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Selective Transduction of Malignant Glioma by Lentiviral Vectors Pseudotyped with Lymphocytic Choriomeningitis Virus Glycoproteins

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ABSTRACT

Malignant gliomas are the most frequent primary brain tumors and have a dismal prognosis due to their infiltrative growth. Gene therapy using viral vectors represents an attractive alternative to conventional cancer therapies. In a previous study, we established lentiviral vectors pseudotyped with lymphocytic choriomeningitis virus (LCMV) glycoproteins (GPs) and demonstrated transduction of human malignant glioma cells in culture. In the current approach, we compared the transduction efficacy of LCMV-GP- and vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vectors for malignant glioma cells and normal brain cells *in vitro* and *in vivo*. LCMV-GP pseudotypes transduced almost exclusively astrocytes, whereas VSV-G pseudotypes infected neurons as well as astrocytes. LCMV-GP pseudotypes showed an efficient transduction of solid glioma parts and specific transduction of infiltrating tumor cells. In contrast, VSV-G-pseudotyped lentiviral vectors transduced only a few tumor cells in solid tumor parts and infected mostly normal brain cells in infiltrating tumor areas. In conclusion, lentiviral vectors pseudotyped with LCMV glycoproteins represent an attractive option for gene therapy of malignant glioma.

OVERVIEW SUMMARY

In the present study the level of gene transfer, using lentiviral vectors pseudotyped with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) glycoproteins, into malignant glioma cells and normal brain tissue was investigated. VSV-G-pseudotyped vectors transduced glial tumor cells with low efficiency *in vivo* and infected mainly normal brain cells in infiltrating tumor areas. In addition, these vectors exhibited a high tropism for neurons and astrocytes on injection into normal rat brain. In contrast, LCMV-GP-pseudotyped vectors transduced rat glioma cells with high specificity and efficiency in both solid and infiltrating tumor areas. In normal brain, astrocytes were the predominant cell population transduced by these vectors *in vitro* as well as *in vivo*; infection of neurons was scarce. These data show that LCMV-GP-pseudotyped lentiviral vectors specifically target glial tumor cells *in vivo* and cause minimal toxicity to normal brain cells in infiltrating tumor areas.

INTRODUCTION

MALIGNANT GLIOMAS, comprising the most frequently encountered primary brain tumors, still have a dismal prognosis despite advances in neurosurgery, radiation, and chemotherapy. Gene therapy based on viral vectors is an attractive alternative therapeutic approach. Vectors derived from the murine leukemia virus (MLV) are the most frequently used

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retroviruses for gene therapy of brain tumors (Benedetti *et al*., 1999; Lam and Breakefield, 2001; Tamura *et al*., 2001; Wang *et al*., 2003). However, the instability of the glycoprotein impedes clinical application as the vectors cannot be concentrated by ultracentrifugation. This is required to achieve vector titers that are sufficient for *in vivo* application. Exchange of the retroviral envelope protein for the rhabdoviral G protein of the vesicular stomatitis virus (VSV) improved vector stability and broadened the host range (Emi *et al*., 1991; Burns *et al*., 1993; Yee *et al*., 1994). Use of these vectors in a rat glioma model has shown high transduction and therapeutic efficiency after transfer of the herpes simplex virus thymidine kinase gene (HSV-*tk*) and ganciclovir treatment (Galipeau *et al*., 1999). However, a major disadvantage of the VSV glycoprotein (VSV-G) is its cytotoxicity, thus preventing establishment of stable recombinant packaging cell lines (Burns *et al*., 1993; Chen *et al*., 1996). In previous studies, we developed oncoretroviral vectors pseudotyped with glycoproteins (GPs) from the lymphocytic choriomeningitis virus (LCMV) (Miletic *et al*., 1999; Beyer *et al*., 2001). These vectors have a broad host range and can be concentrated by ultracentrifugation. In addition, LCMV-GP is not cytotoxic, and stable recombinant packaging cell lines can be established.

Despite these improvements, the restricted infection of dividing cells by retroviral vectors still reduces transduction efficacy, as in human glioma most of the tumor cells are not dividing in a given treatment window. Therefore, a vector system is required for transduction of both dividing as well as nondividing tumor cells. In this regard, lentiviral vectors are attractive candidates. These vectors also transduce nondividing cells and can be pseudotyped by VSV-G, thereby increasing both vector stability and host range (Naldini *et al*., 1996b). In infection studies with lentiviral VSV-G pseudotypes in the normal rat brain, neurons were transduced at high levels (Naldini *et al*., 1996a). Consequently, these vectors may cause serious side effects in gene therapy of malignant glioma.

In one study, we established lentiviral vectors pseudotyped with LCMV-GP and demonstrated transduction of human malignant glioma cells in culture (Beyer *et al*., 2002). In the current approach, we determine the tropism of LCMV-GP pseudotypes for rat malignant glioma cells *in vitro* and *in vivo*. The transduction efficacy of solid and infiltrating glioma by LCMV-GP- and VSV-G-pseudotyped vectors is compared. Furthermore, transduction of normal brain cells *in vivo* and *in vitro* by both pseudotypes is analyzed. LCMV-GP pseudotypes show specific and efficient transduction of rat gliomas and infect mostly astrocytes on injection into normal rat brain. In contrast, VSV-G pseudotypes transduce glioma cells with much lower efficiency and infect predominantly normal brain cells in infiltrating tumor areas. In normal rat brain neurons are the major cell population transduced by VSV-G pseudotypes.

MATERIALS AND METHODS

Cell lines

9L rat gliosarcoma, 293T human kidney, and TE671 human fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA). G62 human glioma cells were kindly provided by M. Westphal (University Hospital Eppendorf, Hamburg, Germany). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum and penicillin–streptomycin in a humidified atmosphere of 5% CO_2.

Transduction of 9L cells with DsRed

DsRed in the pMP71 vector backbone was kindly provided by N. Dinauer (Georg-Speyer-Haus, Frankfurt, Germany). For transduction of 9L cells with DsRed, 9L cells were seeded in 24-well plates at a density of 5×10^4 cells per well. After 4 hr, retroviral supernatants packaging the pMP71DsRed vector were added. Plates were centrifuged for 1 hr at $1000 \times g$. Transduction of cells was repeated 16 hr after the first transduction. DsRed expression was confirmed by fluorescence microscopy (Nikon Eclipse TE300; Nikon, Düsseldorf, Germany). For isolation of DsRed-expressing single clones, 10^2 transduced 9L cells were plated in 10-cm dishes and grown into colonies. DsRed-positive colonies were identified by fluorescence microscopy and transferred into separate wells of 24-well plates. DsRed expression levels of isolated clones were determined by flow cytometry on a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA). For *in vivo* tumor implantation a clone was selected containing 95% DsRed-positive cells.

Transient production of lentivirus vector pseudotypes

Lentiviral vectors were produced by transient transfection of 293T cells. Volumes of 5×10^6 cells were seeded in 10-cm-diameter culture dishes 16 hr before transfection in DMEM–FBS. One hour before transfection, the culture medium was changed to DMEM–FBS–PS (10 ml/dish), that is, DMEM–FBS with 25 μM chloroquine, penicillin (50 U/ml), and streptomycin (50 μg/ml) (GIBCO/Invitrogen Life Technologies, Grand Island, NY). Five micrograms of pRRL.sinCMVeGFPpre, 5 μg of pRSV-Rev, 15 μg of pMDLg/pRRE (described in Dull *et al*., 1998), and 1 to 2 μg of a pHCMV envelope glycoprotein expression plasmid were then used for the transfection of one culture dish. A combination of 450 μl of the plasmids in distilled deionized H_2O and 50 μl of 2.5 M $CaCl_2$ was mixed well and then added dropwise to 500 μl of 2× HEPES-buffered saline (280 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1). After vortexing, the precipitate was immediately added to the cultures. The medium was changed after 8 hr to 10 ml of DMEM–FBS–PS per dish with 20 mM HEPES. Vector-containing supernatants were collected 24 hr after transfection and every 8 to 16 hr thereafter for a period of 2 days. Cell culture supernatants were pooled and filtered through a 0.22-μm-pore size Millex-GP filter (Millipore, Bedford, MA). For *in vivo* and *in vitro* (cultured brain cells) applications, vector supernatants were concentrated by ultracentrifugation at 19,500 rpm for 2 hr in an SW28 rotor (Coulter Beckman, Fullerton, CA). For *in vitro* experiments on TE671 and various glioma cell lines, vector supernatants were not concentrated.

Vector titration

Lentiviral vector titers were measured by transduction of various cell lines. Serial dilutions of cell supernatants were prepared, and 0.5 ml of each dilution was added to 5×10^4 cells

and seeded in a well of a 24-well plate 4 hr before transduction. Plates were centrifuged for 1 hr at 1000 × *g*. Cells were analyzed 65 hr posttransduction by flow cytometry on a FACSCalibur (BD Biosciences Immunocytometry Systems) for GFP expression. Titers were calculated from dilutions, which resulted in 0.5 to 20% enhanced green fluorescent protein (eGFP)-positive cells, a range of linear relation between vector input and percentage of transduced cells, because multiple vector integrations into the target cell are generally not expected.

Rat hippocampal neuron culture

Primary hippocampal neuronal cell cultures were prepared as described previously (Neumann *et al*., 1995). Briefly, hippocampi were isolated from whole brains of embryonic day 16 Wistar rats, and the meninges were removed. The trimmed tissue was dissociated by trituration through a fire-polished Pasteur pipette. Cells (5×10^4/ml) were plated into four-well chamber slides that had been pretreated with poly-L-ornithine (0.5 mg/ml; Sigma, St. Louis, MO) in 0.15 *M* boric acid. Cells were cultured in chemically defined medium containing basal medium Eagle (BME; GIBCO-BRL/Invitrogen Life Technologies, Gaithersburg, MD) with 2% (v/v) B27 supplement (Invitrogen Life Technologies) and 1% (v/v) glucose (45%; Sigma).

Rat astrocyte-enriched glial cell culture

Hippocampi of embryonic day 16 Wistar rats were isolated and dissociated into single-cell suspensions as described for neuronal hippocampal preparations. Cells were plated in 50-ml tissue culture flasks that had been pretreated with poly-L-lysine (5 μg/ml; Sigma). Cells were cultured in serum-containing medium with minimal essential medium (MEM) with D-valine (Invitrogen Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal calf serum (FCS; PanSystem, Würzburg, Germany), and 1% L-glutamine. Astrocyte-enriched glial cells were cultured for 10–20 days and then plated in BME with B27 supplement (2%, v/v; Invitrogen Life Technologies) and 1% (v/v) glucose (45%; Sigma) at a density of 2×10^4/ml in 4-well chamber slides before transduction. In total, 94% (±3% SD) of cells were astrocytes as determined by immunolabeling with rabbit antibodies directed against glial fibrillary acidic protein (GFAP, 10 μg/ml; DakoCytomation, Glostrup, Denmark).

Transduction of cultured brain cells

Fourteen days after plating, neurons and astrocytes were transduced with lentivirus-pseudotyped vectors carrying the eGFP marker gene. Two days after transduction, cells were fixed in 4% paraformaldehyde and immunostained with monoclonal mouse anti-β-tubulin III antibody (Sigma) for neurons and monoclonal rabbit anti-GFAP antibody (DakoCytomation) for astrocytes. The cells were incubated overnight at 4°C with the primary antibody. Cy3–goat anti-mouse and Cy3–goat anti-rabbit were used as secondary antibodies for 2 hr at room temperature. The relative numbers of transduced (eGFP positive) and untransduced (eGFP negative) neuronal (β-tubulin III positive) and astrocytic (GFAP positive) cells were determined by fluorescence microscopy and the counting of 10 camera fields for each pseudotype.

Tumor implantation and lentiviral vector delivery

Adult female Fischer 344 rats (Harlan Winkelmann, Borchen, Germany) were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (2 mg/kg). Intracranial 9LDsRed tumors were established by injection of 1×10^5 9LDsRed cells (in 5 μl of phosphate-buffered saline [PBS]) into the right striatum, using a Hamilton syringe in a stereotaxic apparatus (Stoelting, Wood Dale, IL). The coordinates used were 4 mm lateral to the bregma and 5 mm below the dural surface. Six days after tumor implantation, rats were anesthetized, and lentiviral vector pseudotypes with titers ranging from 2×10^6 to 1×10^7 transducing units (TU)/ml were injected, using the same stereotactic coordinates and 1 mm apart (seven different sites). A total volume of 10 μl was injected into each tumor. Fischer rats not receiving tumor cells were anesthetized and lentiviral pseudotypes were injected either into the right striatum or into the right hippocampus. The coordinates used for the hippocampus region were 4.5 mm lateral to the bregma, 5.5 mm posterior to the coronal plate, and 3 mm below the dural surface.

Analysis of rat brains for lentiviral transduction

Seven (tumor-bearing rats) and 14 days (rats without tumor) after lentiviral vector delivery, animals were killed and perfused with 4% paraformaldehyde. Brains were removed, suspended in 30% sucrose for 3 days, and then snap frozen in isopentane chilled with liquid nitrogen. Coronal sections (12 μm) were prepared on a cryostat and immunostained with either rabbit anti-GFAP antibodies (DakoCytomation, Hamburg, Germany) for astrocytes or mouse anti-NeuN antibodies (Chemicon, Hofheim, Germany) for neurons. Primary antibodies were incubated overnight at 4°C. Cy3–goat anti-mouse and Cy3–goat anti-rab-

Table 1. Vector Titers of Pseudotyped Lentiviral Vectors on TE671 and Glioma Cell Lines[a]

	LCMV-GP		*VSV-G*	
Cell line (no. of experiments)	*Pseudotype titer (TU/ml)*	*Titer relative to TE671*	*Pseudotype titer (TU/ml)*	*Titer relative to TE671*
TE671 (4)	$(2.75 \pm 1.07) \times 10^4$	1	$(6.08 \pm 6.00) \times 10^4$	1
9L (4)	$(1.80 \pm 0.78) \times 10^4$	0.65 (±0.03)	$(8.83 \pm 5.81) \times 10^4$	1.91 (±0.63)
9LDsRed (2)	$(1.29 \pm 1.00) \times 10^4$	0.35 (±0.21)	$(8.35 \pm 5.97) \times 10^4$	1.89 (±0.07)

[a]Cell lines were transduced with LCMV-GP- or VSV-G-pseudotyped lentiviral vectors packaging eGFP. Titers were measured by FACS analysis. Results represent the means and standard deviations of at least two experiments.

Table 2. Tropism of Lentiviral Pseudotyped Vectors for Cultured Brain Cells[a]

Cultured cells	*Lentiviral pseudotyped vector (Titer on TE671)*	*Transduced cells/ high-power field (±SD)*	*Total cell number/high-power field (±SD)*	*Ratio (%) (±SD)*
Astrocytes	LCMV-GP (8×10^4)	35.7 (±12.6)	49.0 (±13.3)	71.7 (±10.3)
	VSV-G (3×10^4)	43.5 (±18.0)	49.9 (±16.4)	85.5 (±12.4)
Neurons	LCMV-GP (8×10^4)	0.4 (±0.5)	17.9 (±5.9)	2.2 (±3.0)
	VSV-G (3×10^4)	8.2 (±3.3)	13.8 (±6.1)	62.5 (±16.9)
G62	LCMV-GP (8×10^4)	67.3 (±18.3)	75.5 (±20.3)	89.5 (±6.7)
	VSV-G (3×10^4)	41.1 (±14.5)	72.3 (±12.2)	56.9 (±17.0)

[a]Cultured rat astrocytes or neurons were transduced with 3×10^4 to 8×10^4 eGFP TU of LCMV-GP- or VSV-G-pseudotyped lentiviral vectors. Cells were analyzed by fluorescence microscopy after immunostaining with monoclonal anti-GFAP antibodies for astrocytes or with anti-β-tubulin III antibodies for neurons. Results represent the mean cell numbers and standard deviations from 10 randomly selected camera fields (0.75 mm^2).

bit (Dianova, Hamburg, Germany) were applied as secondary antibodies for 2 hr at room temperature. The sections were examined under a fluorescence microscope (Zeiss, Jena, Germany) and analyzed by confocal scanning laser microscopy (Leica, Bensheim, Germany). Transduction efficiencies in infected tumor areas were determined by counting, by three independent investigators in a double-blinded fashion, transduced tumor areas in 10 sections per group.

RESULTS

LCMV-GP- and VSV-G-pseudotyped lentiviral vectors transduce 9L tumor cells in vitro

We compared the transduction efficiencies of both VSV-G- and LCMV-GP-pseudotyped lentiviral vectors in the rat glioma cell lines 9L and 9LDsRed (used for tumor implantation) *in vitro*. The human epithelial cell line TE671, which could be transduced by both VSV-G- and LCMV-pseudotyped vectors as shown in a previous study (Beyer *et al*., 2002), was used as infection control. We performed end-point dilutions on 9L, 9L-DsRed, and TE671 cells, and measured the percentage of transduced cells by flow cytometry analysis. Both vectors transduced 9L cells, but VSV-G pseudotypes did so with higher efficiency (Table 1). The relative transduction compared with TE671 was 0.65 for LCMV pseudotypes and 1.91 for VSV-G pseudotypes. In addition, transduction efficiencies for 9L and 9LDsRed tumor cells did not differ significantly *in vitro* (Table 1).

VSV-G pseudotypes transduce cultured neurons and astrocytes more efficiently than do LCMV-GP pseudotypes

To analyze the tropism of both pseudotyped vectors for normal brain cells *in vitro*, we infected cultured neurons and astrocytes derived from embryonic day 16 Wistar rats by end-point dilutions. The human glioma cell line G62, which could be transduced by both pseudotypes in a previous study, was used as infection control. VSV-G pseudotypes transduced GFAP-positive astrocytes and β-tubulin III-positive neurons at a higher level than did LCMV-GP pseudotypes (Table 2). In particular, the transduction efficiency for neurons was markedly different: VSV-G pseudotypes transduced 62.5%, whereas LCMV-GP pseudotypes infected only 2.2%, of counted neurons. In addition, LCMV-GP pseudotypes showed a higher transduction efficiency for the human glioma cell line G62 (89.5%) than for cultured astrocytes (71.7%) and neurons (2.2%). In contrast, VSV-G pseudotypes transduced astrocytes (85.5%) and neurons (62.5%) at a higher level than G62 cells (56.9%).

VSV-G and LCMV-GP pseudotypes show a different tropism for normal brain cells in vivo

We confirmed the *in vitro* results by analysis of the *in vivo* tropism for normal brain cells. For this purpose, we injected LCMV-GP and VSV-G pseudotypes either into the striatum or the hippocampus of Fischer rats. The relative proportion of transduced cell types was analyzed by immunofluorescence staining with cell type-specific markers and confocal mi-

FIG. 1. Neurons and astrocytes were transduced by VSV-G-pseudotyped lentiviral vectors *in vivo*. Normal rat brain was infected with LCMV-GP- or VSV-G-pseudotyped vectors expressing eGFP. Transduction of neurons and astrocytes was analyzed, after staining with antibodies against NeuN and GFAP, by confocal laser scanning microscopy on day 14. (**A**) Transduction of striatal astrocytes by LCMV-GP-pseudotyped vectors. (**B**) Striatal astrocytes expressing GFAP. (**C**) Merge of (**A**) and (**B**). (**D**) In regions with high neuron density (hippocampus) neurons were not transduced, but single astrocytes were. (**E**) Hippocampal neurons expressing NeuN. (**F**) Merge of (**D**) and (**E**). (**G**) Transduction of striatal neurons by VSV-G-pseudotyped vectors. (**H**) Striatal neurons expressing NeuN. (**I**) Merge of (**G**) and (**H**). (**K**) Tranduction of hippocampal neurons by VSV-G-pseudotyped vectors. (**L**) Hippocampal neurons expressing NeuN. (**M**) Merge of (**K**) and (**L**). (**N**) Hippocampal astrocytes transduced by VSV-G-pseudotyped vectors. (**O**) Hippocampal astrocytes expressing GFAP. (**P**) Merge of (**N**) and (**O**). Original magnification, ×40.

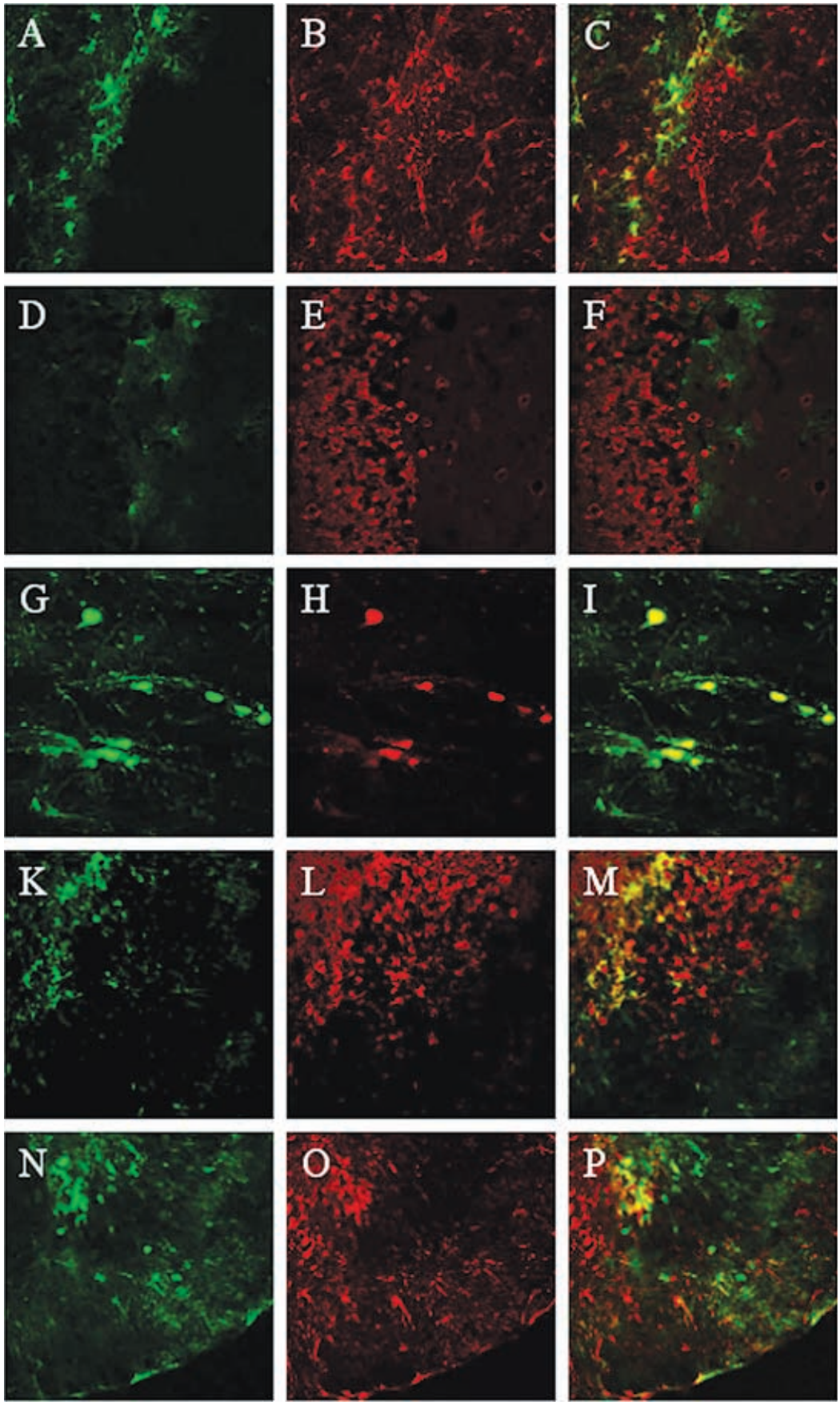
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TABLE 3. TRANSDUCTION EFFICIENCY OF LENTIVIRAL PSEUDOTYPED VECTORS FOR 9LDsRed CELLS *in Vivo*[a]

Lentiviral pseudotyped vector	*Tumor area*	*Cell type*	*Transduced cells/field (±SD)*	*Total cells/field (±SD)*	*Ratio (%) (±SD)*
LCMV-GP	Solid	9LDsRed	191.3 (±44.4)	276.5 (±51.1)	69.3 (±10.6)
VSV-G	Solid	9LDsRed	19.3 (±6.5)	279.9 (±35.6)	6.8 (±1.9)
LCMV-GP	Infiltrating	9LDsRed	98.4 (±25)	137.9 (±27.4)	71.5 (±10.6)
VSV-G	Infiltrating	9LDsRed	2.6 (±2.1)	125.4 (±31.2)	2.2 (±1.9)
LCMV-GP	Infiltrating	Brain cells	3.7 (±3.4)	95 (±19.4)	4.2 (±4.7)
VSV-G	Infiltrating	Brain cells	35.2 (±11.4)	81.6 (±12.7)	42.9 (±11.4)

[a]9LDsRed tumors were transduced with 2×10^6 to 1×10^7 eGFP TU of LCMV-GP- or VSV-G-pseudotyped lentiviral vector per milliliter. Cells were counted with a fluorescence microscope. Data represent the mean cell numbers and standard deviations from 10 microscopic fields (magnification, ×200) of solid and infiltrating tumor areas, respectively.

croscopy. LCMV-GP pseudotypes transduced almost exclusively astrocytes in both brain regions, as shown by staining with antibodies against GFAP (Fig. 1A–C). This observation could be confirmed even in areas with high neuron density (Fig. 1D–F). Transduction of neurons was scarce in the striatum and the hippocampus. In contrast, VSV-G pseudotypes infected neurons with high efficiency. The estimated ratio of cells transduced by VSV-G pseudotypes was 3:1 (neurons:astrocytes) in both brain regions (Fig. 1G–I, K–M, and N–P).

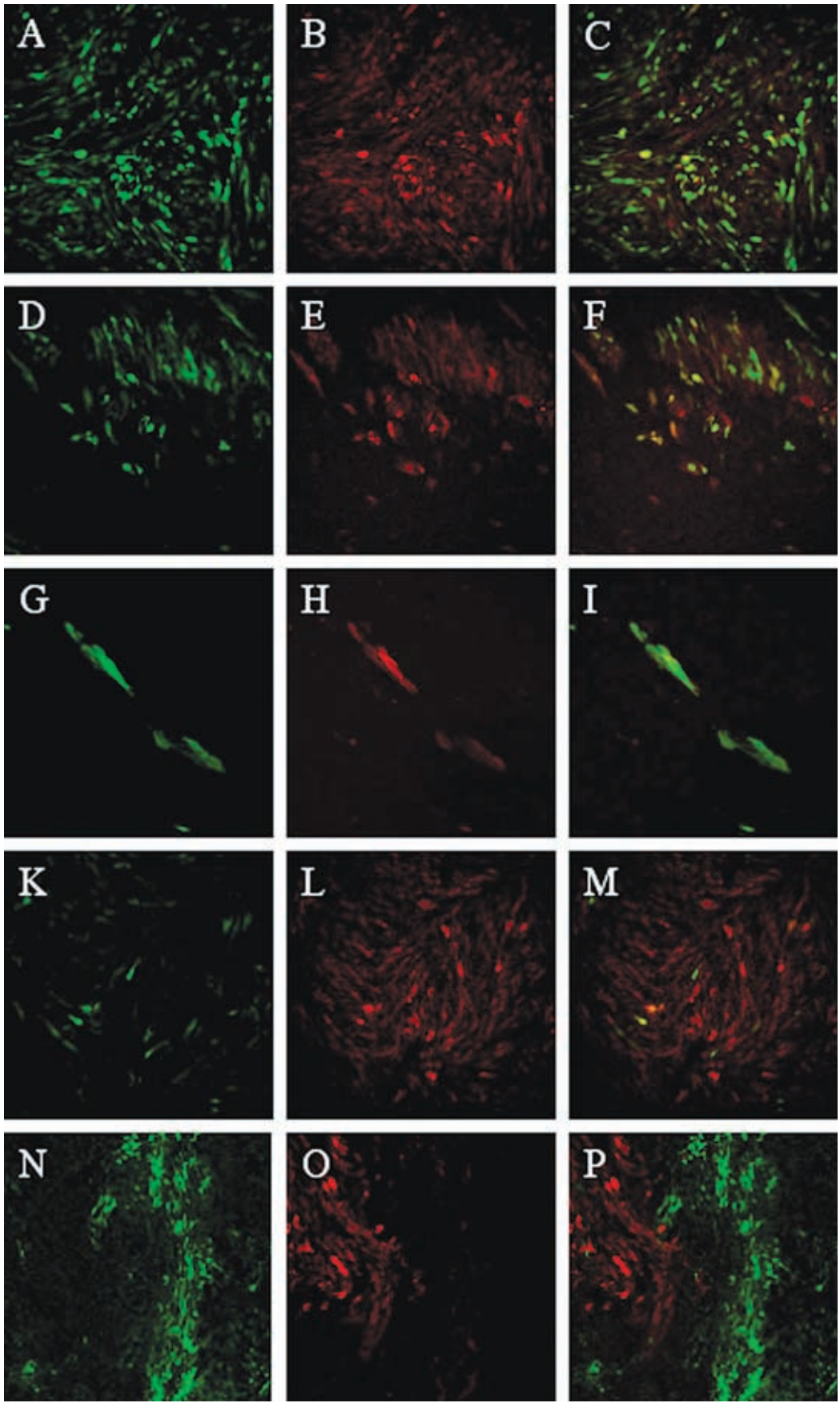
LCMV-GP pseudotypes show specific and efficient transduction of glial tumor cells in vivo

We wanted to exclude the possibility that the established cell line 9LDsRed differed from its parental cell line 9L *in vivo*. Two weeks after intracerebral tumor cell implantation in Fischer rats, established tumors were screened by histology for size and by light microscopy (9L) or fluorescence microscopy (9LDsRed) for their ability to infiltrate into the brain parenchyma. 9L and 9LDsRed tumors showed no difference in size and contained infiltrating tumor cells. With the DsRed marker even single tumor cells could be detected, migrating into the brain parenchyma. To analyze the transduction of 9LDsRed tumors *in vivo* by both pseudotypes, two groups of female Fischer rats, bearing 9LDsRed tumors, were injected with LCMV-GP and VSV-G pseudotypes. To measure the transduction of solid tumor and infiltrating glioma cells, injections were placed in the center of the tumor and 1 mm apart. Transduction efficiencies in infected tumor areas were determined quantitatively. LCMV-GP pseudotypes showed an effective transduction of solid tumor: 69.3 ± 10.6% of tumor cells were GFP positive in tumor areas injected with vector supernatants (Table 3 and Fig. 2A–C). In the infiltrating areas of 9LDsRed tumors, LCMV-GP pseudotypes specifically transduced the glioma cells (Fig. 2D–F): whereas 71.5 ± 10.6% of tumor cells were transduced, only 4.2 ± 4.7% of normal brain cells were GFP positive (Table 3). Even single infiltrating tumor cells were infected by this vector pseudotype (Fig. 2G–I). Only a few reactive astrocytes around the tumor bed were GFP positive, as revealed by staining with antibodies against GFAP (Fig. 3A–C). Neurons were not infected in infiltrating tumor areas. VSV-G pseudotypes showed a different transduction pattern: solid tumor was transduced to a much lower extent (6.8 ± 1.9%) than with the LCMV-GP pseudotypes (Table 3 and Fig. 2K–M). In contrast, many normal brain cells including neurons and reactive astrocytes in the infiltration area and surrounding brain parenchyma were GFP positive (42.9 ± 11.4%), whereas only rarely were tumor cells (2.2 ± 1.9%) transduced (Table 3 and Fig. 2N–P).

DISCUSSION

In human glioblastoma, recurrent tumors often develop from infiltrating tumor cells, which cannot be eliminated by current therapies. These cells represent a major therapeutic target. In this study, LCMV-GP pseudotypes showed a specific and efficient transduction of solid as well as infiltrating rat glioma, whereas VSV-G pseudotypes preferentially transduced normal brain cells. Retroviral vectors based on MLV have shown promising results for gene therapy of brain tumors (Tamura *et al*., 2001; Wang *et al*., 2003). However, the low or absent effect in clinical trials (Rainov, 2000) could be due to the restricted transduction of dividing tumor cells. In contrast to commonly used retroviral vectors, lentiviral vectors introduce

FIG. 2. Solid and infiltrating areas of rat glioma were efficiently transduced by LCMV-GP-pseudotyped lentiviral vectors. Intracranial 9LDsRed gliomas were infected with LCMV-GP- or VSV-G-pseudotyped lentiviral vector expressing eGFP 7 days after tumor implantation and analyzed by confocal laser scanning microscopy on day 14. (**A**) Solid tumor (69.3 ± 10.6%) transduced by LCMV-GP-pseudotyped vectors. (**B**) Solid tumor expressing DsRed. (**C**) Merge of (**A**) and (**B**). (**D**) Infiltrating glioma parts transduced by LCMV-GP-pseudotyped vectors. (**E**) Infiltrating glioma parts expressing DsRed. (**F**) Merge of (**D**) and (**E**). (**G**) Single infiltrating tumor cells transduced by LCMV-GP-pseudotyped vectors. (**H**) Single infiltrating tumor cells expressing DsRed. (**I**) Merge of (**G**) and (**H**). (**K**) Solid tumor (6.8 ± 1.9%) transduced by VSV-G-pseudotyped vectors. (**L**) Solid tumor expressing DsRed. (**M**) Merge of (**K**) and (**L**). (**N**) Infiltrating glioma parts transduced by VSV-G-pseudotyped vectors. (**O**) Infiltrating glioma parts expressing DsRed. (**P**) Merge of (**N**) and (**O**). Original magnification: (**A–F**) ×20; (**G–I**) ×40; (**K–P**) ×20.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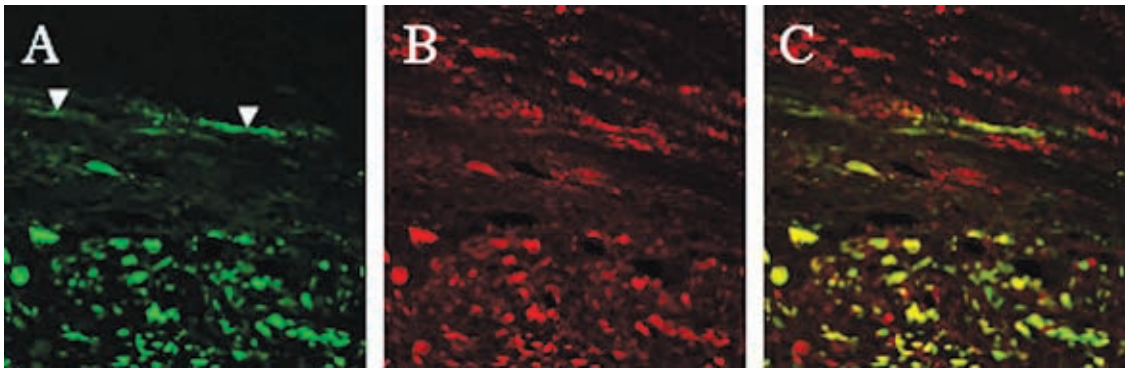

FIG. 3. Only a few reactive astrocytes in infiltrating tumor areas were transduced by LCMV-GP pseudotypes. Intracranial 9LDsRed gliomas were infected with LCMV-GP- or VSV-G-pseudotyped lentiviral vector expressing eGFP 7 days after tumor implantation. Transduction of neurons and astrocytes in infiltrating tumor areas was analyzed, after staining with antibodies against GFAP and NeuN, by confocal laser scanning microscopy on day 14. (**A**) Tumor cells and single astrocytes (arrowheads) transduced by LCMV-GP pseudotypes. (**B**) Reactive astrocytes expressing GFAP. (**C**) Merge of (**A**) and (**B**). Original magnification, ×40.

their genome not only into dividing, but also into quiescent, cells. This can be a major advantage with respect to gene therapy for tumors in general, as within a short treatment window most tumor cells do not divide. Consequently, we decided to use lentiviral pseudotypes as a new approach.

A major problem regarding lentiviral vectors, however, might be the transduction of nontumoral, quiescent cells. The cells of normal brain, in particular neurons, are in a postmitotic state and can be infected by lentiviral vectors. When lentiviral vectors are used, the specificity for tumor cells should be determined by the virus glycoprotein, which is one of the major determinants of virus tropism. Lentiviral vectors are generally pseudotyped with VSV-G, which mediates effective transduction of neurons *in vivo* (Naldini *et al*., 1996a). However, the transduction of malignant glioma with these pseudotypes has not been examined yet. In a previous study, we showed that lentiviral VSV-G pseudotypes transduce human glioma cells in culture (Beyer *et al*., 2002). Also, the 9L rat glioma cell line studied here was highly susceptible to VSV-G pseudotypes. We therefore expected efficient transduction of tumor cells as well as normal brain cells with the VSV-G pseudotypes in the 9L rat glioma model. Surprisingly, the infection of normal brain cells at the tumor border was more efficient than transduction of tumor cells. Also, within solid tumor, transduction of tumor cells was not as efficient as with LCMV pseudotypes. *In vitro* and *in vivo* we could demonstrate a strong transduction of neurons and astrocytes with VSV-G pseudotypes. Although the transduction of malignant glioma by lentiviral and retroviral VSV-G pseudotypes has not been compared directly, two studies using retroviral VSV-G pseudotypes have shown higher transduction efficiency in experimental brain tumors (Galipeau *et al*., 1999; Lee *et al*., 2001) as compared with our study with lentiviral VSV-G pseudotypes. The discrepancy may be explained by different virus strains pseudotyped with VSV-G: as retroviral vectors infect dividing cells, their target in the brain of tumor-bearing animals consists only of tumor cells. Lentiviral vectors infect both nondividing as well as dividing cells and, therefore, they can also infect normal brain cells. Moreover, our data and Naldini *et al*. (1996b) demonstrated that normal brain cells, in particular neurons, present the major target for VSV-G lentiviral pseudotypes. Altogether, these data show that VSV-G-pseudotyped lentiviral vectors are not suitable vectors for gene therapy of brain tumors.

In contrast, LCMV-GP pseudotypes showed a completely different transduction pattern. The vector infected solid and infiltrating tumor with high efficiency and specificity. The level of transduction of infiltrating tumor cells was always much higher than that of reactive astrocytes. *In vivo* tropism of lentiviral LCMV-GP pseudotypes for normal brain cells has been studied, but not in great detail (Watson *et al*., 2002; Wong *et al*., 2004). Both groups showed that VSV-G pseudotypes transduced normal brain cells more efficiently than did LCMV-GP pseudotypes. However, the transduced cell populations were not determined precisely. In our experiments, astrocytes were the predominant transduced cell population in the brain of healthy Fischer rats. The same results were achieved with cultured brain cells.

α-Dystroglycan was found to be a cellular receptor for LCMV (Cao *et al*., 1998). In the CNS, α-dystroglycan is expressed on neurons and astrocytes (Moukhles and Carbonetto, 2001; Zaccaria *et al*., 2001). This is in accordance with studies showing that LCMV infects primarily glial cells, from where it spreads to neurons during persistent infection (Joly *et al*., 1991; Oldstone and Rall, 1993). In particular, Bonthius *et al*. (2002) have demonstrated that glial cells are the first cells of the brain parenchyma to be infected with LCMV. These findings are also in accordance with the susceptibility of astrocytes to LCMV-GP pseudotype transduction found in our study. The scarce transduction of neurons, however, is somewhat surprising. A problem may be that the vectors used are replication defective, while spread of virus via direct cell contact between glial and neuronal cells is required for infection of neurons with LCMV. In addition, a yet unidentified second receptor for LCMV may be required for infection, which is not expressed at sufficient levels on neurons. This hypothesis is supported by two studies showing that some LCMV variants inefficiently interact with

α-dystroglycan and also infect α-dystroglycan-negative cells (Sevilla *et al*., 2000; Smelt *et al*., 2001).

In previous studies (Miletic *et al*., 1999; Beyer *et al*., 2001, 2002), we characterized the major features of both lentiviral and oncoretroviral LCMV-GP pseudotypes: the glycoprotein is stable, allowing concentration by ultracentrifugation for high vector titers. The vector can be frozen and thawed without significant loss of virus titer. Moreover, stable packaging cell lines producing LCMV-GP-pseudotyped retroviral vectors have been established. This is an important advantage over VSV-G-pseudotyped vectors, because cytotoxicity of the VSV glycoprotein impedes generation of packaging cell lines constitutively expressing VSV-G. Stable packaging cell lines, however, are an important tool in gene therapy of solid tumors, as they can be implanted into the tumor bed, which allows more efficient delivery of the vector than does direct injection of vector preparations.

Several LCMV strains have been identified that carry glycoprotein variants with altered tissue tropism (Southern *et al*., 1984; Fazakerley *et al*., 1991). Matloubian *et al*. have shown that single amino acid changes in the LCMV glycoprotein can alter LCMV tropism (Matloubian *et al*., 1993). By pseudotyping viral vectors with LCMV glycoproteins from these strains, or even by inserting specific mutations into LCMV glycoproteins, the spectrum of gene therapeutic targets could be broadened. In conclusion, although further studies with therapeutic genes are warranted, lentiviral vectors pseudotyped with LCMV glycoproteins represent an attractive option for gene therapy of malignant glioma.

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