Local tumor irradiation augments the antitumor effect of cytokine-producing autologous cancer cell vaccines in a murine glioma model

Katalin Lumniczky,¹ Szilvia Desaknai,¹ Laszlo Mangel,² Bela Szende,³ Hirofumi Hamada,⁴ Egon J Hidvegi,¹ and Geza Safrany¹

¹Department of Molecular and Tumor Radiobiology, National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary; ²Department of Radiation Therapy, National Institute for Oncology, Budapest, Hungary; ³First Institute of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary; and ⁴Department of Molecular Medicine, Biomedical Research Center, Sapporo Medical University, Sapporo, Japan.

The combined therapeutic effect of cytokine-producing cancer cell vaccines and local radiotherapy was studied in a mouse glioma 261 (Gl261) brain tumor model. Brain tumor-bearing mice were treated with cytokine (IL-4, IL-6, IL-7, GM-CSF, TNF- α , LIF, LT) producing vaccines made by *in vitro* transduction of Gl261 cells with the corresponding adenoviral vectors. Vaccines producing either IL-4 or GM-CSF cured 20–40% of mice. The antitumor effect strongly depended on the secreted cytokine level. Vaccination therapy induced specific activation of cytotoxic T lymphocytes measured by cell-mediated cytotoxicity assay. Brain tumors were heavily infiltrated by CD4⁺ lymphocytes after treatment with IL-4- or GM-CSF-secreting cells. GM-CSF vaccination induced moderate CD8⁺ infiltration, as well. Depleting either CD4⁺ or CD8⁺ lymphocyte subsets abolished the anticancer effect of GM-CSF-expressing cells. Strong synergism was observed by combining cytokine vaccination (GM-CSF, IL-4, IL-12) with local tumor irradiation: about 80–100% of the glioma-bearing mice was cured. The high efficiency of combined treatment was maintained even under suboptimal conditions when neither of the modalities cured any of the mice alone. This suggests that vaccination therapy might open a new potential in the clinical treatment of high-grade gliomas when applied as adjuvant to existing treatment modalities.

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In humans, gliomas constitute about 30–45% of all intracranial tumors. Glioblastoma multiforme is the most frequent form of glioma and is characterized by very rapid local growth and invasiveness. Gliomas do not metastasize, but their local growth is almost always fatal. Recent advances in neurosurgical techniques, radiation therapy, and chemotherapy have failed to improve substantially the prognosis of this malignant tumor. The life expectancy of patients with glioblastoma is usually less than 1 year from the time of diagnosis and the 5-year survival rate is only about 5%.^{1,2} Therefore, it is necessary to develop new therapeutic modalities.

There are several immune therapy approaches that might increase the immunogenicity of the tumors.^{3,4} One possibility is the introduction of cytokine-encoding genes into the tumor cells. This can be achieved either by direct intratumoral injection of viral vectors or by *ex vivo* modification

of the malignant cells. The direct intratumoral vector injection is much simpler than the *ex vivo* modification, but there are certain risks arising from the introduction of a large number of viral particles into the human body. During the *ex vivo* approach, most of the tumor is removed by surgery and a primary cell culture is established from the malignant tissue. Then cytokine-encoding vectors are introduced into the *in vitro* growing tumor cells and cell division is stopped by high-dose irradiation of the culture. Finally, the cytokine-expressing irradiated tumor cells are used to vaccinate the same patient from whom the original tumor was removed. It is expected that the host immune system is activated by the vaccine, and it will attack both the residual tumor cells at the site of the surgery and the cells at distant metastases. The key requirement of this protocol is the presentation of tumor-associated antigens in the micro-environment of cytokine secretion.^{5,6}

The central nervous system (CNS) has long been considered as an immunologically privileged site. Several factors may contribute to this status, including an endothelial structure that restricts passage of macromolecules, viruses, and cells, the absence of lymphatic drainage, and finally the inadequate expression of MHC molecules.

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Address correspondence and reprint requests to: Dr Geza Safrany, Department of Molecular and Tumor Radiobiology, National Research Institute for Radiobiology and Radiohygiene, Anna u. 5, Budapest H-1221, Hungary. E-mail: safrany@hp.osski.hu

Despite difficulties generating an adequate immune response in the CNS, there are evidences that T lymphocytes can penetrate the blood–brain barrier and mediate an efficient tumor growth inhibitory effect.⁷

Formerly, we studied the therapeutic effect of autologous cancer cell vaccines producing either IL-2 or IL-12 in the mouse Gl261 brain tumor model. Both vaccines exhibited antitumor effects: about 30–40% of mice was cured. In both cases, the therapeutic effect of the vaccines strongly depended on the expressed cytokine level.⁸ In the present paper, we report the anticancer effect of GM-CSF- and/or IL-4-secreting glioma vaccines. The novel therapeutic approaches should be used in combination with existing treatment modalities. Therefore, special emphasis was put on the combined antiglioma effect of cytokine vaccination and local tumor irradiation.

Materials and methods

Cell lines, recombinant adenoviruses

Hybridoma cell lines GK1.5 and 53-6.72, secreting monoclonal antibodies against CD4⁺ and CD8⁺ lymphocyte subsets, were bought from ATCC (Manassas, VA, USA) and maintained according to the supplier's suggestion. The Gl261 mouse glioma tumor was originally induced by intracerebral injection of a chemical carcinogen into C57Bl/6 mice⁹ and maintained on its syngeneic host by alternating intracranial and subcutaneous implantation of small tumor pieces. We have established a permanent cell line (Gl261) from the tumor. The cells were maintained in DMEM culture medium supplemented by 10% fetal calf serum. Detailed characterization of the cell line will be published elsewhere.

The adenoviral vectors AdexCAmIL-2, AdexCAmIL-4, AdexCAmIL-6, AdexCAmIL-7, AdexCAmIL-12p35, AdexCAmIL-12p40, AdexCAmGM-CSF, AdexCAmLT, AdexCAmLIF, and AdexCAmTNF- α encoding the corresponding cytokines were created and very generously provided by Dr. Hamada.¹⁰ Note that the two subunits of the mouse IL-12 gene (p35 and p40) were present in two different constructs. During vector creation, the E1A, E1B, and E3 regions of adenovirus type 5 were removed, rendering the vector replication incompetent in most mammalian cells. The E1A–E1B region was replaced with a cloning cassette containing the appropriate cytokine gene. Cytokine expression was driven by an artificial promoter composed of the core promoter region of the chicken β -actin gene and of the enhancer region of the major early promoter of the cytomegalovirus. Transformed human embryonic kidney 293 cells (ATCC) were used for vector propagation and virus isolation.¹⁰

Brain tumor model and treatment by cytokine-secreting autologous mouse glioma vaccines and/or by radiation therapy

Animal studies were done according to Hungarian national regulations. Logarithmically growing Gl261 cells were harvested and suspended in Hank's balanced salt solution. Brain tumors were induced by intracranial injection of $1-2 \times 10^4$ Gl261 cells in 10- μ L final volume. Cells were

implanted through Fissura petrosquamosa into the right cerebral hemisphere of anesthetized 8- to 12-week-old C57Bl/6 mice at 4 mm depth below the surface of the skull using a 250- μ L Hamilton syringe. When radiation therapy was combined with GM-CSF vaccination, 1×10^5 Gl261 cells were transplanted.

Vaccination with cytokine-producing cells was performed 3 days after tumor transplantation. To create the cytokine-producing vaccines, *in vitro* growing Gl261 cells were transduced at different multiplicity of infection (MOI) with the corresponding adenoviral vectors.¹⁰ Cytokine production was measured in the culture supernatant 48 hours later by conventional ELISA kits (Endogen, Woburn, MA, USA and BioSource Europe, Nivelles, Belgium) and cells were irradiated with 20 Gy ⁶⁰Co- γ radiation (Gammatron-3 radiation source, dose rate: 0.4781 Gy/min; Siemens, Erlangen, Germany) to stop cell division. Cells were harvested by trypsin treatment and 1×10^6 cells were injected subcutaneously to vaccinate tumor-bearing mice. Mice were killed when they were moribund or all mice were killed 100 days after tumor induction. According to our experience, if a mouse survived for 100 days, it was completely tumor-free for at least a year. All mice were carefully autopsied. At least three independent experiments including five to seven mice were performed for each treatment.

For local radiotherapy, the head of anesthetized mice was irradiated with 6 Gy x-ray radiation (THX-250 Therapeutic x-ray SOURCE, dose rate: 1.003 Gy/min; Medicor, Budapest, Hungary) 3 days after tumor transplantation. A lead tube covered the other part of the body to protect it from radiation. For the combined modality treatment, vaccination with cytokine-producing cells was performed 1 hour after irradiation.

Statistical analysis was done by Mantel–Cox test. A *P* value of <.02 was considered significant.

Monoclonal antibody production and depletion of CD4⁺ and CD8⁺ lymphocyte subsets

Mouse hybridoma 53-6.72 cells were maintained under *in vitro* conditions as recommended by ATCC and anti-

Figure 1 Cytokine levels secreted by gene-modified Gl261 cells. *In vitro* growing Gl261 cells were transduced at different MOI by adenoviral vectors encoding either IL-4 or GM-CSF. Cytokine concentration was measured in the culture supernatant 24 hours after transduction by conventional ELISA kits.

Figure 2 Brain tumor treatment by cytokine-secreting autologous cancer cells improves survival. Glioma-bearing mice were treated with cytokine-secreting vaccines 3 days after intracranial transplantation of GL261 cells. For vaccine preparation, *in vitro* growing GL261 cells were transduced at the indicated MOI by adenoviral vectors encoding the corresponding cytokines. Cells were irradiated and harvested 48 hours later and 1×10^6 cells were used for subcutaneous vaccination of brain tumor-bearing mice. **A:** Treatment by GM-CSF-secreting vaccines. $P < .008$ for 10 MOI GM-CSF vaccine *versus* the GL261 vaccine. **B:** Vaccination by IL-4-producing cells. $P < .02$ for 100 MOI IL-4 vaccine *versus* the GL261 vaccine. **C:** Treatment by a vaccine secreting both IL-4 and GM-CSF. $P < .001$, $< .016$, and $< .0008$ for the GM-CSF, IL-4, and dual cytokine-secreting vaccines *versus* the GL261 vaccine, respectively.

CD8⁺ antibody was purified by DE52 (Whatman, Springfield, UK) ion exchange chromatography as described.¹¹ To isolate anti-CD4⁺ antibodies, GK1.5 cells

were intraperitoneally injected into nude mice and the collected ascites fluid was directly used after appropriate dilution.¹¹

The CD4⁺ and CD8⁺ lymphocyte subsets were depleted by treating C57Bl/6 mice with corresponding antibodies.¹¹ After pretreatment with monoclonal antibodies, brain tumors were induced and vaccination with GM-CSF-expressing cells was performed as described above.

Cell-mediated cytotoxicity assay

Healthy mice were vaccinated with either irradiated GL261 cells or with mIL-4- or mGM-CSF-producing GL261 cells at therapeutic concentrations (see below). The spleens were collected at different intervals after vaccination (3 days, 1, 2, and 3 weeks) and lymphocytes isolated. The lymphocytes were preincubated in the presence of IL-2 with irradiated GL261 cells for 5 days and then mixed with ⁵¹Cr-labelled GL261 or B16 melanoma cells at different effector-to-target ratios. The amount of radioactivity released in the medium was used for the evaluation of lymphocyte activation.¹²

Immunohistochemistry

Brain tumor-bearing mice were vaccinated with IL-2-, IL-4-, IL-12-, and GM-CSF-secreting irradiated GL261 cells. Mice were killed and brain tumors removed 10 days after vaccination. The tumors were fixed in 4% buffered formaldehyde, embedded in paraffin, and 8- μ m thin sections were cut. Sections were routinely stained in H&E. Immunohistochemical reactions¹³ were performed on fixed sections using polyclonal antibodies directed against CD4⁺ and CD8⁺ cells (Sc-7219 and Sc-7188; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were used in 1:100 dilutions. Diaminobenzidine (DAB) was applied to visualize the reaction. Normal mouse lymph node was used as positive control. Negative controls were

Figure 3 Induction of CTL in mice after vaccination by cytokine-expressing autologous cancer cells. Healthy mice were vaccinated either by irradiated GL261 or by IL-4- or GM-CSF-secreting GL261 cells. CTL activity, against ⁵¹Cr-labeled GL261 cells was measured 3 weeks after vaccination, as described in *Materials and methods*. In the experiment marked with B16, vaccination was performed with GM-CSF-secreting cells, but the targets were ⁵¹Cr-labeled murine B16 melanoma cells.

Figure 4 Immune histochemistry of brain tumors after treatment with GM-CSF-expressing cancer cells. Brain tumor-bearing mice were vaccinated either with Gl261 cells (**Panels A and C**) or GM-CSF-secreting Gl261 cells (**Panels B and D**). Tumor infiltration by CD4⁺ (**Panels A and B**) and CD8⁺ (**Panels C and D**) lymphocyte subsets was investigated 10 days after vaccination by immune histochemistry as described in *Materials and methods*. N, normal brain tissue; T, brain tumor. The arrows show the tumor infiltrating CD4⁺ and CD8⁺ lymphocytes, respectively.

achieved by omitting the primary antibody. Light green was applied as counterstain.

Results

Transduction efficiency and cytokine production in Gl261 cells

First, the transduction efficiency of adenoviral vectors was investigated in Gl261 cells. A construct (Adex1CALacZ) encoding the bacterial *lacZ* gene was introduced into *in vitro* growing Gl261 cells at different MOI and the presence of

lacZ activity measured.¹⁰ At ten MOI, about 50–70% of the cells was transduced. The transduction efficiency increased linearly up to 100 MOI when all of the cells were already infected.⁸ This proved that adenoviral vectors could transduce Gl261 cells with high efficiency.

Next, cytokine-encoding adenoviral vectors (Adex-CAMGM-CSF, AdexCAMIL-4) were introduced into Gl261 cells at different MOI and cytokine expression was measured in the culture medium by conventional ELISA kits. Both GM-CSF and IL-4 levels increased linearly in the medium at least up to 500 MOI (Fig 1). As mentioned before, at 100 MOI, the vector already infected

all of the cells. The linear increase in the cytokine level beyond 100 MOI suggests that the cells were infected not only by one, but also by several virus particles. The data also show that altering the MOI during transduction could very easily modify the cytokine production of the cancer cell vaccines.

Tumor growth inhibition by cytokine-producing autologous glioma cell vaccines

The therapeutic effect of cytokine-producing autologous cancer cell vaccines was investigated in a mouse glioma 261 brain tumor model. Brain tumors were transplanted by intracranial injection of $1-2 \times 10^4$ GI261 cells. Three days later, tumor-bearing mice were vaccinated with GI261 cells expressing various levels of different cytokines. For vaccine preparation, *in vitro* growing GI261 cells were transduced at different MOI with adenoviral vectors encoding various cytokines (IL-4, IL-6, IL-7, GM-CSF, LIF, LT, and TNF- α). Two days later, cytokine-producing cells were irradiated, harvested, and used for subcutaneous vaccination of brain tumor-bearing mice.

The therapeutic effect of the vaccines strongly depended both on the type and on the level of the secreted cytokine. Vaccines expressing IL-6, IL-7, LIF, LT, and TNF- α were inefficient to prolong the survival of glioma-bearing mice when produced by transduction at 10, 50, 100, or 200 MOI (data not shown). However, vaccines producing either GM-CSF or IL-4 cured a substantial portion of the mice (Fig 2A and B). In these cases, the survival rate depended very strongly on the produced cytokine level. For the GM-CSF-expressing vaccine, the highest survival rate (30–40%; Figure 2A) was obtained when 10 MOI was used for transduction during vaccine preparation. This vaccine produced about 10 ng of GM-CSF/ 1×10^6 cells/24 h (Fig 1). Vaccines, secreting higher levels of GM-CSF (50 or 100 MOI), were much less efficient (Fig 2A).

IL-4-secreting vaccines were effective, if cells were transduced at 100 MOI with the adenoviral vector. This vaccine expressed about 50 ng of IL-4/ 1×10^6 cells/24 h (Fig 1) and prolonged the lifespan and/or cured a significant portion of mice (Fig 2B). Vaccines produced at lower MOI were inefficient.

Because both IL-4- and GM-CSF-producing vaccines were able to cure mice, we studied the combined effect of these two vaccines. Glioma-bearing mice were treated with GI261 cells that were co-transduced with IL-4 (100 MOI) and GM-CSF (10 MOI) encoding adenoviral vectors. The therapeutic effect of the double cytokine-secreting vaccine was similar to the GM-CSF-producing vaccine (Fig 2C).

Vaccination by cytokine-producing cells activates cell-mediated antitumor immune response

Three lines of experiments were performed to study the activation of the immune system against the tumor after vaccination with cytokine-producing GI261 cells. First, we

investigated the activation of cytotoxic T lymphocytes (CTL) against glioma cells. Healthy mice were vaccinated with IL-4- or GM-CSF-expressing GI261 cells at therapeutic cytokine concentrations as described above. Mice were killed at different intervals after vaccination and lymphocytes were prepared from the spleen. The CTL response was measured as described in *Materials and methods*. CTL activation was not detected during the first week, and then it gradually increased and peaked 3 weeks after vaccination (Fig 3). The CTL response was much higher for the GM-CSF-producing vaccine than for the IL-4-secreting vaccine.

The involvement of CD4⁺ and CD8⁺ lymphocyte subsets in the antitumor response has been also studied. Glioma-bearing mice were vaccinated with IL-2-, IL-4-, IL-12-, or GM-CSF-expressing cells. The controls were vaccinated with irradiated GI261 cells. Mice were killed 10 days later, brain tumors removed, and the infiltration of tumors by CD4⁺ and CD8⁺ lymphocyte subsets was investigated by immune histochemistry. When the tumor-bearing mice were treated with GM-CSF-producing cells, the brain tumors were heavily infiltrated by CD4⁺ lymphocytes (Fig 4A and B). Similar data have been obtained with IL-2-, IL-4-, and IL-12-secreting cells (data not shown). The GM-CSF-producing vaccine induced moderate tumor infiltration by CD8⁺ lymphocytes (Fig 4C and D), as well. CD8⁺ infiltration was not detected with other vaccines (not shown).

The role of the CD4⁺ and CD8⁺ lymphocytes in the antitumor response was further investigated by depleting these cells before tumor induction. Healthy mice were pretreated with antibodies against either CD4⁺ or CD8⁺ lymphocyte subsets.¹¹ Brain tumors were induced by intracranial injection of GI261 cells and 3 days later, glioma-bearing mice were vaccinated with GM-CSF-producing autologous cancer cells. Depletion of either CD4⁺ or CD8⁺ lymphocytes equally prevented the antitumor effect of the vaccine (Fig 5).

Figure 5 Depletion of CD4⁺ and CD8⁺ lymphocytes eliminates the tumor growth-inhibitory effect of GM-CSF-producing vaccines. Glioma-bearing mice were vaccinated with autologous cancer cells transduced at 10 MOI by AdexCamGM-CSF. In experiments labeled as GM-CSF+anti-CD4⁺ and GM-CSF+anti-CD8⁺, the corresponding lymphocyte subsets were eliminated by pretreatment with appropriate monoclonal antibodies before tumor transplantation. $P < .006$ for 10 MOI GM-CSF vaccine versus the GI261 vaccine.

Combination of antiglioma vaccination with local tumor irradiation considerably improves survival

As mentioned above, vaccination with certain cytokine-expressing autologous cancer cells was able to cure about 20–40% of glioma-bearing mice (Fig 2). We have investigated whether the combination of the vaccination protocol with local radiation therapy could further improve survival. The tumor was induced by intracranial transplantation of $1-2 \times 10^4$ GL261 cells. Three days later, the head of the glioma-bearing mice was irradiated with 6 Gy x-ray radiation. One hour after irradiation, mice were

Figure 6 Combination of local tumor irradiation with vaccination by cytokine-producing cancer cells improves survival. **A:** Vaccination with IL-4– or IL-12–producing cells. Brain tumors were induced by intracranial transplantation of 1×10^4 GL261 cells. Glioma-bearing mice were vaccinated with IL-12– or IL-4–secreting cells and/or irradiated with 6 Gy x-ray radiations. $P < .01$ and $< .02$ for the IL-12 and IL-4 vaccines *versus* the GL261 vaccine. $P < .000$ and $< .000$ for the combined treatments with irradiation and vaccination with IL-12– or IL-4–secreting cells *versus* the GL261–vaccinated and -irradiated group, respectively. **B:** Vaccination with GM-CSF–secreting cells. Brain tumors were induced by intracranial transplantation of 1×10^5 GL261 cells. Mice were treated with GL261 cells, with GM-CSF–producing GL261 cells transduced at 200 MOI, and with the 1:3 mixtures of transduced and nontransduced cells, respectively. The combined modality groups were vaccinated as shown, and local irradiation was performed with 6 Gy x-ray. $P < .002$ for the combined treatment with radiation and vaccination with GM-CSF–secreting cells and $< .000$ for the treatment with radiation and vaccination with 1:3 mixture of GM-CSF–secreting and nonproducing cells *versus* the GL261–vaccinated and -irradiated group, respectively.

vaccinated with either IL-4– or IL-12–producing⁸ GL261 cells at therapeutic cytokine concentrations. The combination of radiotherapy with vaccination by cancer cells secreting either IL-4 or IL-12 was very efficient. The tumor was completely eliminated in about 80% of glioma-bearing mice by the combined modality treatments (Fig 6A).

To investigate the limits of the combined protocol, brain tumor was induced by 10-fold higher cell number (1×10^5) as usual. This cell dosage killed the untreated mice very rapidly. Three days later, the head of the mice was irradiated as mentioned before. Within 1 hour, mice were vaccinated with GM-CSF–expressing GL261 cells transduced at 200 MOI. Vaccination alone with cells expressing such a high level of GM-CSF has not improved survival rate at all (Fig 6B). To slightly reduce the secreted cytokine level, GM-CSF–producing cells were mixed in 1:3 ratios with nontransduced cells. Vaccination with this mixture moderately improved life-span, but none of the mice was cured of the tumor. Radiation alone slightly increased survival rates, but again, none of the mice survived. However, the combination of vaccination therapy with local irradiation considerably enhanced survival. About 80% of the mice was completely cured of the tumor if they were irradiated and vaccinated with cells secreting high levels of GM-CSF. When the secreted GM-CSF levels were slightly lower (1:3 mixtures of GM-CSF transduced and nontransduced cells), all of the mice recovered from the brain tumor (Fig 6B).

Discussion

High-grade gliomas belong to the most aggressive malignant diseases with very poor prognosis. The induction of the immune system against cancer cells might open new potentials in the treatment of brain tumors.¹⁴ Recently, the potential tumor growth inhibitory effect of various immunostimulatory protocols has been studied by a number of investigators in intracranial tumor models. The models included intracerebrally implanted murine melanomas,^{15–17} as well as rat^{18–26} and murine glioma cells.^{27–32} The immunostimulatory genes were delivered by several vehicles, including retroviral vectors, vaccinia virus, plasmids, and liposomes. Interestingly, adenoviral vectors were rarely used for this purpose.³³ Our data showed that the advantage of the adenoviral vector system is its high transduction efficiency. At rather low MOI, both murine GL261 cells, as well as primary human glioma cells (latter is not shown), could be transduced with 100% efficiency by adenoviral vectors. The other benefit of the adenoviral vector system is that at high MOI, the transduced cells may contain many viral particles. The cytokine level produced by the vaccine is linearly related to the number of viral particles per cell or to the applied MOI. With adenoviral vectors, it is easy to alter the cytokine level secreted by the gene-modified cancer cells.

A high number of cytokine genes (IL-2, IL-4, IL-6, IL-7, IL-12, GM-CSF, LIF, LT, and TNF- α) have been used by

us for the genetic modification of the *ex vivo* growing tumor cells. Others have not tested such a high panel of cytokines against gliomas. We have found that from the wide panel of cytokines, only IL-2,⁸ IL-4,⁸ IL-12,⁸ and GM-CSF-secreting vaccines prolonged lifetime and cured substantial proportion of glioma-bearing mice. This is in good agreement with previous findings that GM-CSF,^{15–17,28,30} IL-4,^{15,18,19,22,25} IL-12,^{21,24} or IL-2^{21,23,31,33} production by cancer cells is in association with prolonged antitumor effect.

Our data suggested that the anticancer effect strongly depended not only on the type, but also on the quantity of the secreted cytokine. The optimal cytokine concentrations were about 10, 50, 60, and 30 ng/1×10⁶ cells/24 h for vaccines producing GM-CSF, IL-4, IL-2,⁸ or IL-12,⁸ respectively. In the case of IL-4-, IL-2-,⁸ or IL-12-expressing⁸ vaccines, the therapeutic effect increased at higher cytokine concentrations. In contrast, the antitumor effect of the GM-CSF-producing vaccine decreased at higher cytokine levels (Fig 2A). Most strikingly, the GM-CSF vaccine produced at 200 MOI (80 ng GM-CSF/1×10⁶ cells/24 h) was ineffective when applied alone. When the cytokine concentration secreted by the latter vaccine was lowered, by mixing GM-CSF-producing cells with nonsecreting cells in 1:3 ratios, the lower cytokine concentration resulted in a slightly improved anticancer effect. The increased tumor growth-inhibitory effect of the mixture was maintained when vaccination therapy was combined with radiation therapy (Fig 6B). The scientific background of the strong dependence on the cytokine concentration is not completely clear. It is well known, however, that a certain cytokine can influence a wide range of immune stimulatory mechanisms and usually different optimal concentrations are necessary for various target cells.³⁴ Probably, only the activation of certain target cells will result in long-lasting antitumor immunity. It is also possible that supraoptimal doses may prove inhibitory for a certain action.³⁴ We think that the strong dependence of the anticancer effect on the cytokine level might explain the very moderate success of clinical trials using autologous cancer cell vaccines for the treatment of human malignancies.

Wakimoto *et al*¹⁵ used a vaccine secreting both GM-CSF and IL-4 for the treatment of intracranial transplanted B16 melanoma and reported that the tumor growth-inhibitory effect was superior to a single cytokine-secreting vaccine. In the GL261 mouse glioma model, we were unable to detect an increased survival for this combination. However, we cannot exclude that under suboptimal cytokine concentrations, the dual cytokine secretion is beneficial.

There are controversial data in the literature about the role of CD4⁺ and CD8⁺ lymphocyte subsets in the anticancer effect of cytokine-modified cells. Several authors suggested that both cell types play an important role in the tumoricidal effect.^{15,17,18,23} Others support the preferential role of either CD4⁺¹⁹ or CD8⁺ cells.^{16,26,27,29} According to our finding, both CD4⁺ and CD8⁺ cells are necessary for tumor eradication, at least for the GM-CSF-secreting vaccine. Pretreatment of brain tumor-bearing mice with monoclonal antibodies against either CD4⁺ or CD8⁺ lymphocytes

completely abolished the antitumor effect of the GM-CSF vaccine (Fig 5). Moreover, when glioma-bearing mice were treated with GM-CSF-producing cells, the intracranial tumor was heavily infiltrated by CD4⁺ and moderately by CD8⁺ cells (Fig 4). We should mention that 10 days after treatment, the IL-2-, IL-4-, or IL-12-producing vaccines induced only CD4⁺ infiltration at the tumor site. It is possible that CD8⁺ infiltration could be detected at other time points for these vaccines, as well.

The most important finding of our work is that local radiation therapy might be very efficiently combined by vaccination with cytokine-expressing autologous cancer cells for the treatment of experimental brain tumors. Recently, some other investigators also suggested that the combination of gene therapy with tumor irradiation might be beneficial. The intratumoral administration of a secreted angiostatin-like molecule into glioma xenografts by adenoviral injection had only marginal influence on tumor growth alone. However, its combination with radiation therapy resulted in a synergistic effect probably through the inhibition of tumor vascularization.³⁵ Others detected the enhanced radiosensitivity of glioma cells after transduction with wild-type p53 encoding vectors.^{36–38} The radiosensitising effect of p53 is probably established through its apoptotic effect and p53 might also suppress tumor vascularization.

Li *et al*³⁹ and Staba *et al*⁴⁰ reported that the combination of radiation with intratumoral administration of a cytokine (TNF- α)-encoding vector substantially slowed down tumor progression. We have found that cytokine-expressing vaccines might cure only 30–40% of brain tumor-bearing mice. Local radiation therapy alone hardly increased lifespans. However, the combination of these two modalities improved survival rates up to 80–100% (Fig 6). One simple explanation for the synergistic effect of vaccination and radiation therapies is that there is a continuous competition between tumor growth and tumor eradication by the activated immune system. Local irradiation decreases the tumor burden, so the activated immune system could overcome the decreased tumor mass. Another possibility is that after irradiation, the primary tumor cells die by necrosis. The necrotic death might lead to the liberation of immunogenic molecules that further enhances immune response.

The presented data indicated that the secreted cytokine level strongly influenced the antitumor effect of the cancer cell vaccines. Therefore, we suggest that future clinical trials should focus on the determination of the optimal cytokine concentrations by measuring the activation of the immune system. It is also possible that cytokine levels should be adjusted to the individual needs of the cancer patient. Our most important finding is that vaccination with cytokine-expressing cancer cells could be combined successfully with local irradiation of the tumor even under suboptimal therapeutic conditions. This suggests that vaccination therapy might open a new potential on the clinical treatment of gliomas when applied as an adjuvant treatment to existing therapeutic modalities, namely to surgery and radiation therapy, to eradicate residual tumor cells.

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