting and promising approaches to treat malignant brain tumors. Moreover, the broad scope of various strategies illustrates the expansive knowledge acquired during the last few years. However, despite all inventiveness, a breakthrough in the therapy of malignant glioma was not achieved. Therefore, the combination of immunotherapy and chemotherapy has been studied in order to design therapies with presumably increased antitumoral effectiveness. There are a considerable number of reports on synergistic interactions between anticancer drugs and cytokines in vitro or in animal models. Most of these studies have been performed with interferons or TNF-α [138, 139]. Moreover, some reports on the synergy of cytotoxic drugs and the death ligands, CD95 ligand and Apo2 ligand, have been published [140–142].

These approaches are based on the rationale that tumor cells, being rather resistant to many cytotoxic agents, have to be attacked on several different fronts to achieve an optimal antitumoral effect. First, administration of anticancer drugs may lead to direct cytotoxicity and to a reduction of tumor burden. Second, the immunologic antitumoral response can be strengthened actively or passively by immunotherapeutic measures. Thus, it is believed that tumor cells, once damaged by toxic effects of antitumor drugs, are prone to elimination by the immune system because their immune-evasive and immune-suppressive mechanisms may no longer be functional due to the drug-induced cell damage. Moreover, intracellular mechanisms of synergistically interacting signalling cascades of cytokines and anticancer drugs have been proposed. For example, it has been demonstrated that both cytostatic drugs [e.g., 143] and TNF-α-induced signalling [144] involve activation of caspases,

Table 4. Clinical trials of immunochemotherapy for malignant glioma.

	Carmustine	Cisplatin	Cyclophos-phamide	Eflornithine	Etoposide	Nimustine	Tamoxifen	Vincristine	IFN-α	IFN-β	Granulocyte-colony-stimulating factor
Boiardi et al. 1991 [146]		X			X					X	
Brandes et al. 1997 [147]	X								X		
Buckner et al. 1995 [148]	X								X		
Buckner et al. 1998 [149]				X					X		
Chang et al. 1998 [150]							X		X		
Jereb et al. 1989 [151]		X						X	X		
Jereb et al. 1994 [152]		X						X	X		
Newton et al. 1995 [153]			X		X						X
Rajkumar et al. 1998 [154]	X								X		
Silvani et al. 1990 [155]		X			X					X	
Yoshida et al. 1994 [156]						X				X	

the proteases establishing the intracellular death machinery of cells. An interaction of the signalling pathways of interferons and cytotoxic drugs is not yet proven but conceivable. Interferons activate the Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) [145], which may interfere with subcellular targets of anticancer drugs.

Clinical trials. Table 4 provides a survey of studies on combined immunochemotherapies for malignant glioma. Most of the trials have been carried out with regimes involving IFN-α that was generally well tolerated. Although some encouraging results are reported, e.g., the combination of carmustine and IFN-α [147], the patient numbers have been too small for reliable conclusions to be drawn. However, most of the combined immunochemotherapies have not led to a more efficient treatment of malignant glioma and, to date, appear not to be superior to conventional chemotherapy. Thus, the outlook with the established and the innovative therapies outlined above remains depressing. Neither common chemotherapies nor novel drugs, neither immunotherapies nor combined immunochemotherapies have achieved a real breakthrough in glioma treatment. The prognosis for patients with malignant glioma is virtually unchanged. In view of this situation, one question remains to be answered: what is the reason for the powerful resistance of glioma to this considerable variety of refined therapeutic strategies, employing so many different tumoricidal mechanisms? The development of efficacious treatments for this tumor entity will depend on insight into the cellular and molecular basis of glioma pathophysiology. Therefore, some important properties of malignant glioma and why they impede therapeutic interventions will be discussed (table 5).

Reasons for the failure of current treatments

Hypoxia, hypoperfusion, and increased pressure due to edema

Perfusion in central zones of gliomas is often decreased partially by disruption of blood vessels and partially by increased tissue pressure. The hypoperfusion leads not

Table 5. Pathophysiologic properties of malignant glioma possibly impeding therapeutic interventions.

Properties
Hypoxia
Hypoperfusion
Increased pressure due to edema
Heterogeneity of glioma
Blood-brain barrier
Immune privilege of the brain
Glioma-associated immunosuppression
Lack of tumor-specific antigens
Insufficiency of antigen presentation
Resistance of glioma cells (intrinsic and extrinsic) to apoptosis

Table 6. Liposolubility of common anticancer drugs.

Class	Name of drug	Liposolubility
Alkylating agents		
Nitrogen mustards	ifosfamide	+
	melphalan	+ +
	DAG (dianhydrogalactitol)	+ +
	DBD (dibromodulcitol)	+ +
Metal salts	cisplatin	–
	carboplatin	–
Nitrosoureas	carmustine	+ + +
	nimustine	+ +
	lomustine	+ + +
	MeCNU	+ + +
Benzquinone	AZQ	+ +
Alkaloids		
Vinca alkaloids	vincristine	–
Podophyllotoxins	teniposide	(+)
	etoposide	(+)
Antimetabolites		
Pyrimidine analogs	5-fluorouracil	+ +
	cytarabine	+ +
Folic acid analogs	methotrexate	(+)
Antibiotics	adriamycin	–
	idarubicin	+
	bleomycin	–
	mitoxantrone	(+)
Others	hydroxyurea	+ +
	procarbazine	+ +
	dacarbazine	(+)
	temozolomide	+ +

only to an insufficient supply of nutrients, resulting in shortcomings in the energy metabolism of the cell, but also to marked hypoxia and a pH shift. Therefore, necrotic areas are neuropathologic hallmarks of grade IV gliomas. The necrosis of tumor tissue develops despite commonly vigorous neovascularization. Clearly, hypoperfusion is a direct obstacle to the transport of antitumor drugs to glioma cells. Thus, increased tissue pressure in gliomas reduces drug delivery by decreased blood flow and decreased diffusion from blood vessels within the tumor. It is also conceivable that hypoperfusion and increased tissue pressure may inhibit the migration of immue effector cells into the tumor. Glioma cells in hypoxic and hypoperfused tumor areas often exhibit cell cycle arrest, thereby impeding the efficacy of cell-cycle-specific anticancer drugs. Moreover, the decreased metabolism of glioma cells at perinecrotic sites and the shifted tissue pH may also add to the weakened antitumor action of various drugs.

Cellular heterogeneity of gliomas

Malignant gliomas are highly heterogeneous tumors. Therapeutic interventions face not only the interindivid-

ual differences of gliomas but also the heterogeneous cell populations within the same tumor [157]. Even in long-term-cultured glioma cell lines, supposedly of clonal origin, cellular and molecular heterogeneicity has been demonstrated [158]. This property of malignant gliomas is a major impediment for all therapies targeting common characteristics of tumor cells, including immunotherapy targeting of putative tumor antigens.

Blood-brain barrier

Probably the most important cause of insufficient drug availability in the CNS after systemic drug therapy is the presence of the blood-brain barrier which is morphologically based on the tight junctions of endothelial cells. The blood-brain barrier is characterized by a paucity of transport vesicles in the endothelial cells and the presence of a drug efflux system such as the transport protein MDR1 P-glycoprotein. Access of hydrophilic substances into the CNS is hampered by this barrier. Essentially, only water and small, inert, and lipophilic molecules up to a molecular weight of 200 Da may passively penetrate the blood-brain barrier. However, other factors such as specific transport mechanisms (e.g., glucose) or concentration gradients for certain substances are also important. Therefore, the physicochemical properties of a substance do not strictly predict its blood-brain barrier penetration in vivo. The penetration of a given cytotoxic drug into the CNS cannot be precisely predicted nor is there a good overall correlation between the clinical efficacy of a cytotoxic drug and its blood-brain barrier penetration. Moreover, a variety of agents that do not readily cross the intact blood-brain barrier also have therapeutic activity against gliomas and other types of primary brain tumors. This could be explained by the finding that the morphological correlates of the barrier are disrupted in necrotic and perinecrotic zones of gliomas, enabling hydrophilic agents to penetrate into tumor tissue too. Unfortunately, the blood-brain barrier may be completely intact in the CNS areas where migrating glioma cells infiltrate the healthy brain tissue. Therefore, only the tumor cells responsible for the nearly 100% recurrence rate of glioma are protected from cytotoxic drugs by a functional blood-brain barrier. Yet, these infiltrating glioma cells should be the main target of systemic drug therapy. The major tumor cell burden in the center of a glioma can be eliminated fairly well by neurosurgery and radiotherapy. Similarly, the common chemotherapy drugs may reach the necrotic sector of a tumor rather easily but only poorly the actively infiltrating cells in the periphery. Taken together, the mainstays of glioma therapy fail to target those glioma cells representing the infiltrating border which are mainly responsible for the recurrence and the poor prognosis for patients with malignant brain tumors. Glioma cells can be found at a distance of 3–5 cm from the macroscopic tumor margin. Extracellular-matrix-targeting enzymes like metalloproteinases may play an important role in the invasive growth pattern of glioma. Successful adjuvant therapies will need to control these infiltrating cells at the tumor margin. Therefore, it is still critical to consider the ability of drugs to penetrate the blood-brain barrier when searching for novel drugs suitable for the treatment of malignant glioma. In general, small molecules which are liposoluble and non-ionized may exhibit the best penetration into the CNS. Table 6 lists the liposolubility of various anticancer drugs.

Since insufficient drug availability is one major impediment for glioma treatment, a strategy to overcome this problem would be to render the blood-brain barrier more permeable. The opening of the blood-brain barrier was first tried with hypertonic solutions and later with the bradykinin agonist RMP-7 [159–161]. However, the initial optimism soon gave way to a more critical assessment because of increased toxicity and limited response to this therapy. Thus, future studies will have to provide sound evidence that this mode of application is superior to common approaches before a broader employment of these techniques may be recommended.

Immune privilege of the brain

The presence of the blood-brain barrier and the highly limited participation of the brain in the lymphatic system signify the specific immunological mechanisms that are active in the CNS. For many years it was believed that the immune privilege of the brain prevents any interaction between glioma and immune cells. However, recent evidence suggests that considerable trafficking of activated T cells takes place to and from the brain [162]. As demonstrated in animal models, T cells that have been primed against glioma cells in the periphery can circulate into the CNS and exert strong antitumoral effects on glioma growth [163–165]. However, the shortcomings of afferent and efferent immune reactions in the brain have also been demonstrated. Tumor-infiltrating T cells can kill glioma xenografts in the liver of animals, but not intracranially [127]. Rats generate cytotoxic T cells when challenged with subcutaneously implanted syngeneic glioma cells, but not when the cells are implanted into the brain [166]. Furthermore, a gene transfer for IL-2 into glioma cells leads to rejection of these cells after peripheral subcutaneous implantation, but not after intracerebral injection [91]. These differences between peripheral and central immunologic mechanisms are not completely understood, but need to be elucidated in order to develop effective immunotherapy for glioma.

Glioma-associated immunosuppression

Malignant glioma patients exhibit signs of local and systemic immune deficiency. This immunosuppression is presumably based on glioma-derived cytokines which counteract various components of the central as well as peripheral immune system [89]. Lymphocytes from healthy probands become immunocompromised if incubated together with supernatant obtained from cultured glioma cells. The T cell compartment appears to be affected most severely by the glioma-cell-mediated suppression. Histopathological analyses have shown that the extent of glioma infiltration by lymphocytes does not correlate with a better prognosis for glioma patients [167]. This finding supports the hypothesis that tumor-infiltrating T cells show considerable functional deficits [168, 169]. The range of cytokines possibly involved in glioma-caused immunosuppression is not completely known. TGF-β plays a major role, and prostaglandin E and IL-10 have also been shown to exert immunosuppressive actions [170].

TGF-β has various, but mostly suppressive, effects on the immune system. It inhibits the proliferation and activity of lymphocytes and influences cell cycle events and mitosis of immune cells, partially by inducing cell cycle arrest. Increased concentrations of TGF-β are found in glioma tissue and in the cerebrospinal fluid of glioma patients. In vitro long-term-cultured glioma cell lines as well as ex vivo glioma cells produce TGF-β. Importantly, TGF-β can inhibit the INF-γ-induced expression of MHC class II molecules on astrocytoma cells [97, 98]. Therefore, the putative glioma antigens may be recognized less effectively by CD4 + T cells. Also discussed is whether TGF-β can downregulate MHC expression on immune cells [98, 171, 172]. Furthermore, TGF-β has been demonstrated to induce apoptotic cell death of lymphocytes [173, 174], suggesting that tumor-infiltrating cytotoxic lymphocytes could be killed by glioma-derived TGF-β in vivo. Regarding immunotherapeutic strategies, an essential finding is that TGF-β effectively inhibits the activity of NK, LAK, and cytotoxic T cells [94]. However, TGF-β-induced effects other than immune suppression could also be important for the promotion of glioma growth. For example, a role for TGF-β as an autocrine growth factor of glioma cells has been suggested [175]. Moreover, involvement of TGF-β in tumor neovascularization has also been proposed [176].

Given these immunosuppressive mechanisms, one promising therapeutic approach would be the antagonism of immune evasive strategies of gliomas. 9L rat glioma cells acquired immunogenicity after TGF-β antisense gene transfer and subcutaneous implantation. This led to the regression of established wild-type 9L gliomas [93, 177]. In addition, treatment with antisense TGF-β oligodeoxynucleotides sensitizes glioma cells to the actions of LAK cells [178]. A refined approach to knock out

the glioma-induced immunosuppression is transfection of glioma cells with decorin, a small glycoprotein that binds to and inhibits TGF-β. In an animal model, glioma cells expressing ectopic decorin exhibited significantly decreased tumorigenicity [179].

Immunosuppressive effects are also assigned to the autocrine insulin-like growth factor (IGF). The immunogenic phenotype of glioma can be reversed by IGF in the rat C6 model. Thus, gene transfer of antisense IGF [164, 180] or antisense IGF receptor [165] rendered glioma cells susceptible to immune rejection in syngeneic animals. Moreover, the administration of antisense-IGF-transfected C6 glioma cells initiated the rejection of established wild-type C6 tumors [164]. However, to the best of our knowledge, these striking observations have not been translated into further studies either in laboratory animals or human patients. Moreover, the significance of the findings has been questioned since the model was not truly syngeneic [181].

Other candidates for immunosuppressive factors may include prostaglandin E and IL-10. Prostaglandin E, produced by glioma cells, is capable of inhibiting the activity of immune cells [171, 182–185]. IL-10, which can be isolated from glioma culture supernatant, has been shown to downregulate the expression of MHC class II molecules on monocytes [186]. Further investigations will have to show whether these cytokines could be a promising target for therapeutic purposes. Other, so far unidentified cytokines produced by tumor-infiltrating lymphocytes have been suggested to stimulate the proliferation of glioma cells [187]. However, alternative mechanisms of immune suppression may be more essential, e.g., the defective expression of IL-2 receptors by tumor-infiltrating T cells leading to insufficient activation of immune reactions.

A different but nevertheless clinically relevant immunosuppression is that induced by steroid application for symptomatic relief of brain edema. In general, steroids such as dexamethasone are immunosuppressants by de-
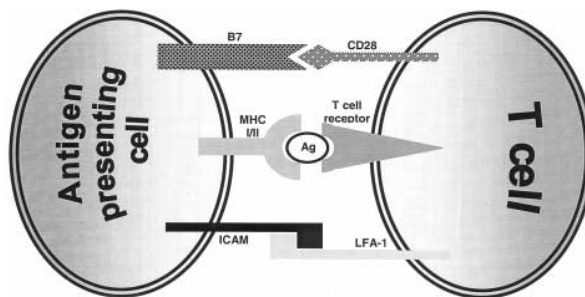

Figure 2. Afferent immune mechanism. Cellular interactions between a T cell and an antigen-presenting cell. Note that these mechanisms may be suppressed in glioma patients (see text).
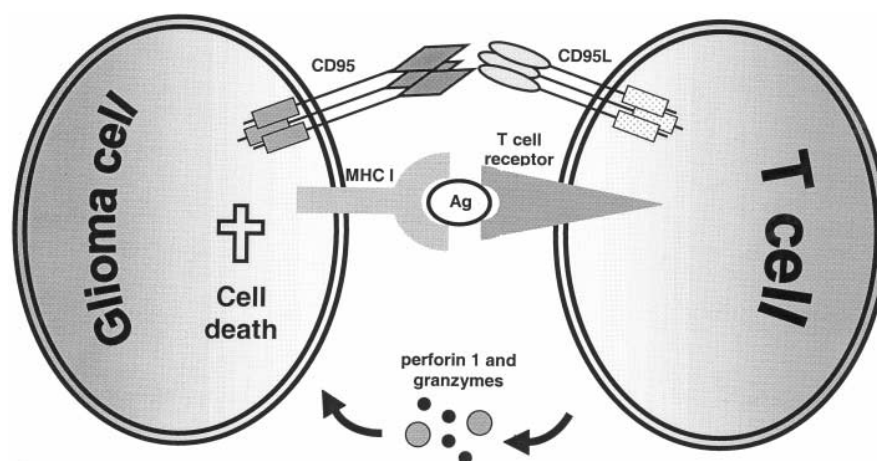
Figure 3. Efferent immune mechanism. Recognition and subsequent killing of a tumor cell by an activated cytotoxic T cell. This mechanism may be defective due to glioma-associated immune depression (see text).

creasing the migration of leukocytes into inflamed tissues, reducing blood counts of certain leukocyte subsets, especially T cells, lowering IgG levels, and impairing cutaneous delayed-type hypersensitivity responses. Importantly, dexamethasone has been demonstrated to inhibit the cytotoxic effects of several common anticancer drugs on cultured glioma cells [2]. Moreover, the steroid-induced stabilization and impermeabilization of the blood-brain barrier is rather undesirable because it hampers access of drugs into the CNS. The consequences of steroid administration to glioma patients are therefore somewhat difficult to predict: on the one hand, it reduces the leakiness of the blood-brain barrier and thus *decreases* drug delivery to the tumor, but on the other hand, it reduces intracranial pressure and thus possibly *increases* drug delivery to the tumor. In the clinical management of glioma patients, steroid medication is often imperative. Taken together, it would seem to be appropriate to avoid simultaneous application of steroids and proapoptotic agents, and reduce the steroid dose to the minimum.

Lack of tumor-specific antigens
Peripheral immune cells of patients bearing malignant glioma are often chronically activated [188, 189], suggesting that the tumor-specific immune responses are not completely inhibited. Since specific immune reactions depend on tumor antigens it has been surmised that peripheral lymphocytes are activated by hitherto unidentified glioma antigens. To date, no glioma-specific immunologic epitope has been characterized. The first specific tumor antigen described was the melanoma antigen (MAGE-1) on malignant melanoma [190].

MAGE-1 is also expressed on some cultured glioma cell lines [191]. A tumor-antigen-targeting immunotherapy for malignant glioma must be based on the recognition of antigens that are expressed preferentially in glioma cells. Candidate proteins comprise, in particular, the proteins that are responsible for the process of malignant transformation, because normal cells should not express or only moderately express these gene products. In preclinical trials, p53 [192, 193] and EGF receptor [194] have been employed for this strategy. However, the identification of a specific glioma antigen would be the most important step forward to a specific antiglioma immunotherapy.

Insufficiency of antigen presentation
Besides glioma-derived immunosuppressive factors and the lack of tumor-specific antigens, a further mechanism is involved in the development and progression of malignant glioma. Presentation of antigens is a fundamental immunologic process that enables cytotoxic T cells to recognize and kill target cells. The priming of specific T cells against glioma cells requires an antigen-specific interaction between T cell and tumor cell. This specific killing of target cells must be preceded by the presentation of the antigen by a professional antigen-presenting cell to CD4 + T cells. Here, the antigens are presented together with MHC class I and II molecules (fig. 2). In the peripheral immune system, antigen presentation is achieved by professional antigen-presenting cells including dendritic cells, macrophages, and B cells. In the CNS, where these cells are probably missing [96], other cell types have been suggested to accomplish antigen presentation, especially microglial cells, but also possi-

bly astrocytes, endothelial cells, or capillary pericytes. Integrins such as leukocyte function antigen-1 (LFA-1), expressed by T cells, play a major role in the process of antigen recognition by mediating adhesion between immune cells and antigen-presenting cells. Typically, LFA-1 binds to adhesion molecules such as ICAM-1. Moreover, the clonal expansion of antigen-specific naive T cells requires the expression of co-stimulatory signals. The best characterized co-stimulatory molecule for antigen-presenting cells is B7. The receptor for B7 on T cells is CD28, a member of the immunoglobulin superfamily. The interactions of the co-stimulatory molecules are decisive for the induction of immune responses versus an anergic reaction [195]. Importantly, glioma cells express the co-stimulatory B7 molecule neither in vivo nor in vitro. One can speculate that the downregulation of B7 supports glioma growth by promoting T cell anergy or by impeding efficient T cell priming. A therapeutic approach to restore the mechanisms underlying antigen recognition is gene transfer for B7 into glioma cells. These experiments have been carried out in vitro [196] and in an animal model in which glioma growth inhibition was demonstrated [197]. Alternatively, the recognition of tumor cells can be enhanced by MHC class II transactivators [198]. Class II activator (CIITA) proteins are regulators of the transcriptional activation of class II MHC genes. IL-1β inhibits the transcription of the CIITA gene and thereby reduces subsequent class II MHC expression on astrocytoma cells [199]. This mechanism could lead to insufficient recognition of glioma antigens by immune cells. Taken together, the presentation of antigens in the CNS appears to be limited, weakening immune responses to glioma cells.

Another possibly defective immune mechanism leading to an insufficient prevention of tumor growth may be the process of T-cell-mediated cytotoxicity based on the interaction of T cell receptors and target cell antigens (fig. 3). This process is highly regulated by a variety of immune modulatory cytokines including interferons, IL-1, IL-2, and TFN-α. Moreover, the efficacy of the antigen recognition and the subsequent

elimination of a cell depends on the presence of MHC I on the surface of the target cell. The T-cell-mediated elimination of virally infected or tumor cells is generally accomplished by two alternative mechanisms, namely CD95/CD95 ligand interactions or perforin [200]. Defects in CD95- or perforin-initiated pathways are supposed to hamper the elimination of malignant target cells (see below).

Intrinsic resistance to apoptosis

Probably the most essential obstacle to virtually all chemo- and immunotherapeutic strategies is the resistance of glioma cells to a wide variety of cytotoxic agents and to the actions of immune effector cells. An extrinsic resistant phenotype can be discriminated from an intrinsic one. The extrinsic resistance of tumor cells may be caused by preceding selection of more resistant clones due to the influence of damaging agents. Thus, previously irradiated tumors may be less sensitive to chemotherapeutic treatment than tumors which have not been irradiated. In contrast, intrinsic resistance emerges as a direct consequence of the process of malignant transformation and dedifferentiation. The phenotype of resistance is thought to depend on the pattern of genes expressed. These genes that may carry mutations are typically responsible for cellular events such as proliferation, DNA repair, cell cycle regulation or cell-cell adhesion. Important genes involved in glioma resistance to cytotoxic drugs are, for example, efflux pumps such as multidrug resistance and cellular detoxification systems like glutathione-S-transferase. Furthermore, DNA repair genes like O6-methylguanine-DNA methyltransferase are essential for glioma cells to survive DNA damage due to cytostatic drugs [201, 202]. Some of the most important cellular resistance mechanisms are listed in table 7.

Presumably even more vital for the survival of glioma cells after attack by diverse damaging agents are mutations or amplifications affecting the expression or functional activity of tumor suppressor genes or oncogenes. Mutations in such genes may promote or inhibit the ability of glioma cells to undergo apoptotic cell death. Apoptosis is an active form of cell death involving the initiation of intact intracellular signalling pathways. Decreased or increased expression of genes such as Bcl-2, p53, p21, p16, MDM2, Rb, and PTEN, may not only play a role in the development of gliomas but may also have a major impact on the resistance of glioma cells to therapy. Of great putative importance has been the tumor suppressor gene p53 which is regarded as a guardian of the cell cycle. Dis-

Table 7. Types of cellular resistance to common anticancer drugs.

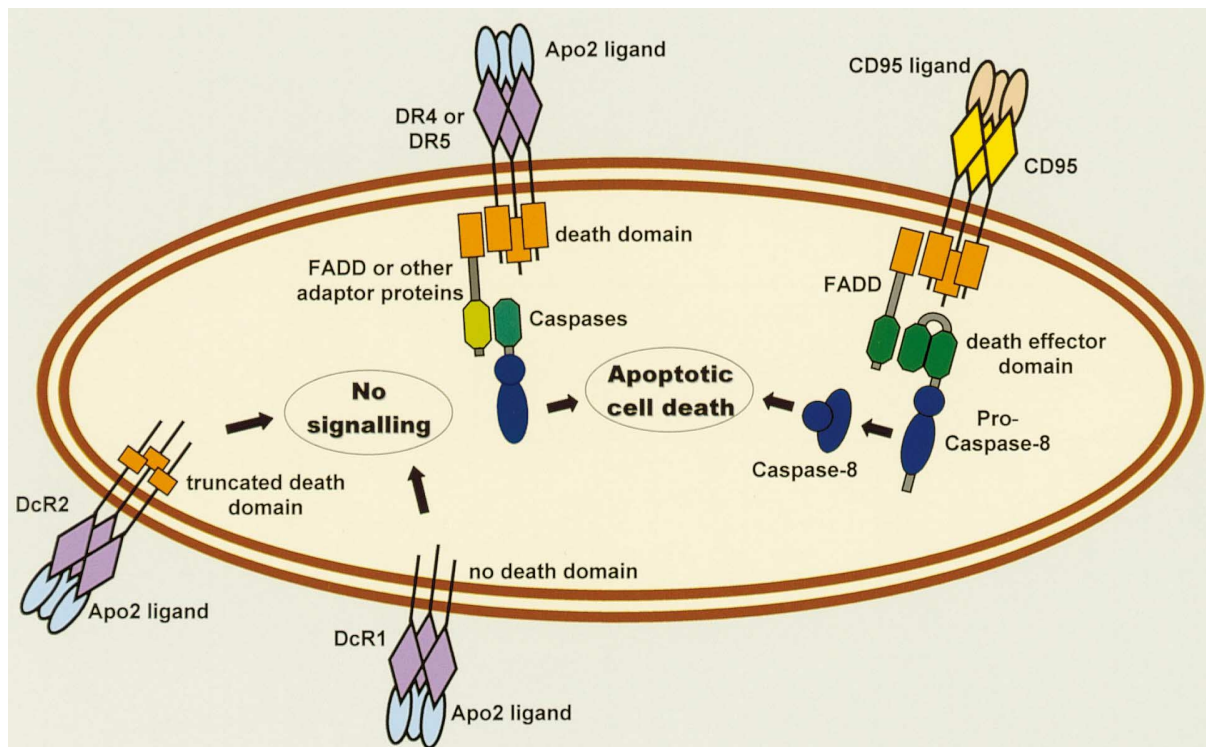| Increased	Decreased
Export from cells by enhanced expression of the multidrug resistance MDR1 gene coding for P-glycoprotein	expression of topoisomerase II
Conjugation with glutathione by enhanced expression of glutathione-S-transferase	apoptosis
DNA repair, e.g., by enhanced expression of O6-alkylguanine-DNA alkyltransferase	

Figure 4. Apoptotic signalling upon activation of death receptors by Apo2L and CD95L, and modulation of sensitivity to apoptotic cell death by decoy receptors.

ruption of p53 can disable the intracellular death machinery, and affected glioma cells can lose their ability to die by apoptosis after DNA damage [reviewed in refs 203, 204]. Surprisingly, our own in vitro studies failed to define an important role for p53 in determining cellular responses to chemotherapy [203, 205]. Another candidate is Rb, designated the master brake of cell cycle progression. Defective Rb functioning may lead to uncontrolled proliferation of glioma cells [206, 207]. PTEN, a recently discovered gene, codes for a tyrosine phosphatase and appears to be involved in the phosphatidylinositol-3,4,5-trisphosphate signalling pathways, a major growth control pathway in the cell. Following mutations in the PTEN gene, these intracellular mechanisms, acting both to stimulate cell growth and to block apoptosis of glioma cells, can become deregulated [208–211]. Conversely, reconstitution of wild-type PTEN in PTEN-mutant glioma cells may restore responsiveness to radiotherapy [212]. Enhanced expression of the antiapoptotic Bcl-2 gene induces resistance of glioma cells to cytotoxic drugs and other proapoptotic agents [213, 214]. Thus, defects in the apoptotic machinery of glioma cells seem generally to be a major impediment for a wide variety of therapeutic approaches. Moreover, the acquisition of antia-

poptotic properties may be crucial for tumorigenesis as well as immune evasion of tumor cells due to enhanced resistance to immune effector cells. Specifically, the expression of antiapoptotic proteins of the Bcl-2 and inhibitor-of-apoptosis (IAP) family, by virtue of their ability to inhibit caspases, the major effectors of apoptotic cell death, may inhibit both drug- and immune-mediated cell death. Therefore, future therapeutic approches will mainly have to overcome these molecular mechanisms of resistance. During the last decade, apoptosis-regulating systems have been discovered which exhibit cellular and molecular mechanisms that could be employed to overcome some of the above-mentioned problems of current therapeutic strategies. In our hands, CD95 ligand and Apo2 ligand have turned out to be the most promising candidates for a possible death receptor/ death ligand-based immunotherapy of malignant glioma. All investigations into these newly discovered cytokines are preclincal. The great expectations placed in these experimental therapies are based mainly on the ability of the death ligands to induce the apoptotic cell death of tumor cells rapidly, definitely and, at least in part, selectively.

Death ligands: an example for promising future strategies

Apoptosis-inducing ligands

CD95 ligand. CD95/CD95L interactions, leading to apoptotic cell death, were first observed a decade ago. The ligand, CD95L (FasL, Apo1L), binds to its cognate receptor, CD95 (Fas, Apo1), to signal apoptotic cell death. CD95-induced apoptosis fulfills several biological functions. The CD95 and perforin systems are essential for T-cell-mediated cytotoxicity. Thus, the CD95 system plays a central role in the regulation of immune reactions, in maintaining immune privilege at distinct sites, and in killing virally infected and tumor cells [reviewed in refs 215, 216].

The intracellular mechanisms of CD95-induced apoptosis have been extensively investigated. After the binding of CD95L to CD95 and the induced conformational change of the intracellular death domains, the adaptor protein FADD (Fas-associated protein with death domain) binds CD95 via interaction of the death domains (fig. 4). Through self-association of the death effector domains, caspase-8 is coupled to the complex. The complex comprising CD95, FADD, and caspase-8 is designated the death-inducing signalling complex (DISC). The active subunits of caspase-8 induce the stepwise cleavage and thereby activation of several effector caspases. However, the precise order of activation of the diverse caspases remains to be elucidated and may be cell type specific. Numerous substrates of caspases have already been identified. Despite extensive knowledge about target proteins of caspases, some crucial downstream steps on the way to the final completion of cell death remain obscure. Specifically, the essential proteins that need to be cleaved by caspases for death to occur have not been identified.

CD95-induced apoptosis is based on a signalling cascade that can be influenced by various other intracellular signalling factors. A multitude of metabolic pathways can interact with the execution of the apoptotic death program, among which, the Bcl-2 family is of outstanding importance [reviewed in ref. 213]. Bcl-2 inhibited CD95-induced apoptosis in glioma and other cell lines [214, 217]. Quite recently, a soluble decoy receptor that binds to and inhibits CD95L was identified [218]. This antagonistic receptor, termed DcR3, was predominantly expressed in malignant tissue, suggesting a novel immune-evasive strategy of CD95-positive tumors.

In the last few years, a refined immune-evasive mechanism used by tumor cells has been revealed, the so-called counterattack [reviewed in ref. 219]. Glioma cells can kill attacking immune cells by utilizing the CD95 system. The CD95L expressed on the glioma cell surface can trigger apoptosis through interacting with CD95 on the cytoplasmic membrane of cytotoxic T cells [220–222].

Apo2 ligand. Recently, another apoptosis-inducing system has emerged which seemed to be quite different from the CD95 system in that the ligand, Apo2L (TRAIL), was shown to bind to at least four distinct receptors [reviewed in ref. 223]. Apo2L, a type II protein, triggers apoptotic cell death when binding to one of the agonistic receptors, DR4 or DR5. In contrast, no cell death signal is transmitted when expression of the decoy receptors DcR1 and DcR2 dominates.

Ectopic expression of the death receptors DR4 and DR5 results in the induction of apoptotic cell death. DR4 (= TRAIL-R1) as well as DR5 (= TRAIL-R2) harbor a death domain (fig. 4). The two decoy receptors DcR1 (= TRID = TRAIL-R3) and DcR2 (= TRUNDD = TRAIL-R4) antagonize Apo2L-induced apoptosis by binding the ligand without transmitting the death signal [224]. DcR1 is a membrane-bound protein similar to DR4, but lacks a cytoplasmic tail and thus a signalling domain. Experimental overexpression of DcR1 renders cells resistant to Apo2L-induced cell death. DcR2 is similar to DcR1 but harbors a truncated cytoplasmic death domain. Because no proapoptotic signalling is possible through this truncated sequence, DcR2 is acting as an antagonistic receptor, too. Apo2L-mediated apoptosis depends critically on the activity of caspases in all cell lines examined so far.

The biological function of the Apo2L system is still a matter of controversy. Apo2L, like CD95L, induces caspase-dependent apoptosis; however, it has also been reported that NF-κB, which is a transcription factor with proliferative properties, can be activated by Apo2L in some cell types. Therefore, physiological functions of Apo2L other than merely induction of apoptosis need to be considered.

Expression of receptors

Normal as well as malignant CNS tissue exhibits a remarkable expression pattern of CD95 and CD95L. CD95 is not expressed in normal brain tissue with the exception of endothelial cells which have proven to be resistant to CD95 agonists [225–227]. However, there have very recently been reports on constitutive CD95 expression on astrocytes in vitro which, under certain circumstances, are considered as a useful model for reactive astrocytes in vivo [228, 229]. Moreover, CD95 expression was also detected in reactive gliosis associated with multiple sclerosis and Alzheimer dementia [230, 231]. In contrast to normal CNS tissue, the majority of both human glioma cell lines and ex vivo glioma cells express CD95 [108, 222, 232–234].

Unlike CD95L, Apo2L is expressed in many tissues and, interestingly, its expression pattern coincides to a great extent with the expression of the proapoptotic receptors DR4 and DR5 [235]. Since co-expression of ligand and receptor does not lead to apoptotic cell death under physiological circumstances, powerful antiapoptotic

mechanisms have to be maintained. Antagonistic decoy receptors, like DcR1 and DcR2, protect DR4- and DR5-positive cells from Apo2L-induced apoptosis. Thus, the balance of proapoptotic and antiapoptotic receptors could be decisive for the susceptibility to Apo2L. Furthermore, intracellular antiapoptotic proteins may inhibit Apo2L-triggered cell death in tissue abundant in the agonistic receptors DR4 and DR5. Further investigations will have to unravel the meaning and importance of these remarkable expression patterns as well as the precise mechanisms by which susceptible cells are protected from apoptosis.

When considering a possible Apo2L-based therapy for malignant glioma, it is imperative to take into account the expression of ligand and receptors in normal brain tissue. Whereas it has been shown by immunohistochemistry that virtually all investigated malignant gliomas (23 out of 23) expressed Apo2L [236], Apo2L was not found in normal brain parenchyma [235]. Given that Apo2L is expressed on most human tissues, the expression pattern in the brain is quite unique. Moreover, in brain tissue, no mRNA for DR5, DcR1 or DcR2 has been found [237–239].

Antitumor activity in vitro and in vivo

CD95 ligand. Most of the investigated glioma cell lines and ex vivo glioma cells are susceptible to CD95-mediated cell death [108, 140, 232]. Moreover, CD95 gene transfer into CD95-negative resistant glioma cells rendered them sensitive to CD95 agonists [240]. A further important finding is the efficacy of combined treatment of tumor cells with CD95L and other cytotoxic substances. Human malignant glioma cells are highly susceptible to synergistic induction of cell death by co-treatment with CD95L and diverse anticancer drugs [110, 140, 241–244]. It has been shown that the combined application of CD95L and topoisomerase I or II inhibitors, vincristine, or taxol are the most effective in achieving prominent synergistic activation of apoptotic cell death [110, 140, 242]. The precise mechanisms underlying this synergistic induction of cell death are not completely understood. The synergy of CD95L and taxol, e.g., has been hypothesized to involve the reversal of the inhibitory effect of Bcl-2 on CD95-mediated apoptosis. Because taxol phosphorylates and possibly inactivates Bcl-2, CD95-triggered apoptosis is facilitated [110]. Some cell types respond to cytotoxic drugs with upregulation of CD95 whereby the proapoptotic effects of CD95 agonists may be enhanced [141, 245]. Moreover, it is believed that several cell types directly employ the CD95 system to commit suicide in response to anticancer agents. Thus, treatment of neuroblastoma, hepatoma, and lymphoma cells with anticancer agents

results in autocrine or paracrine cell death by direct CD95L/CD95 interactions [245–247]. However, numerous cell types, including glioma cells, fail to exhibit this path of drug-induced CD95-mediated apoptosis [Glaser et al., unpublished data, 248–250]. Hence, the mechanisms underlying the synergy of CD95 agonists and cytotoxic drugs seem to be cell type specific.

Upon reports that intravenously injected CD95 antibody caused fatal liver toxicity in mice [251], fears arose that CD95L would be too toxic for any therapeutic application. However, since then, many reports have shown that locally administered CD95L is a powerful agent to achieve various desired effects without causing systemic toxicity. Intraperitoneally administered mouse CD95L killed CD95-positive, intraperitoneal Yac-1 lymphomas efficiently [252]. Arai et al. [253] demonstrated that an adenoviral CD95L gene transfer into immunodeficient mice resulted not only in the regression of CD95-positive renal carcinoma xenografts but also in the destruction of CD95-negative colon carcinoma tissue. While CD95-positive carcinoma cells died by apoptosis, the CD95-negative tumor was attacked by locally activated immune cells, mainly neutrophils. Similar results were obtained by implantation of CD95L-transfected tumor cells in syngeneic or nu/nu mice [254]. The local inflammatory response caused rejection of CD95L-expressing lymphoma, hepatoma, and melanoma cells. No in vivo study of direct CD95L gene transfer for treatment of malignant glioma has been published. However, there are

Table 8. Possible mechanisms of immune evasion by malignant glioma.

Insufficient antigen presentation to immune cells because of:
- lack of professional antigen-presenting cells in the CNS
- decreased expression of MHCI or II molecules
- decreased expression of specific glioma antigens

Insufficient T-cell-mediated cytotoxicity due to:
- lack of co-stimulatory molecules
- induction of tolerance and anergy
- decreased CD95 expression
- defective T cell functioning, e.g., due to decreased IL-2R expression

Secretion of immunosuppressive factors by glioma cells:
- TGF-β
- IL-10
- prostaglandin E

Defects regarding the CD95 system:
- lack of CD95 expression on glioma cells
- defective CD95 signalling
- increased expression of CD95L and thereby killing of activated T cells by glioma cells
- expression of the antagonistic CD95L receptor DcR3 on glioma cells

several promising reports on the in vivo treatment of glioma by viral transfer of other proapoptotic genes. Malignant glioma cells both in vitro and in vivo could be effectively killed by gene transfer of caspase-3 [255], caspase-6 [255], FADD [256], and caspase-1 [257].

Apo2 ligand. Most tumor cell lines have proven to be susceptible to Apo2L, including glioma cells [258], lymphoma cells [259], melanoma cells [260, 261] and myeloma cells [262], whereas untransformed cells are resistant [235, 263]. DcR1 is abundantly expressed in normal tissue, but only to a limited extent on malignant cells [238]. Therefore, an Apo2L-based therapeutic approach could be rather specific for malignant tissue. In fact, 8 out of 12 investigated malignant glioma cell lines proved to be positive for DR4, and 11 out of 12 positive for DR5. In contrast, only 4 out of 12 glioma cell lines were DcR1 positive, and 2 out of 12 were DcR2 positive [258]. Most glioma cell lines were susceptible to Apo2L-induced cell death (10 out of 12) [236, 258]. Importantly, the sensitivity to Apo2L-triggered apoptosis was unaffected by the simultaneously expressed Apo2L. This finding is in accordance with observations in the CD95 system and indicates the presence of subcellular inhibitory mechanisms that prevent apoptotic fratricide due to Apo2L expressed on the glioma cell surface. Overall, the expression pattern of the four receptors did not correspond to the sensitivity to Apo2L-initiated apoptosis [258]. This finding is in agreement with reports on the lack of correlations in other malignant tumor cell lines [261]. Thus, Apo2L/receptor interactions represent a complex regulatory system, probably including still unknown agonistic and antagonistic receptors or intracellular factors which control the different pathways of apoptosis.

Recently, the powerful antitumoral effects of Apo2L were demonstrated in vivo for the first time. Walczak et al. [264] showed that the administration of leucine zipper forms of human Apo2L can suppress the growth of a human mammary adenocarcinoma cell line in SCID mice. Importantly, no systemic toxicity was observed. Moreover, we have recently observed that the local administration of soluble Apo2L exerts prominent antitumoral activity against intracranially growing human glioma xenografts in athymic mice [Roth et al., unpublished data]. Two intratumoral injections of Apo2L resulted in long-term survival of treated mice and in the eradication of the glioma xenografts. We did not detect any Apo2L-mediated local toxicity in the brain of treated mice.

Feasibility of a death-ligand-based therapy. A major obstacle to the established chemotherapy for malignant glioma is the resistance of glioma cells to many cytotoxic agents (see above). The most important advantage of a death-receptor-based therapy would be the direct activation of caspases leading to rapid and definite activation of the tumor cell death program. The strategy of employing the intrinsic apoptotic program of tumor cells could overcome many of the known resistance mechanisms such as mutations of tumor suppressor genes. Significantly, death-ligand-induced cell death is executed independently of the potential loss of p53, Rb, or p16. Moreover, the immunosuppressive effects exerted by glioma cells, which can hamper most of the cellular immunotherapeutic approaches, fail to prevent the apoptotic signalling cascade. The CD95-mediated apoptotic cascade is not affected by TGF-β, which is known to suppress numerous other immunologic mechanisms (see table 8).

However, there are also some foreseeable obstacles to a CD95L- or Apo2L-based therapy of malignant glioma. The complete absence of CD95 or DR4/DR5 on the surface of tumor cells would render them resistant to proapoptotic ligands. Nevertheless, it has been shown that CD95 gene transfer in CD95-negative, resistant glioma cells can render them susceptible to CD95 agonists, suggesting intact signalling pathways in many tumor cells even when the receptors are missing [240]. Moreover, a low level of death receptors could be increased by certain cytokines; for example, the expression of CD95 was increased after pretreatment with TNF-α and IFN-γ [108]. However, the expression level of death receptors on tumor cells might not be the most decisive factor for the efficacy of such a therapeutic approach. Since several glioma cell lines are susceptible to death-ligand-induced apoptosis only if protein synthesis is simultaneously inhibited, cytoprotective proteins may be capable of preventing this form of cell death. Therefore, a successful death-receptor-based therapy may also have to neutralize the intracellular antiapoptotic factors. This could be accomplished by combined treatment with drugs either generally affecting RNA or protein synthesis or specifically interacting with known inhibitors of CD95L- or Apo2L-induced apoptosis. Further investigations will have to identify and evaluate the eligible candidates for this kind of combination therapy. For example, combined therapy with CD95L and topotecan administered locally could be a rewarding immunochemotherapeutic treatment strategy [242]. Some of the substances found to act in synergy with CD95L may exert their potentiating effects by blocking the activity of antiapoptotic proteins [110, 140]. In-vivo-confirmed synergy of death ligands and common cytostatic agents would permit the application of lower doses of cytotoxic agents without loss of anticancer effectiveness. Moreover, it is conceivable that cells resistant to one death ligand may, nevertheless, be sensitive to another ligand. Melanoma and myeloma cells are resistant to CD95-mediated apoptosis but susceptible to Apo2L-induced cell death [261, 262].

Some uncertainty remains regarding a potential CD95L-based treatment of human glioma. Although normal brain parenchyma lacks CD95, the reports on CD95 expression on reactive gliosis have to be taken into consideration before a therapeutic application of CD95L is envisaged. Clearly, further investigations have to address the question whether reactive glial tissue that may develop due to the tumor itself or due to therapeutic interventions expresses CD95 and is susceptible to CD95-mediated cell death.

Apo2L could turn out to be a powerful therapeutic tool against malignant glioma, for two reasons. First, Apo2L may be able to kill tumor cells selectively. Second, Apo2L promises to be a quite well tolerated drug without systemic toxicity. However, the expression of agonistic as well as antagonistic Apo2L receptors in the human brain has to be studied thoroughly prior to a clinical application of Apo2L. Thus, the protein expression levels of the agonistic receptors DR4 and DR5 need to be examined urgently. Moreover, Walczak et al. [264] have demonstrated that human astrocytes are susceptible to Apo2L-mediated cell death in vitro, although no neurotoxicity in the murine brain was seen in vivo. In our studies, a simian-virus-transformed human astrocytic cell line was completely resistant to Apo2L [Roth et al., unpublished data]. Further investigations in animal models as well as proper expression studies on human brain tissue will demonstrate whether a CD95L- or Apo2L-based clinical phase I trial is warranted.

Future clinical trials will show which of the numerous chemo- and immunotherapeutic strategies may evolve into a successful therapy for malignant glioma. However, for the time being, we will have to study further the numerous factors influencing glioma development and growth, as well as the mechanisms of resistance to chemotherapy. To be more successful in the management of malignant brain tumors, we will probably have to resort to integrated treatment strategies which combine various approaches to induce tumor cell death.

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