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Immunogene therapy of recurrent glioblastoma multiforme with a liposomally encapsulated replication-incompetent Semliki forest virus vector carrying the human interleukin-12 gene – a phase I/II clinical protocol

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Summary

Glioblastoma multiforme (GBM) is an incurable brain tumor resistant to standard treatment modalities such as surgery, radiation, and chemotherapy. Since recurrent GBM tends to develop predominantly within the infiltrative rim surrounding the primary tumor focus, novel therapy strategies need in addition to focal tumor destruction to target this somewhat diffuse area.

This is a phase I/II clinical study in adult patients with recurrent GBM which is aimed at evaluating biological safety, maximum tolerated dose, and antitumor efficacy of a genetically modified replication-disabled Semliki forest virus vector (SFV) carrying the human interleukin 12 (*IL-12*) gene and encapsulated in cationic liposomes (LSFV–IL12). The vector will be administered in doses of 1×10^7–1×10^9 infectious particles by continuous intra-tumoral infusion, thus exploiting the advantages of convection-enhanced drug delivery in the brain. The present protocol is also designed to investigate systemic and local immune response and to identify factors predicting tumor response to LSFV–IL12 therapy, such as volume of extracellular space of the tumor, volume of contrast enhancing lesion, and immune status of the patients.

SFV, an insect alphavirus, infects mitotic and non-mitotic cells and triggers apoptosis in tumor cells within 48–72 h. Preclinical work with the LSFV–IL12 vector in breast and prostate cancer animal models demonstrated its biosafety and some antitumor efficacy. An ongoing phase I clinical study in patients with melanoma and renal cell carcinoma seems also to confirm the biosafety of intravenously administered vectors.

This protocol will be the first study of SFV–IL12 therapy of human recurrent GBM.

Introduction

Malignant glioma is the second leading cause of cancer mortality in adults under 35 years of age, and the fourth leading cause in those under 54 years. Median survival time in patients with glioblastoma multiforme (GBM), the most common type of malignant glioma, ranges between 40 and 50 weeks, with most patients surviving less than 2 years [1–3]. Despite aggressive multimodal therapy which includes surgical debulking of tumor, post-operative radiation and/or chemotherapy, prognosis in GBM patients still remains poor. Median survival time after GBM recurrence is only 3–6 months depending on age and Karnofsky performance score [4,5]. The main challenges in treating GBM are local recurrences after initial response to treatment, or progressive tumor growth and resistance to adjuvant therapy.

Therefore, alternative therapeutic approaches are necessary, which ideally should be based on specific biological features of malignant glioma and able to target tumor cells infiltrating brain tissue. Numerous gene and immune therapy strategies for cancer are currently being tested in animal models and in early clinical trials. Immunogene therapy with cytokines is one of the promising strategies for malignant

glioma and aims at the activation of tumor specific T-lymphocytes and/or natural killer (NK) cells capable of killing tumor cells in patients with low tumor burden after surgical tumor removal or at protecting patients from recurrence after initial response to treatment [6].

The aim of the present study is to evaluate safety, maximum tolerated dose, and anti-tumor efficacy of a liposomally encapsulated replication-disabled recombinant Semliki forest virus vector (SFV) carrying the human interleukin 12 (*IL-12*) gene. The vector will be administered in escalating doses by continuous intratumoral infusion in patients with recurrent or progressive GBM after standard therapy.

Background and rationale

Cytokine genes previously used for immunotherapy of cancer include IL-2, IL-4, IL-7, IL-12, interferon-(IFN)-α and IFN-γ, GM-CSF, and TNF-α, alone or combined with genes encoding co-stimulatory molecules, such as B7-1 or CD40 [7–9]. Theoretically, cytokine immunotherapy aims at actively stimulating cytotoxic T-lymphocytes (CTL) and/or NK cells to achieve a systemic and/or local cellular antitumor response. Immunotherapy in rodent tumor models with low intrinsic tumor immunogenicity was demonstrated to result in regression of pre-existing tumors and in tumor cures. Furthermore, in some instances cured animals have retained immunological memory and resisted a second challenge with parental tumor cells [10].

IL-12

IL-12 is a heterodimeric protein consisting of two subunits (p35 and p40) and is secreted by antigen presenting cells such as dendritic cells (DC) and macrophages. IL-12 is an important immunoregulatory cytokine which enhances the function of cytotoxic immune cells, including CTL and NK cells, and possesses potent IFN-γ dependent therapeutic activity [11]. In experimental tumor models, recombinant IL-12 treatment has shown a dramatic antitumor effect on transplanted or chemically induced tumors and on tumors arising spontaneously in genetically modified mice [12]. In a recent study, mice bearing GL-26 gliomas were treated with direct intratumoral administration of adenovirus carrying the *IL-12* gene. Survival was significantly prolonged in IL-12 treated animals, and immunohistochemistry demonstrated increased CD4+ and CD8+ T cell infiltration of the tumor compared to controls [13]. The prominent anti-tumor effect of IL-12 may result at least in part from the fact that IL-12 directs differentiation of uncommitted T cells towards the T helper phenotype (Th1), which is critical for cellular anti-tumor immunity. In addition, acting as an endogenous adjuvant, IL-12 may provide an important link between innate immunity and specific adaptive immunity to mediate anti-tumor resistance [14].

The antitumor effect in the case of IL-12 may be more efficient compared with other tested cytokine genes such as IL-2, IL-4, IL-6, IFN-γ, TNF-α, or GM-CSF [10,12]. T cells from draining lymph nodes show increased cytotoxic activity after IL-12 application, and IFN-γ systemic levels are elevated, which together with a cascade of other pro-inflammatory cytokines induced by IL-12 may have a direct toxic effect on tumor cells or may in addition activate antiangiogenic pathways [14–18].

Furthermore, transfer of *IL-12* cDNA was employed for the treatment of cancer in several human clinical trials. Anti-tumor responses were observed in patients with different types of malignant tumors, such as melanoma or renal cell carcinoma [19]. It was also observed that IL-12 production from DC is suppressed in patients with malignant glioma, which suggested that one of the possible mechanisms of immunosuppression in these patients may be IL-12 dysfunction, resulting in failure to promote and maintain antigen specific T cells and/or NK cells [20]. Therefore, local delivery of IL-12 to the tumor site may result in more effective local recruitment and activation of CTL, which are functionally impaired in the microenvironment of malignant gliomas.

SFV vectors and their in vivo *use*

SFV is an RNA virus from the alphavirus family which is pathogenic to insects but not to humans. Favorable features of alphaviruses when used as gene transfer vectors are: rapid production of high-titer virus, broad host range (including a variety of mammalian cell types and dividing as well as non-dividing cells), high RNA replication rate in the cytoplasm, and high transgene expression levels. The main disadvantage of the vector include short duration of transgene expression [21,22], which, on the other hand, may be considered as advantageous under certain circumstances, e.g. for cancer gene therapy applications as well as for vaccine production [23].

SFV vectors have been successfully used for cell culture and *in vivo* expression of a number of human or murine genes, such as *IL-12*, *p53*, or endostatin. It has been also shown that SFV vectors can efficiently infect human prostate cancer cell lines and prostate duct epithelial cells *ex vivo*. Apoptosis was rapidly induced in the majority of cells infected with SFV–LacZ virus [24]. Intratumoral injections of SFV expressing the p40 and p35 subunits of IL-12 showed significant tumor regression and inhibition of tumor blood vessel formation in a mouse B16 melanoma model [15]. Moreover, multiple injections resulted in increased tumor response, but interestingly no antivirus immune response could be detected.

In another study, nude mice with implanted human lung carcinomas were injected with various SFV vectors [25]. It was found that intratumoral injections with SFV–LacZ, SFV–GFP or even empty SFV particles (containing only the SFV replicase genes) resulted in induction of p53-independent apoptosis and in significant tumor shrinkage. Again, repeated injections on 3 consecutive days followed by another series of 3 injections 1 week later turned out to be more beneficial that single applications [25].

Injection of replication-deficient SFV into rat brain resulted in local, high-level transient expression of a reporter gene [26]. It was also shown recently in animal models of malignant glioma that SFV infection of autologous DC is providing a potential mechanism for enhanced immunogenicity and tumor rejection [27]. In addition to its direct cytotoxic effects in tumors, SFV seems to be particularly suitable for induction of systemic immune responses against infected tumor cells and against transgene proteins carried by the vector, while there is little or no immune response against the vector itself. Expression of the *P1A* gene from recombinant SFV vectors resulted in induction of tumor immunity [28]. Intramuscular injection of self-replicating SFV–LacZ RNA protected mice from tumor challenge and prolonged the survival time of animals with established tumors [29]. Furthermore, immunization of mice with SFV particles carrying the human papilloma virus early genes *E6* and *E7* protected 40% of the animals from cervical cancer challenge [30].

These and other studies indicated that recombinant SFV is an expression vector with strong antitumor effect and potentially useful for the construction of human cancer vaccines, and that these properties are combined with a remarkably low complication rate and with a high level of biosafety [21–23].

Recombinant SFV vectors for use in the present study

In this study the SFV vector is rendered replication-deficient by deleting structural genes encoding essential capsid and membrane proteins [31]. The *IL-12* gene is inserted immediately downstream of the *nsP4* virus gene and its expression is driven by the subgenomic SFV 26S promoter located at the 3′ end of the *nsP4* gene. The helper virus used for packaging of SFV–IL12 vector in culture contains the intact SFV structural genes encoding the capsid and membrane proteins required for packaging of infectious particles. RNA coding for both the cloning and helper vectors is transfected into BHK cells either by electroporation or by lipofection for packaging of recombinant SFV particles. High-titer virus stocks (1×10^8–1×10^9 infectious particles/ml) are harvested 24 h post-transfection [31]. Single SFV particles with a diameter of about 35 nm are encapsulated in cationic liposomes with a mean diameter of 90 nm using the proprietary encapsulation technology of Regulon, Inc. (Palo Alto, CA). Liposomally encapsulated SFV–IL12 (LSFV–IL12) has several advantages over therapy with non-encapsulated virus [32]:

1. Recognition of the virus particles by the immune system is greatly reduced, which results in slow clearance and inactivation rates of virus *in vivo* and reduces further any vector inactivating immune response.
2. The SFV vector is protected from inactivation by serum components (complement proteins).
3. The persistence and infectivity period of the vector are prolonged.
4. The native neurotropism of SFV is expanded to other cell types.

Pre-clinical studies with LSFV–IL12

Prior experiments using either LSFV–IL12 or SFV–LacZ particles showed no detectable side effects in the CNS. Lundstrom et al. (1999) reported that neuronal infection by SFV–LacZ vectors injected into the amygdala and striatum of male Wistar rats did not result in any significant neurotoxicity [26].

Liposomally encapsulated SFV–LacZ particles were injected intravenously or intraperitoneally into SCID mice with implanted tumors that were killed 4 days post-injection, and tumors and internal organs were stained with X-gal to detect β-galactosidase (β-gal)

Table 1. Growth of subcutaneous Panc-1 pancreatic cancer in SCID mice after LSFV–IL12 treatment commencing simultaneously with tumor implantation. Tumor size (in mm^2) is obtained by multiplication of the two largest tumor diameters taken at 90° to each other

Day	Control (mm^2)	LSFV–IL12 (mm^2)
0	1	1
3	27	17
7	69	34
10	75	54
14	81	65
17	90	71
21	95	75
24	114	81
28	120	85
31	127	90
35	147	96
38	155	100
42	170	110

expression. Only tumors and a few other specific organs (liver, intestine, kidney) showed expression of β-gal. In addition, no detectable toxicities at levels up to 1×10^8 infectious particles per animal were seen with escalating doses of LSFV–IL12 after intravenous or intraperitoneal injection in either SCID or Balb/c mice. Neither the SFV vector itself nor the expressed transgenic IL-12 seemed to cause any significant side effects or unwanted toxicity [24]. Also SFV–IL12 was shown to cause significant cytotoxicity in solid tumors in preclinical animal models of human breast, lung, prostate, and pancreatic cancer in mice [24,25].

In addition to the published studies, in our own series of animal experiments we used 10 adult male SCID mice per group and implanted subcutaneously on day 0 1×10^7 Panc-1 pancreatic cancer cells per mouse. Intraperitoneal injections of LSFV–IL12 started on the day of tumor cell implantation. Each animal was injected with 1×10^7 LSFV–IL12 particles twice per week for 2 weeks. Tumors were measured in two dimensions and their approximate size was calculated in mm^2 (Table 1 and Figure 1A). There was a statistically significant difference in the size of tumors in animals treated with LSFV–IL12 compared with controls.

In another experiment for treatment of established tumors, 10 SCID mice per group were again implanted subcutaneously with 1×10^7 Panc-1 cells per animal. Intraperitoneal injections of LSFV–IL12 started on day 21 after tumor implantation. Each animal was injected with 1×10^7 LSFV–IL12 particles twice

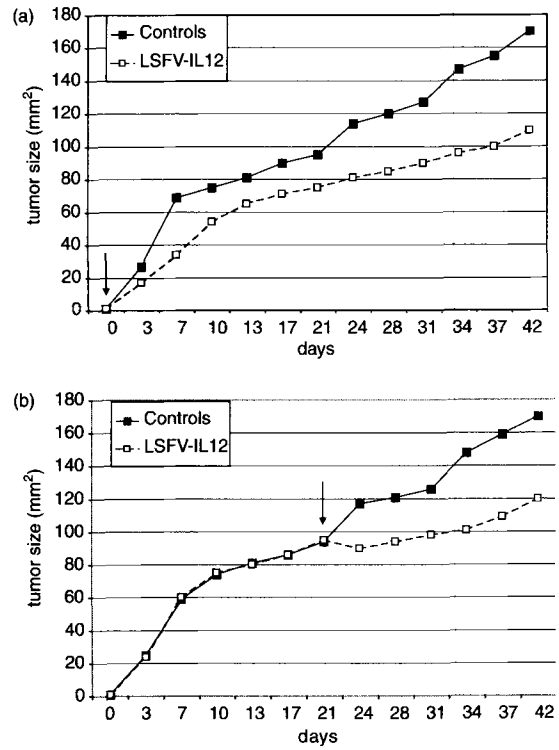

Figure 1. Graphs demonstrating results of LSFV–IL12 treatment in a SCID mouse model of subcutaneous pancreatic cancer. (a) Intraperitoneal injections with LSFV–IL12 were started on the day of tumor implantation (day 0, arrow). The difference in tumor size between the groups is statistically significant ($p < 0.05$). (b) Intraperitoneal injections with LSFV–IL12 were started on day 21 after tumor implantation (arrow). The difference in tumor size from day 21 on is statistically significant ($p < 0.05$).

per week for 4 weeks. A statistically significant difference in the size of tumors was noted in animals treated with LSFV–IL12 after day 21 compared with controls (Table 2 and Figure 1B).

All these experiments add to the existing body of data on *in vivo* biosafety and anti-tumor efficacy of SFV–IL12 vectors and strongly support their use as a locally or systemically administered therapy for human cancer.

Clinical studies with LSFV–IL12

In addition to animal studies, there is an ongoing European phase I study using SFV–IL12 in cancer patients with stage III or IV metastasizing melanoma or renal cell carcinoma (T Boulikas, personal communication). So far, 18 patients have been injected intravenously every 3 days for 4 weeks

Table 2. Growth of subcutaneous Panc-1 pancreatic cancer in SCID mice after LSFV–IL12 treatment commencing on day 21 after tumor implantation. Tumor size (in mm^2) is obtained by multiplication of the two largest tumor diameters taken at 90° to each other

Day	Control (mm^2)	LSFV–IL12 (mm^2)
0	1	1
3	25	24
7	59	60
10	74	75
14	81	80
17	86	86
21	94	95
24	117	90
28	121	94
31	126	98
35	148	101
38	159	109
42	170	120

with LSFV–IL12 in two different concentrations. At 1×10^8 or 1×10^9 liposomally encapsulated virus particles per m^2 body surface, there was no major NCI grade III or IV toxicity to internal organs such as lung, liver, kidney, or the hematopoietic system. Lab tests during and after intravenous virus treatment followed hematological, hepatic, renal, and general chemical functions of the body, and showed no significant changes of the investigated parameters in 18 out of 18 patients. Additional parameters followed in the treated cancer patients included cardiac function and full neurological status.

There was no detectable morbidity except for temporary and mild inflammatory reactions such as itching, flu-like symptoms with slight fever, and cutaneous reactions such as red buttons on the skin in 2 out of 18 patients occurring a few hours after the initial virus injections. Both patients received the highest dose of $1 \times 10^9/m^2$ LSFV–IL12. The side effects were attributed to high systemic levels of IL-12 and were successfully treated by application of oral steroids (dexamethasone). IL-12 concentration in the peripheral blood of the treated patients reached levels up to 10-fold higher compared to baseline levels before treatment. These levels lasted for 3–4 days after virus injection and returned to baseline shortly thereafter.

Intratumoral convection-enhanced infusion of the above replication-disabled and liposomally encapsulated SFV–IL12 in patients with recurrent or progressive GBM is expected to result in short-term expression and secretion of human IL-12 in transduced brain tumor cells and non-neoplastic cells, such as reactive astrocytes and endothelial cells in capillaries. Infection of these cells should result in apoptotic death after 2–4 days. Professional antigen presenting cells, such as DC, and brain microglia should endocytose apoptotic tumor cells and subsequently present tumor-specific antigens in the context of the strong co-stimulatory signals elicited by IL-12, thus eliciting an enhanced CTL response. Secretion of IL-12 should stimulate IFN-γ and TNF-α production that may be detected by assays for these cytokines in the systemic circulation and/or tumor *in situ*. IL-12 expression is expected to result in activation of CTL and/or NK cells capable of rejecting autologous glioma cells. The expression of lymphocytic markers such as CD3, CD4, CD8, CD25, CD56, CD69, Fas, and Fas ligand will be monitored systemically and intratumorally to further elucidate mechanisms involved in anti-tumor immune response *in vivo*.

Study protocol

This is a phase I/II, dose-ranging, parallel-group study in at least 18 patients with recurrent or progressive GBM after previous treatments such as surgery or radiation or chemotherapy. LSFV–IL12 vector suspension will be applied as convection-enhanced intratumoral infusion. The primary endpoint of the study is grade 3 or 4 toxicity due to LSFV–IL12 treatment or radiologically (MRI) defined progression of tumor.

Patient selection and enrollment

Patients must meet specific inclusion criteria in order to be enrolled in the study. Patients will sign an informed consent form approved by the local ethics committee (EC) before any study-related procedures are performed.

To participate in this study, patients must meet all of the following criteria:

1. Male or female patients older than 18 years.
2. Histologically documented supratentorial GBM that has progressed after initial stereotactic biopsy or open surgical resection, and/or external beam radiation therapy, and/or chemotherapy.
3. Unilateral, unifocal tumors with an enhancing volume not higher than 120 ml.
4. Karnofsky performance score ≥ 60 and a life expectancy of at least 3 months.

5. Normal hematological, renal, and hepatic function.
6. Stable dose of dexamethasone for at least 72 h prior to treatment.
7. Able to read, understand, and provide written informed consent before enrolling in the study, and willing to comply with all study procedures.
8. Female patients in the reproductive age using acceptable birth control methods.

Patients who meet any of the following criteria will be excluded from the study:

1. Brain stem or infratentorial tumors, tumors invading midline, or multifocal tumors.
2. Tumors with a clinically significant mass effect (>5 mm midline shift) while on stable dose of steroids.
3. Presence of clinical symptoms caused by increased intracranial pressure and/or brain edema.
4. Presence of an additional immunosuppressive disorder or of iatrogenic immunosuppression (with the exception of steroid use) or of any active infection.
5. Chemotherapy during the preceding 4 weeks before enrollment.
6. Craniotomy within 4 weeks prior to enrollment.
7. Radiation therapy within 8 weeks prior to enrollment.
8. Medically unstable condition because of non-malignant systemic disease, e.g. cardiopulmonary disease.
9. Previous or concurrent malignancies at other sites with the exception of carcinoma *in situ* of the cervix and basal or squamous cell carcinoma of the skin.
10. History or suspicion of inability to comply fully with all procedural aspects of this study.

Investigations prior to treatment

Comprehensive clinical and laboratory examinations should be conducted no longer than 2 weeks prior to LSFV–IL12 treatment, including cranial MRI with and without gadolinium, MR-spectrosocopy (MRS), FDG-PET and thallium-SPECT, and full immunological status by FACS analysis of peripheral mononuclear cells (PMNC). In addition, quantitative assessment of the extracellular space (ECS) of tumors will be carried out by CT using a two-compartment model, and will be correlated subsequently with tumor response and imaging parameters.

Vector infusion

Three dose levels are planned in 6 patients at each dose level. The starting dose of LSFV–IL12 in the lowest dose group will be 1×10^7 infectious particles per ml (median dose group 1×10^8, highest dose group 1×10^9 particles per ml) in 11 ml of 0.9% NaCl. This volume will be infused by a syringe pump into the tumor over 24 h at a rate of 0.5 ml/h with initial volume escalation starting with 0.1 ml/h and increasing the volume by 0.1 ml each hour until reaching the above maximum flow rate.

Investigations after treatment

Control MRI scans with contrast enhancement will be performed within 24 h after end of infusion. Follow-up MRI and MRS with contrast, and serum IL-12 ELISA will be carried out at regular intervals after virus infusion. MRI scans with volumetric analysis will be used to determine tumor response to LSFV–IL12 therapy. In addition, metabolic scans (PET/SPECT) will be carried out at baseline and 8 weeks after virus infusion. Assessment of immune status and possible systemic immune response will be performed by delayed type hypersensitivity (DTH) assay, cytokine ELISA in serum and by FACS analysis of PBMC subpopulations. Also, in selected patients, autologous tumor cells will be co-cultured with PBMC in an IFN-γ ELISPOT assay to assess CTL response.

Follow-up studies and off study

Patients will be considered on study from the time of LSFV–IL12 administration until tumor progression, start of another treatment, or death. Follow-up studies will be performed every 8 weeks until documented tumor progression, until the patients begin some other treatment for their disease, or until death. All drug related toxicities must be followed until resolution ($\leq$grade 2 or $\leq$baseline). All patients will be followed in addition for overall survival.

A comprehensive version of this abbreviated clinical protocol may be obtained from the authors upon request.

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