Induction of a therapeutic antitumor immunological response by intratumoral injection of genetically engineered Semliki Forest virus to produce interleukin-12

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Object. The authors investigated immunogene therapy for malignant glioma to determine whether its therapeutic efficacy could be improved.

Methods. Four groups of 203-glioma-bearing mice were treated with injections of phosphate-buffered saline, Semliki Forest virus (SFV)–LacZ, retrovirus vector DFG–interleukin (IL)–12, and SFV–IL12, respectively.

The results indicated that therapeutic immunization with SFV–IL12 prolonged the survival of mice with established tumors. Semliki Forest virus induces apoptotic death to glioma cells, which facilitates the uptake of apoptotic cells by dendritic cells, providing a potential mechanism for enhanced immunogenicity.

Conclusions. Immunogene therapy with IL-12 via SFV may be an excellent candidate for the development of new cancer vaccines.

KEY WORDS • interleukin-12 • Semliki Forest virus • apoptosis
• immunogene therapy • malignant neoplasm • glioma

The prognosis for patients with malignant glioma has not improved much over the past years. Treatments, including surgery, radiotherapy, and chemotherapy have not changed the prognosis for a patient with these malignant neoplasms. Therefore, the development of novel therapies for malignant glioma is essential. The CNS is an immunologically privileged region, considered to lack lymphoid reactivity and normal immune surveillance. Recently, several groups have reported immunogene therapy protocols in which they used intradermal vaccines of genetically modified tumor cells; the treatment was shown to be effective in rodent brain tumor models.^{9,23}

Interleukin-12 is a heterodimeric protein consisting of two subunits (p35 and p40) and is secreted by antigen-presenting cells such as DCs and macrophages.^{10,28} It enhances the proliferation and cytolytic functions of T cells and NK cells and promotes these effector cells to produce a number of other cytokines, including IFN- γ .⁴ Furthermore, IL-12 directs the differentiation of uncommitted T cells toward the T helper type 1,¹² which is critical for cell-mediated antitumor immunity. The antitumor effects of

IL-12 are mediated by activation of CTLs as well as NK cells^{5,15,30} and by the induction of IFN- γ production by T cells and NK cells.^{3,17} Recently, several investigators have reported potent antitumor effects of IL-12 delivery by using *IL-12* gene-modified tumor cells or systemic administration of IL-12 protein.^{4,17,25} Based on these results, clinical trials of *IL-12* gene therapy in which autologous fibroblasts were used have been completed.²⁷ Partial response was observed in patients with several types of cancer. Delivery to the tumor site of *IL-12* genes that are genetically modified to express gene products that enhance their ability to elicit a response may avoid the requirement for selective recruitment and local activation, which are disordered within the tumor microenvironment. We have observed that IL-12 production on DCs is suppressed in patients with malignant glioma (unpublished data); therefore, we suspect that one of the mechanisms of immunosuppressive properties in malignant glioma patients is IL-12 dysfunction, resulting in failure to promote and maintain antigen-specific T cells. To enhance the immune response further, we evaluated transduction of *IL-12* genes in a murine brain tumors model. We investigated whether *IL-12* gene transduction into tumor may serve a possible strategy to treat malignant glioma.

Viral vectors commonly used for therapeutic DNA delivery include replication-deficient forms of adenovirus and retrovirus. Although retroviruses have the advantage of mediating stable gene transfer with a low potential for

Abbreviations used in this paper: BHK = baby hamster kidney; CNS = central nervous system; CTL = cytotoxic T lymphocyte; DC = dendritic cell; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescent-activated cell sorter; FCS = fetal calf serum; IL = interleukin; IFN = interferon; NK = natural killer; PBS = phosphate-buffered saline; SFV = Semliki Forest virus.

immunogenicity, this vector delivery system has several therapy-related problems including difficulty in producing high titers of retrovirus, the fact that only actively dividing cells are capable of being infected, and the possibility of insertional mutagenesis.¹⁶ Whereas the adenovirus vector system is capable of delivering genes with high efficiency to a wide spectrum of nondividing cells *in vivo*,⁷ unfortunately, it has produced only transient expression with different gene products. Transient expression may result from a strong immune response of host cells against the adenovirus.⁸ The SFV, a member of the Alphavirus, has received considerable attention for use as a virus-based expression vector. The SFV expression system differs from currently available viral delivery systems in that it is an RNA virus, known to generate high levels of protein expression *in vitro*. The SFV is less pathogenic to humans.¹⁹ It is a self-amplifying expression vector that produces high-titered stocks of infectious particles, infects nondividing cells, and generates high-level expression of transgenes.²⁴

We have previously reported that genetically modified DC-pulsed tumor complementary DNA via SFV markedly enhanced antitumor immune responses in murine glioma models.³² In the studies presented here we evaluated the efficacy of genetically modified SFV to produce IL-12. The vaccines were studied in a model of active immunogene therapy for CNS tumors.

MATERIALS AND METHODS

Tumor Cell Lines and Animal Models

The 203-glioma cell line is derived from C57BL/6 mice. Baby hamster kidney cells were grown in minimum essential medium containing 10% FCS. All cell lines were shown to be free from mycoplasma contamination. In all experiments we used 6 to 12-week-old female C57BL/6 mice, which were maintained in a virus-free environment and treated in accordance with the National Institutes of Health standards.

Generation of SFV-IL12 Particles

The plasmid pSFV3, pSFV3-LacZ contains an SP6 promoter, a 7-kb fragment encoding the SFV RNA replicase, and a subgenomic promoter that is bound by the RNA replicase to synthesize a large quantity of subgenomic RNA. A helper plasmid, pSFV–Helper 2, which contains the genes for the structural proteins (capsid, E3, E2, 6K, and E1) required for packaging of the viral genome, was used for construction of the recombinant envelope gene. Retrovirus vector DFG-IL12 was kindly provided by Dr. Nishioka.¹⁸ The *IL-12* gene was amplified using DFG-IL12 as a template and put into pSFV. The SFV-IL12 RNA was transcribed *in vitro* and capped using SP6 RNA polymerase and a capping analog. The BHK cells were transfected with SFV-IL12 RNA and helper virus RNA, respectively. For cotransfections of helper and expression RNA into BHK cells, electroporation was performed. Electroporated cells were transferred to 10 ml of minimum essential medium containing 5% FCS and incubated for 12 hours. Cells were then washed with PBS and incubated in 10 ml of minimum essential medium without FCS. After 24 hours, culture supernatants were harvested,

and aliquots were stored at -80°C . Recombinant SFV particles were produced. Retroviral particles were also generated as described elsewhere.¹⁸ Infectivity of recombinant viruses to BHK and 203 glioma was determined by transfer of the SFV and retroviral particles that can transduce the β -galactosidase gene. Viral supernatant dilutions were added to adherent cells (2×10^5) in 6-well plates. After 1 hour of incubation at 37°C , cells were washed with PBS and incubated in growth medium at 37°C for 24 hours. Viral infection was evaluated by X-galactosidase staining. Briefly, cells were fixed in PBS containing 0.5% glutaraldehyde for 15 minutes followed by washing with PBS three times. Then cells were stained with PBS containing 1 mg/ml X-galactosidase, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM MgSO_4 at 37°C for 2 hours. The activity of IL-12 was measured using ELISA, and biological activity to stimulate IFN- γ production from ConA-treated splenocytes was also confirmed. The splenocytes ($1 \times 10^5/\text{ml}$) was stimulated by supernatant of transfectant, and the IFN- γ production was also estimated by ELISA.

In Vitro Cytotoxicity Assay

In vitro cell-mediated cytotoxicity assays were performed using standard procedures as previously described.³¹ In this study, splenocytes obtained in immunized and control mice were restimulated *in vitro* for 5 days on monolayers of mitomycin C-treated 203-glioma cells. Target cells included 203-glioma cells.

Implantation of Brain Tumors

The glioma cells were harvested by trypsinization and washed twice in Dulbecco PBS. The cells (500 cells in a volume of 5 μL) were then implanted into the right-sided caudate nucleus of the brain of the C57BL/6 mice by stereotactic injection, as previously described.³¹ The mice were examined macro- and microscopically. Intratumoral cells were evaluated using anti-CD8 antibody and fluorescein isothiocyanate labeled anti-rat immunoglobulin G.

Cell Death and Uptake Analysis

The cell death and uptake analysis was performed as previously described.³³ Briefly, the self-replicating SFV was transcribed *in vitro* from the pSFV-Lac Z or IL-12 plasmid and then transfected into the 203-glioma cells. As a control, the plasmid pLac Z-C1 was also transfected into the 203-glioma cells. Viable cells were counted every 24 hours. In uptake experiments, 203-glioma cells were transfected with SFV-IL12 and then labeled 24 hours later with PKH26-GL. Labeled cells (1×10^6) were cocultured with 1×10^6 bone marrow-derived immature DCs prepared by culture of bone marrow cells for 4 days with granulocyte-macrophage colony-stimulating factor and IL-4. Cells were then stained with fluorescein isothiocyanate-labeled anti-I-A^b monoclonal antibody and analyzed by FACS.

Statistical Analysis

Survival estimates and median survivals were determined using the method of Kaplan and Meier. Survival data were compared using the Wilcoxon test. The Student t-test was used for calculating the significance of

Fig. 1. Graph showing that 203-glioma cells can be efficiently transduced with an SFV-IL12 vector and produce IL-12. The production of IL-12 by IL-12 gene-modified glioma cell is shown. The glioma cells were transduced with DFG-IL12, SFV-LacZ, and SFV-IL12, respectively. After transduction on Day 5, supernatant in the glioma cell culture was collected and assayed in an IL-12 ELISA.

other data. Statistical significance was considered to be less than 0.05.

Sources of Supplies and Equipment

The 203-glioma cell line was kindly provided by Riken Cell Bank (Tsukuba, Japan). The BHK cells were acquired from Invitrogen (San Diego, CA). The minimum essential medium was purchased from GIBCO-BRL (Gaithersburg, MD). We obtained the mice from Jackson Laboratory (Bar Harbor, ME).

The plasmid pSFV3, pSFV-LacZ was acquired from Life Technologies (Gaithersburg, MD), as was the capping analog. The retrovirus vector DFG-IL12 was kindly provided by Dr. Nishioka (Department of Surgery, School of Medicine, University of Pittsburgh Cancer Institute, University of Pittsburgh). Cells were incubated in minimum essential medium obtained from GIBCO-BRL. The IL-12 activity was measured by an ELISA kit produced by R&D systems (Tokyo, Japan). The anti-CD8 antibody and fluorescein isothiocyanate-labeled anti-rat immunoglobulin G were obtained from Pharmingen (La Jolla, CA), as was the anti-I-A^b antibody. The XTT assay kit (Boehringer Mannheim, Tokyo, Japan) was used to count viable cells. In uptake experiments, glioma cells were transfected and later labeled with PKH26-GL obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Dendritic Cell Generation and Infection With Recombinant Viruses

As described previously,² after 7 days of culture, DC aggregates were observed, and these cells were major his-

topatibility complex class I, major histocompatibility complex class II, CD80, and CD86+, respectively. Infectivities of recombinant viruses were determined by transfer of the pSFV-lacZ, DFG-lacZ that can transduce the β -galactosidase gene. Viruses had a very high infectious titer against BHK cells (5×10^7 lacZ cfu/ml), and 203-glioma (4×10^7 lacZ cfu/ml). After completion of transduction procedures performed on Days 3 and 7, the concentration of IL-12 p70 heterodimer released into the culture media was measured in an ELISA. As shown in Fig. 1, accumulation of IL-12 was observed in cultures of SFV-IL12- and DFG-IL12-transduced 203-glioma cells but not in cultures of PBS- and SFV-LacZ-transduced cells. Interleukin-12 production from IL-12-transduced 203-glioma cells ranged from 150 to 180 ng/10⁶ cells/48 hours for DFG-IL12 and 650 to 750 ng/10⁶ cells/48 hours for SFV-IL12. The IL-12 protein produced by the gene-modified 203-glioma cells was confirmed to be biologically active and capable of stimulating IFN- γ production from ConA-treated splenocytes (data not shown).

Prolonged Survival of Mice Bearing CNS Glioma Cells and Treated Using IL-12 via SFV

When considering the clinical application of a tumor vaccination strategy, it is realistic to treat animals with tumor present at the time of vaccination. Thus 500 viable 203-glioma cells were implanted in the brain of naïve mice, and treatment was undertaken 7 days later. Animals were separated into groups and received three intratumoral injections spaced 1 week apart with PBS, SFV-LacZ, DFG-IL12, and SFV-IL12, respectively. The titer of these virus was all 1×10^8 particles/ml. Mice in the first three groups had median survivals of 20 to 35 days. Mice treated with IL-12 via SFV had a significantly longer median survival of 90 days ($p < 0.05$); seven of 14 of these mice were alive at Day 90 when the experiment was stopped.

Tumor-Specific Immune Response Identified After Treatment With SFV-IL12

We have investigated whether intratumoral injection of IL-12 via SFV is capable of inducing tumor-specific CTLs. Standard cytotoxicity assays were performed using splenocytes harvested from treated animals 7 days after the third intratumoral injection and were restimulated for 5 days in vitro with irradiated 203-glioma cells. Cytotoxic activity was tested against glioma cells (Fig. 2). As shown in Fig. 3, SFV-IL12 immunization induced 203 glioma cell-specific CTL responses that were statistically significant compared with animals immunized using either SFV-LacZ, DFG-IL12, or PBS ($p < 0.01$).

Intratumoral Infiltration of CD8 T Cells Evident in SFV-IL12-Treated Mice

Examination of the brains of the SFV-IL12-treated mice that survived past 90 days revealed no evidence of tumor, suggesting eradication of established lesions in these long-term survivors. Immunohistochemical analysis of mice treated with SFV-IL12 documented an increased intratumoral infiltration of CD8 T cells (Fig. 4). This suggests the presence of the CTL response in vivo.

Fig. 2. Graph showing induction of specific lytic activity against tumor cells after immunization with SFV-IL12. Mice were immunized three times with either PBS, SFV-LacZ, DFG-IL12, and SFV-IL12. Seven days later, splenocytes were isolated and restimulated for 5 days with irradiated 203-glioma cells. Cytotoxic activity was then measured by Cr release assay by using glioma as the targets. Error bars indicate one standard deviation of the mean.

Apoptosis Evident After Transfection With SFV and Uptake of SFV-Induced Apoptotic Cells by DCs

After being transfected with SFV-IL12, cells rounded up, shrank, and stopped dividing after 24 hours. At 48 hours, these cells showed nuclear fragmentation characteristic of apoptosis (data not shown). Nearly all of the cells were dead by 96 hours. The 203-glioma cells transfected with a conventional DNA plasmid encoding LacZ remained nearly as proliferative as nontransfected control cells (Fig. 5 upper). In the uptake experiment, cells were evaluated at 3 and 24 hours by using FACS analysis for PHK (red) and I-A^b (green). Double-positive cells were counted as a fraction of total FACS events. Uptake of SFV-induced apoptotic cells by DCs was also evident (Fig. 5 lower).

DISCUSSION

In this study, we demonstrated the prolongation of survival in tumor-bearing animals and induction of CTL response by using our vaccine strategy. The treatment of tumor-bearing mice with SFV-IL12 led to a dramatic reduction in the mortality rate at 90 days. At 90 days, 50% of the treated animals were still alive, and histological analysis indicated that SFV-IL12 therapy led to the eradication of established tumors in those long-term survivors. Systemic immune responses, as demonstrated by CTL activity, were significantly higher and tumor specific when SFV-IL12 was used.

Fig. 3. Graph showing that the treatment of established 203-glioma with SFV-IL12 prolongs survival. Intracranial tumor challenge with glioma cells was performed, and 4 days later, the mice were treated with a total of three immunizations spaced 1 week apart. Mice were evaluated daily until death. Median survival, was as follows: PBS group (10 mice) 20 days; SFV-LacZ group (12 mice), 20 days; DFG-IL 12 group (12 mice), 35 days; and SFV-IL12 group (14 mice), 90 days.

Recent advances in tumor immunology have made it possible to evaluate new cytokine-based anticancer therapies.^{6,22} Among many cytokines evaluated as anticancer agents, IL12 confers potent antitumor immunity in murine tumor models.^{4,17,20,26,29} Systemic administration of IL-12 also was associated with severe dose-dependent toxicity in patients during the first clinical trial. The localized transfer of cytokine genes may circumvent some of the toxicity of systemic IL-12 delivery and provide adequate local cytokine levels for immune cell activation.^{17,20,26} In a recent report the authors showed that IL-12 acts directly on DCs to promote nuclear localization of nuclear factor κ B and primes DCs for IL-12 production.¹¹ This explains why the IL-12 gene is more effective in tumor treatment application. Cytokine and chemokine production induced by IL-12 stimulation at the tumor site might be responsible for these effects through alteration of the tumor microenvironment. A number of individual cytokine genes have also been shown to be effective in gene therapy studies in mouse tumor systems.

Semliki Forest virus is being developed as a vector for expression of heterologous genes and has many advantages for expression vector systems. In SFV systems, because helper RNA does not contain a packaging signal, it will not form a defective interfering particle or be packaged with recombinant RNA. Furthermore, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule, without a DNA intermediate.²⁴ This behavior is in contrast with retroviruses, which must enter the nucleus and integrate into the host genome to initiate

Fig. 4. Photomicrograph showing that 203-glioma vaccinated with SFV-IL12 demonstrates increased intratumoral infiltration of CD8 T cells. Intratumoral cells were evaluated using anti-CD8 antibody and fluorescein isothiocyanate-labeled antirat immunoglobulin G original magnification $\times 400$.

vector activity. Thus, retrovirus vectors have applications for long-term expression of foreign genes, whereas SFV is useful primarily for transient high-level expression. Furthermore, although adenovirus vectors can express high levels of foreign genes, these systems are more complex than SFV and express many highly antigenic virus-specific gene products including structural proteins.²¹ In contrast, current SFV systems express only the four viral replicase proteins (ns P1–4) required for RNA amplification in the transduced cells. The self-replicating RNA produced more than 200% the amount of antigen produced by a conventional DNA immunogen.³³ This system has been found to express significant quantities of heterologous proteins *in vitro*¹³ and *in vivo*.¹⁹ As we have shown, DCs acquire antigen from apoptotic cells for further presentation to T cells.^{1,32} The SFV induces apoptosis in infected cells.^{14,33} Thus, it is possible that both processes, activation and apoptosis, as a result of the replicase activity upon SFV-IL12 infection, play an important syngeneic role in the initiation of specific immune responses against tumor cells. The other mechanisms underlying the enhanced immunogenicity is the enhanced uptake of antigen by DCs and other professional antigen-presenting cells. The results of this study suggest that intratumorally injected non-transduced immature DCs can acquire and process tumor antigens *in situ*, migrate to lymphoid organs, and initiate a significant tumor-specific immune response. In summary, we found that intratumoral injection with SFV-IL12 mediates an effective antitumor response that is superior to that observed in retroviral transduced IL-12 and is capable of inducing systemic antitumor immunity. Thus, the self-

Fig. 5. Graphs showing induction of apoptotic death of host cells by SFV and its effect on the uptake by DCs. *Upper:* The SFV induces host cell death. The SFV-IL12 was transfected into 203-glioma cells; a control plasmid containing the *LacZ* gene under the control of the cytomegalovirus promoter was also transfected into glioma cells. Viable cells were counted every 24 hours to determine their survival. *Lower:* Uptake of SFV induced apoptotic cells by DCs. Unmanipulated or SFV-IL12-transfected glioma cells were labeled with PKH26-GL and then fed to immature DCs. Cells were evaluated at 3 and 24 hours by using FACS analysis for PKH and I-A^b. Double-positive cells were counted as a fraction of total FACS events.

replicating SFV system could serve as a powerful tool in treating malignant brain tumors.

CONCLUSIONS

The use of SFV-IL12 for the treatment of experimental brain tumors prolonged survival in tumor-bearing animals and induced CTL response. Thus, the self-replicating SFV system may be a novel approach for the treatment of malignant glioma.

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