

Antiangiogenic Gene Therapy in a Rat Glioma Model Using a Dominant-Negative Vascular Endothelial Growth Factor Receptor 2

MARCIA R. MACHEIN,¹ WERNER RISAU,² and KARL H. PLATE¹

ABSTRACT

Malignant gliomas are a prominent target for cancer gene therapy approaches because of their poor prognosis despite all currently available therapies. Gene therapy strategies developed to interfere with the normal function of vascular endothelial growth factor receptors have been successfully used in different experimental models to block tumor angiogenesis and to inhibit tumor growth. In this study we examined whether retroviruses encoding a mutant VEGF receptor 2 (VEGFR-2) could suppress tumor angiogenesis and thereby prolong the survival of rats bearing syngeneic intracerebral glioma tumors. Survival time of rats with intracerebral tumors was significantly prolonged in a dose-dependent manner when retroviruses carrying a VEGFR-2 mutant were cotransplanted with tumor cells. No effect on survival was observed in rats that received virus-producing cells or virus supernatant intracerebrally after 5 days of tumor injection. In established subcutaneous tumors treatment with multiple injections of virus-producing cells also inhibited tumor growth in a dose-dependent manner. After implantation of tumor cells stably transfected with a truncated form of VEGFR-2, rats exhibited a rate of survival similar to that of animals treated with high numbers of virus-producing cells encoding the truncated form of VEGFR-2. Morphologically, tumors showed signs of impaired angiogenesis, such as extensive necrosis and reduced tumor vascular density. These results suggest a dual mode of function of truncated VEGFR-2, namely dominant-negative inhibition of VEGFR-2 function and VEGF depletion by receptor binding. We further explored the safety of retrovirus-mediated gene transfer. Although virus sequences were found in different tissues after intracerebral injection of virus-producing cells, no morphological changes were observed in any tissue after a follow-up time of 6 months. Our results indicate that VEGFR-2 inhibition is useful for the treatment of malignant gliomas.

OVERVIEW SUMMARY

Approaches to block VEGF/VEGFR pathways have been successfully used to inhibit tumor growth. The present study was undertaken to evaluate the effect of retroviruses encoding dominant-negative VEGFR-2 on the survival of rats bearing 9L intracerebral tumors and on growth of 9L subcutaneous tumors. The effect on tumor growth was dose dependent, with the maximal effect achieved when virus-producing cells were coimplanted in eightfold excess over tumor cells. Established tumors growing subcutaneously could be suppressed with multiple injections of virus-producing cells, also in a dose-dependent manner. No effect on survival was

observed in rats that received virus-producing cells or virus supernatant after 5 days of tumor injection. In summary, our data suggest that antiangiogenic gene therapy using a dominant-negative VEGFR-2 strategy is a feasible approach to control brain tumor growth if gene delivery can be improved.

INTRODUCTION

GLIOMAS REPRESENT one-third of all primary brain tumors. They have an extremely poor prognosis despite the use of surgery, radiation therapy, and chemotherapy (Black,

¹Department of Neuropathology, Freiburg University Medical School, D-79106 Freiburg, Germany.

²Department of Molecular Cell Biology, MPI for Physiological and Clinical Research, W.G. Kerckhoff-Institute, D-61231Bad Nauheim, Germany.

1991). The development of new therapeutic strategies such as gene therapy may therefore be of great promise. One potential gene therapeutic approach for malignant tumors involves inhibition of tumor angiogenesis (Plate, 1996; Kong and Crystal, 1998).

Angiogenesis relies on the concept that the growth of solid tumors is dependent on their ability to satisfy their demand for nutrients and oxygen (Folkman, 1995a; Folkman *et al.*, 1966). To meet this demand tumors must be able to generate new blood vessels, through a process termed *angiogenesis* (Folkman, 1995b).

Angiogenesis is a complex process that includes activation, proliferation, and migration of endothelial cells, disruption of the capillary basal membrane, and formation of vascular tubes and networks (Folkman, 1995a). In tumors, angiogenesis can be triggered by various proteins secreted by tumor cells or macrophages (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). Vascular endothelial growth factor (VEGF) appears to be the most relevant angiogenic factor in solid tumors (Kim *et al.*, 1993; Senger *et al.*, 1993; Saleh *et al.*, 1996). Consistent with this concept, VEGF is upregulated in most human tumors (Plate *et al.*, 1992; Brown *et al.*, 1993a,b, 1995; Senger *et al.*, 1993; Suzuki *et al.*, 1996). The mechanism of VEGF upregulation is not fully understood but growing evidence suggests that hypoxia is the major driving force (Shweiki *et al.*, 1992; Damert *et al.*, 1997).

VEGF binds to endothelial cells via interaction with high-affinity tyrosine kinase receptors Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2), found almost exclusively on endothelial cells (Shibuya *et al.*, 1990; De Vries *et al.*, 1992; Terman *et al.*, 1992; Millauer *et al.*, 1993). The selective expression of VEGF receptors ensures that VEGF action is confined to the endothelial cells.

The *fms*-like tyrosine receptor (Flt-1, VEGFR-1) consists of seven IgG-like repeats in the ligand-binding domain, a single transmembrane domain, and a tyrosine kinase domain (Shibuya *et al.*, 1990). The function of Flt-1 is still speculative. Although necessary for normal vascular development (Fong *et al.*, 1995), there is no evidence of the participation of Flt-1 in endothelial cell migration or proliferation (Waltenberger *et al.*, 1994).

Fetal liver kinase (Flk-1, VEGFR-2) is the mouse homolog of kinase insert domain-containing receptor, KDR, in humans (Matthews *et al.*, 1991; Terman *et al.*, 1992; Millauer *et al.*, 1993). VEGFR-2 has a similar arrangement of extracellular, transmembrane, and intracellular domains than VEGFR-1. In contrast to VEGFR-1, VEGFR-2 is phosphorylated and VEGFR-2-expressing cells proliferate after addition of VEGF (Millauer *et al.*, 1993; Waltenberger *et al.*, 1994).

Previously, upregulation of VEGF and VEGF receptors during glioma development and progression has been described. VEGF receptors were not detected in normal human brain vascular cells, whereas expression was increased in the tumor vasculature (Plate *et al.*, 1993, 1994; Plate and Risau, 1995). Thus, the upregulation of VEGF receptors appears to be a critical event that regulates glioma angiogenesis.

The relevance of the VEGF/VEGFR-2 ligand/receptor system for tumor angiogenesis was confirmed in different experimental settings (Millauer *et al.*, 1994, 1996; Strawn *et al.*, 1996; Skobe *et al.*, 1997). Millauer *et al.* (1994) used retrovirus-mediated gene transfer of a dominant-negative VEGFR-2 to block

endogenous wild-type receptor function. Although an effect of dominant-negative VEGFR-2 on tumor growth was observed, further investigations are warranted to develop this laboratory model into clinically applicable gene therapy. In this study we explored the efficacy of dominant-negative VEGFR-2 receptor antiangiogenic therapy in a syngeneic subcutaneous and intracerebral rat glioma model.

In addition, we investigated the safety of retrovirus-mediated gene transfer encoding a truncated VEGFR-2.

MATERIALS AND METHODS

Recombinant vectors

Production of LXflk-1TM vector has been previously described (Millauer *et al.*, 1994). In brief, the flk-1TM mutant lacks 561 C-terminal amino acids of the intracellular kinase domain. The flk-1TM cDNA was subcloned into pLXSN retroviral plasmid. Expression of the cDNAs is driven by the 5' Moloney murine sarcoma virus long terminal repeat (LTR). The vector also contains an internal simian virus 40 (SV40) promoter that drives a neomycin resistance gene (*neo^r*) (Miller and Rosman, 1989).

Cells and cell cultures

GPE LXflk-1TM is a GP+E86 ecotropic virus-packaging cell line that produces virus carrying flk-1TM (Millauer *et al.*, 1994). GPE LXNULL is a similar virus-packaging line with pLXSN vector without transgene, and is used as control. GPE LXNULL virus-producing cells were obtained by calcium phosphate transfection of GP-E86 as described (Ausubel, 1997). Individual colonies were harvested and tested for virus production and vector integration by Southern blot analysis. Titers of virus-producing cell lines were about 1×10^6 colony-forming units (CFU)/ml.

GS-9L (rat gliosarcoma) cells were a gift of T. Budd (St. Lawrence University, New York). GS-9L-flk-1TM cells were engineered by infecting GS-9L with virus supernatant containing flk-1TM. Transfected cells were selected in G418 (Pansystems, Aidenbach, Germany) (1 mg/ml). Clones with high expression levels of flk-1 were screened by Northern and Western blot. Expression of cell surface receptors was determined by an ^{125}I -labeled VEGF binding study as described (Clauss *et al.*, 1996). Growth curves *in vitro* of GS-9L-flk-1TM were determined as described (Saleh *et al.*, 1996) and were similar to those of parental cells.

Virus-producing cells were cultured in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) containing G418. GS-9L and GS-9L-flk-1TM cells were cultivated in 10% FBS-RPMI (Life Technologies). For *in vivo* injections, cells were trypsinized, centrifuged, rinsed twice with phosphate-buffered saline (PBS), counted (Casy 1; Schärfe Systems, Reutlingen, Germany), and resuspended in PBS for inoculation.

Preparation of flk1-TM virus-containing concentrated supernatant

Viral stock was obtained from cultures that had been grown to near 80% confluence in selection medium, then maintained

in medium lacking G418 for 24 hr. The supernatant from GPE LXflk-1TM virus-producing cells was harvested, filtered through a 45- μ m pore size filter, and stored at -70°C . To concentrate the virus supernatant, stocks were thawed and centrifuged for 20 min at 14,000 rpm in a JA-14 rotor (Beckman Instruments, Palo Alto, CA) at 4°C . The supernatant was transferred into new sterile bottles and centrifuged for 16 hr at 14,000 rpm. The supernatant was discarded and the pellet was resuspended in 0.1% of the original volume in DMEM. The virus titer was 4.5×10^7 CFU/ml.

Intracerebral tumor implants

All procedures with animals were performed according to the institutional guidelines for use of laboratory animals. Female Fischer 344 rats (150–180 g) (Charles River Laboratories, Wilmington, MA) were used for syngeneic GS-9L implants. Transplantation has been previously described (Plate *et al.*, 1993). For coimplantation experiments, tumor cells (4×10^5) were stereotactically inoculated either alone or mixed with virus-producing cells in a final volume of 20 μ l. Virus-containing concentrated supernatant (VCS, 10 μ l) was coinoculated with 4×10^5 GS-9L. The rats were divided into groups described in Table 1.

To determine the efficacy of an antiangiogenic therapy using the VEGFR-2 dominant strategy in established tumors, 4×10^5 GS-9L tumor cells were implanted intracerebrally. Five days later, 3×10^6 GPE LXflk-1TM (five animals), or VCS (five animals) in a final volume of 10 μ l, was stereotactically injected at the tumor inoculation site using the same coordinates. At this time point, two additional animals were sacrificed to confirm tumor growth. Control animals ($n = 5$) were injected with PBS alone.

To determine the toxicity of virus-producing cells in the brain and peripheral organs, 4×10^5 GPE LXflk-1TM were implanted intracerebrally. Three and 6 months after cell injection, rats (two animals at each time point) were sacrificed and tissues were collected as described below.

Animals were observed daily for the development of symptoms associated with progression of intracerebral tumors. Animals displaying advanced symptoms (loss of appetite, leaning, dehydration, and/or significant weight loss) were sacrificed by cardiac perfusion with PBS. The brains were removed and analyzed histologically. From two animals of each group, the brains were divided into two parts so that both parts contained tumor tissue. One part was fixed in 4% paraformaldehyde for 5 days, and reserved for histological examination. The other part of the brain and heart, kidney, liver, lung, and spleen were immersed in cryovials and immediately frozen in liquid nitrogen. Rats without symptoms after 3 months of tumor cell implantation were excluded from the study.

Subcutaneous tumor implants

To investigate the influence of tumor size on tumor growth inhibition after GPE LXflk-1TM administration, six groups of 344 Fischer rats ($n = 6$ for each group) were injected subcutaneously with 10^6 GS9L tumor cells in 500 μ l of PBS in the right flanks. GPE LXflk-1TM cells (10^6) were inoculated every 3 days, with the beginning of treatment being at a different time point for each group: day 0 (group II), day 3 (group III), day 9 (group IV), day 18 (group V), and day 36 (group VI). Control tumors were injected with PBS alone (group I). On day 39, rats were sacrificed and tumors were removed for histological analysis. To quantify tumor growth, two perpendicular diameters of the resultant subcutaneous tumors were measured with calipers every 3 days. The inhibition was calculated by comparing the tumor size of groups injected with GPE LXflk-1TM with that of the control group.

DNA isolation and analysis

DNA was isolated from tissue by overnight incubation at 55°C with proteinase K (0.5 mg/ml; Boehringer GmbH, Mannheim, Germany) in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS). Each

TABLE 1. SURVIVAL OF FISCHER 344 RATS IN COIMPLANTATION AND LATE THERAPY EXPERIMENTS

Group	Ratio of tumor cells to virus producer cells	Number of rats	Mean survival time ^a
Coimplantation experiments			
Control (GS-9L alone)	—	13	12 (1.5)
GS-9L/GPE LXNULL	1:1	5	12 (0.8)
GS-9L/GPE LXflk-1TM	1:0.01	10	15 (1.2)
GS-9L/GPE LXflk-1TM	1:0.1	15	26 (12)
GS-9L/GPE LXflk-1TM	1:1	11	32 (10.3)
GS-9L/GPE LXflk-1TM	1:8	9	41 (8.1)
GS-9L/VCS	—	10	24 (3.5)
GS-9L-flk-1TM	—	5	39 (7.4)
GPE LXflk-1TM	—	4	Sacrificed without symptoms (3 and 6 months)
Late therapy experiments			
Control (PBS)	—	5	12 (0.7)
GPE LXflk-1TM (3×10^6)	—	5	12 (2.1)
VCS	—	5	11 (2.0)

^aStandard deviation in parentheses.

mixture was subsequently extracted two times with equal volumes of buffered phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated with 2 vol of ethanol and 0.5 vol of 7.5 M ammonium acetate, pelleted at 12,000 × g in a microcentrifuge for 10 min, rinsed twice with 70% ethanol, and dried at room temperature in a laminar flow hood. The DNA pellet was resuspended in 80 μl of water with RNase A (0.1 mg/ml; Sigma, St. Louis, MO) and incubated at 37°C for 30 min. To remove digested RNA, DNA was again precipitated as described above. The DNA pellet was rehydrated in 80 μl of water. OD₂₆₀ readings were taken to determine the DNA concentration. A 1-μg sample was run on a 0.8% agarose gel to confirm visually the concentration of samples and DNA purity.

Southern blot analysis

Sample DNA (10 μg/lane) was digested by overnight incubation with *Eco*RI (10 IU/μg; Boehringer GmbH) and electrophoresed in a 1% agarose gel in 1× TAE buffer at 35 V. DNA was transferred by a standard Southern procedure to a Duralon membrane (Stratagene, La Jolla, CA). After UV cross-linking, membranes were hybridized for 1 hr in Quick Hyb (Stratagene) at 68°C with randomly primed, ³²P-labeled, 760-bp *neo*^r fragment.

Detection of *neo*^r gene by PCR

For amplification of the *neo*^r gene, the following oligonucleotide primers were used as described (Kiem *et al.*, 1994):

Sense primer Neo R 350: 5' AAGAGACAGGATGAAG-GATCG 3'

Antisense primer Neo R 1150: 5' CAGAAGAAGCTCGT-CAAGA 3'

These sequences amplified an 800-bp fragment. DNA samples (1 μg) were amplified by using 2.5 U of *Taq* polymerase (Qiagen, Hilden, Germany) per 50-μl reaction mixture with 150 ng of each primer. The thermocycler profile used included initial denaturation at 94°C for 4 min, followed by 30 cycles each consisting of 94°C for 1 min, 50°C for 2 min, and 72°C for 4 min. Aliquots (10 μl) were separated in a 1% agarose gel and transferred to nylon membrane (Duralon-UV; Stratagene) by a Southern blotting method (Ausubel, 1997). *neo*^r-specific polymerase chain reaction (PCR) products were detected by hybridization of a ³²P-labeled DNA probe. This probe was synthesized from a plasmid fragment containing the *neo*^r sequence by PCR amplification. Primers used in this synthesis were as follows:

Sense primer Neo R 450: 5' ACAAACAGACAATCGGCT-GCT 3'

Antisense primer Neo R 1027: 5' GCCAACGCTATGTCCT-GATA 3'

The resultant 577-bp fragment would hybridize to *neo*^r fragments amplified by the previous primers, but should not hybridize to the primers themselves.

RNA probe generation by *in vitro* transcription

To determine the infection rate of tumor/endothelial cells in tumor and normal tissue, *in situ* hybridization was conducted

using a *neo*^r probe and an Flk-1 probe. A 760-bp *neo*^r PCR fragment in PGEM-T (Promega, Madison, WI) and a 2.6-kb *Eco*RI–*Bam*HI fragment of the murine flk-1 cDNA encoding part of the extracellular domain in PGEM3 (Promega) were used to generate nonradioactive antisense and sense RNA probes by *in vitro* transcription. After linearization of the plasmid, single-stranded “runoff” transcripts were synthesized from the SP6/T7 polymerase promoters by using digoxigenin-labeled UTP as a substrate according to the manufacturer instructions (DIG RNA-labeling kit; Boehringer GmbH). After determination of concentration and labeling efficiency, digoxigenin-labeled probes were stored at –20°C or directly used for *in situ* hybridization analysis.

In situ hybridization

Paraffin-embedded 4-μm sections were deparaffinized, followed by digestion with proteinase K (10 mg/ml) for 15 min at 37°C. After postfixation with 4% paraformaldehyde, sections were acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min at room temperature. Prehybridization solution containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% deionized formamide, 2% SDS, 5× Denhardt's solution, 10% dextran sulfate, and yeast tRNA (0.5 mg/ml) was applied for 1.5 hr at 48°C. The RNA sense/antisense probe concentration for hybridization was 0.1–0.5 ng/ml of initial transcript. Tissue sections were incubated in a humidified chamber under glass coverslips at 48°C overnight. Posthybridization stringency washes at 48°C included 2× SSC for 30 min, 2× SSC plus 0.1% SDS for 5 min, 0.1× SSC plus 0.1% SDS for 15 min. Each wash was carried out twice. After RNase A treatment (2.5 mg/ml in 2× SSC) for 5 min at 37°C hybridized probes were detected by anti-DIG antibody conjugated to alkaline phosphatase (Boehringer GmbH) diluted 1:500 for 1 hr at room temperature. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution was used as color substrate in 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5. Color reaction times ranged from 6 to 8 hr, after which slides were rinsed in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Immunohistochemistry

Immunohistochemical stainings were performed with von Willebrand factor (Dakopatts, Copenhagen, Denmark) (diluted 1:100), a rat-specific CD3 marker (IF4; Serotec, Oxford, England) (diluted 1:1000), and a rat-specific microglia-macrophage marker (Ed-1; Serotec) (diluted 1:1000). Staining was carried out using immunoperoxidase staining kits for rabbit and mouse immunoglobulins (Vector Laboratories, Burlingame, CA) according to the manufacturer instructions. The immunoperoxidase reaction was visualized with 3,3'-diaminobenzidine-HCl (DAB) buffer tablets (Merck, Darmstadt, Germany) or 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) and 0.006% H₂O₂. The slides were briefly counterstained with hematoxylin and mounted. Negative controls included incubations in which the primary antibodies were omitted.

Combined *in situ* hybridization and immunohistochemistry

To visualize whether endothelial cells were transduced *in vivo* with GPE LXflk-1 TM, we performed *in situ* hybridiza-

tion as described, followed by immunostaining with von Willebrand factor. After *in situ* hybridization color development, slides were blocked with serum (Vector Laboratories) and immunohistochemistry using von Willebrand factor (Dakopatts) was performed as described.

Statistical analysis

Statistical analysis was performed using Statistica (StatSoft) software for Kaplan–Meier plots. The log-rank test was used for statistical analysis of survival data. $p < 0.001$ was considered significant.

RESULTS

We first compared the survival time of animals coimplanted with GPE LXflk-1TM in different ratios (tumor cells/virus-producing cells) with the survival of control groups (GS-9L alone and GS-9L with GPE LXNULL). The virus-producing cells GPE LXNULL were used as control to exclude unspecific responses in tumor growth due to infection with retroviruses. The results are summarized in Table 1. Animals without treatment developed neurological symptoms within 12–14 days after tumor cell injection. In animals inoculated with tumor cells and GPE LXflk-1TM (1:0.1, 1:1, and 1:8 ratios) survival was longer than in control rats (Fig. 1). No survival benefit was observed

in animals coimplanted with GS-9L/GPE LXflk-1TM at a ratio of 1:0.01. The effect on survival of tumor-bearing rats was dose dependent, with a maximum achieved when the virus-producing cells were in eightfold excess relative to the tumor cells. Rats treated with flk-1TM virus particle-containing producer cell supernatants (VCS) immediately after tumor cell implantation also exhibited prolonged survival, but to a lesser extent when compared with rats inoculated with virus-producing cells.

We then investigated whether the VEGFR-2 dominant-negative strategy was able to prolong survival in rats with established GS-9L tumors. Animals treated with GPE LXflk-1TM or VCS 5 days after tumor cell inoculation did not show any significant change in survival time, compared with the control group (Table 1; Fig. 1).

We evaluated whether the VEGFR-2 dominant-negative strategy could inhibit tumor growth in established tumors in a subcutaneous tumor model. Rats coinoculated with GS-9L and GPE LXflk-1TM at a 1:1 ratio developed small tumors with 92% tumor growth inhibition. When GPE LXflk-1TM was injected commencing on day 3, 67.5% suppression of tumor growth was observed; 45% tumor growth suppression was observed when the treatment was started on day 9 after tumor cell inoculation. No effects on tumor growth were observed when the tumors were inoculated with GPE LXflk-1TM commencing on days 18 and 36 after tumor cells injection (Fig. 2).

We investigated further the participation of VEGF sequestration in the dominant-negative strategy by inoculating intra-

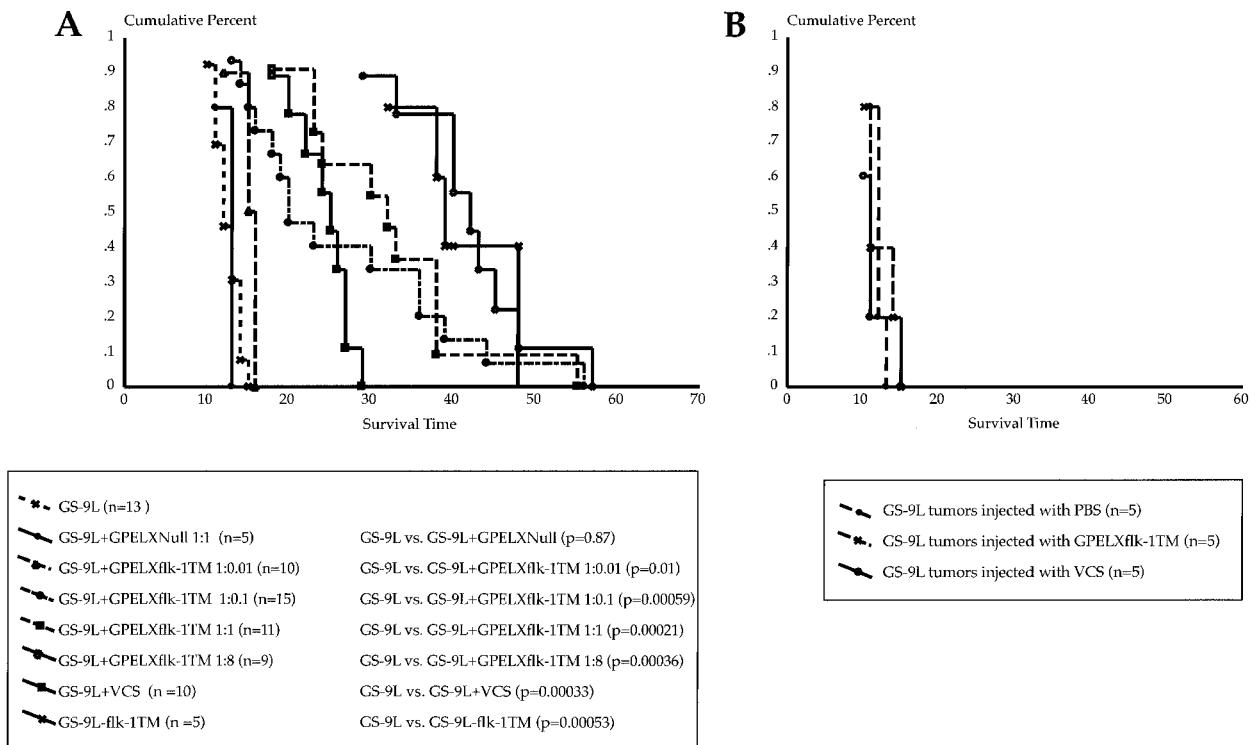


FIG. 1. (A) Kaplan–Meier survival curves of Fischer 344 rats after brain grafting of GS-9L cells alone, or after coimplantation with GPE LXNULL, with different ratios of GPE LXflk-1TM, or with VCS. Survival of rats implanted with GS-9L-flk-1TM was also evaluated. Differences in survival were evaluated by the log-rank test. (B) Kaplan–Meier survival curves of rats with GS-9L tumors treated with a single injection of GPE LXflk-1TM or VCS. Control animals were injected with PBS alone. Intracerebral implantation of GS-9L tumor cells (4×10^5) on day 0. Virus-producing cells (3×10^6) or VCS was injected 5 days after tumor cell inoculation. No significantly prolonged survival rates were observed in the late treatment groups.

cerebrally GS-9L stably transfected with flk-1TM. These cells exhibited growth rates similar to that of the parental cell line *in vitro* (data not shown); therefore, the effect on tumor growth *in vivo* cannot be attributed to alteration in tumor cell mitogenesis due to expression of VEGFR-2 or retrovirus integration. GS-9L flk-1TM binds VEGF as determined by a ^{125}I -labeled VEGF-binding assay (data not shown). Rats implanted with GS-9L flk-1TM showed significantly prolonged survival, similar to that obtained in rats coimplanted with an eightfold excess of virus-producing cells relative to tumor cells (Fig. 1).

Histologically, control animals showed large tumors with well-vascularized tissue. Necrotic areas were absent. In contrast, GPE LXflk-1TM-coinoculated tumors showed large areas of necrosis with reduced tumor vascularization (Fig. 3). Rats injected with GS-9L stably transfected with flk-1TM developed large tumors, with central necrosis. Vascularization of these tumors was greatly impaired, since only a few vessels around necroses were observed (Fig. 3). Little immunoreactivity was observed for monocyte-macrophage and T cell markers in all tumors studied (data not shown).

Detection of *neo^r* and VEGFR-2 in tumors

To detect infection rates of treated tumors we performed *in situ* hybridization with *neo^r* and VEGFR-2. The efficacy of gene transfer was proportional to the number of implanted virus-producing cells in the coimplantation experiments. Using the gene marker *neo^r*, we observed that the majority of transduced cells

were tumor cells. At the time point of tumor removal, endothelial cells were sporadically positive for *neo^r* (Fig. 4a and b). Dominant-negative VEGFR-2-inhibited tumors expressed *neo^r* in tumor cells (Fig. 4c and d), which matched the result obtained with a VEGFR-2-specific probe (flk-1) (Fig. 4e and f). Endogenous VEGFR-2 expression in tumor endothelial cells was confirmed by *in situ* hybridization (data not shown). *In situ* hybridization of GS-9L flk-1TM tumors with an flk-1 probe confirmed VEGFR-2 expression (Fig. 4g and h). In tumors treated with GPE LXflk-1TM and VCS 5 days after tumor cell implantation, transduction efficiency was low, with only focal *neo^r*-positive signals (data not shown).

Detection of gene marker in peripheral organs

To determine whether transduction of peripheral organs occurs after intracerebral inoculation of virus-producing cells, genomic DNA was isolated from tumors (as positive control), contralateral brain, heart, liver, lung, kidney, and spleen after the animals died. Using Southern blot analysis for *neo^r* gene, we could detect specific hybridization signals only in treated tumors (data not shown). In addition, we performed PCR analysis for *neo^r* to detect retrovirus sequences that were present but below the level of Southern blot detection sensibility. We amplified *neo^r* marker gene in all organs studied. Variability in hybridization signals existed between animals, but since this was not a quantitative study, the differences may have been due to DNA loading or variations in amplification efficiency. In rats

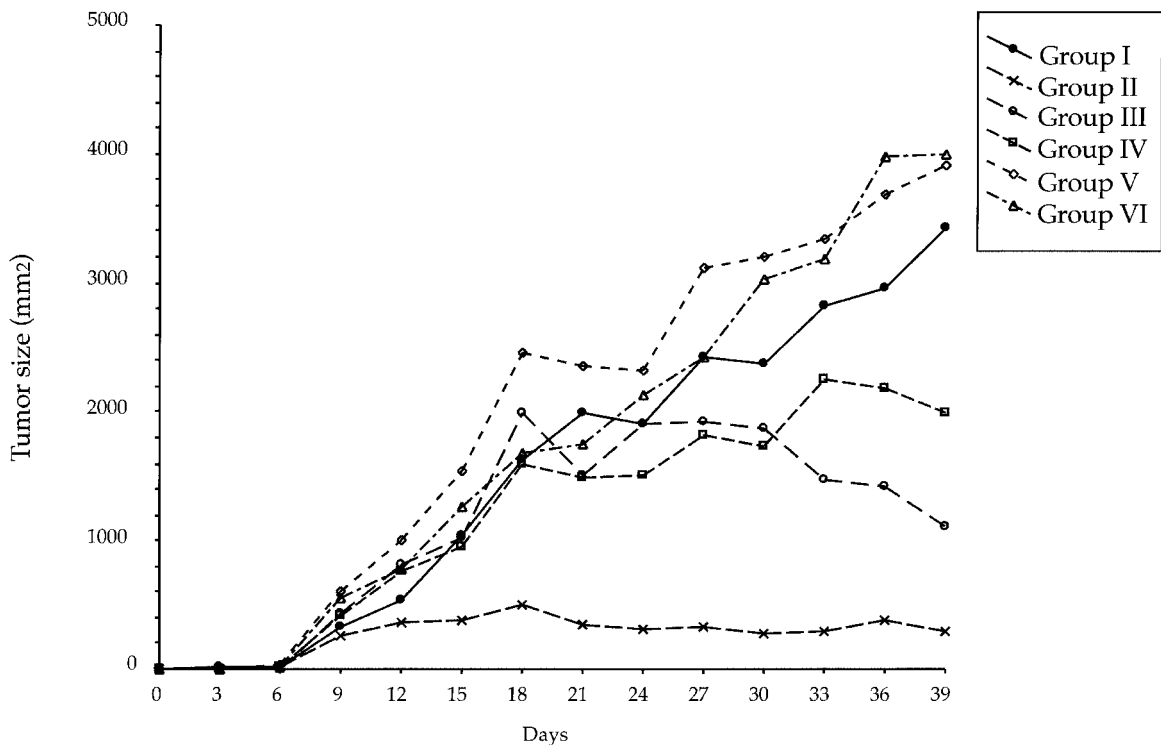


FIG. 2. Growth of GS-9L subcutaneous tumors ($n = 6$ for each group) treated with GPE LXflk-1TM. Tumors were inoculated with 10^6 GPE LXflk-1TM cells every 3 days, with the beginning of treatment being at different time points for each group: day 0 (group II), day 3 (group III), day 9 (group IV), day 18 (group V), and day 36 (group VI). Tumors were removed on day 39. Control tumors were injected with PBS (group I).

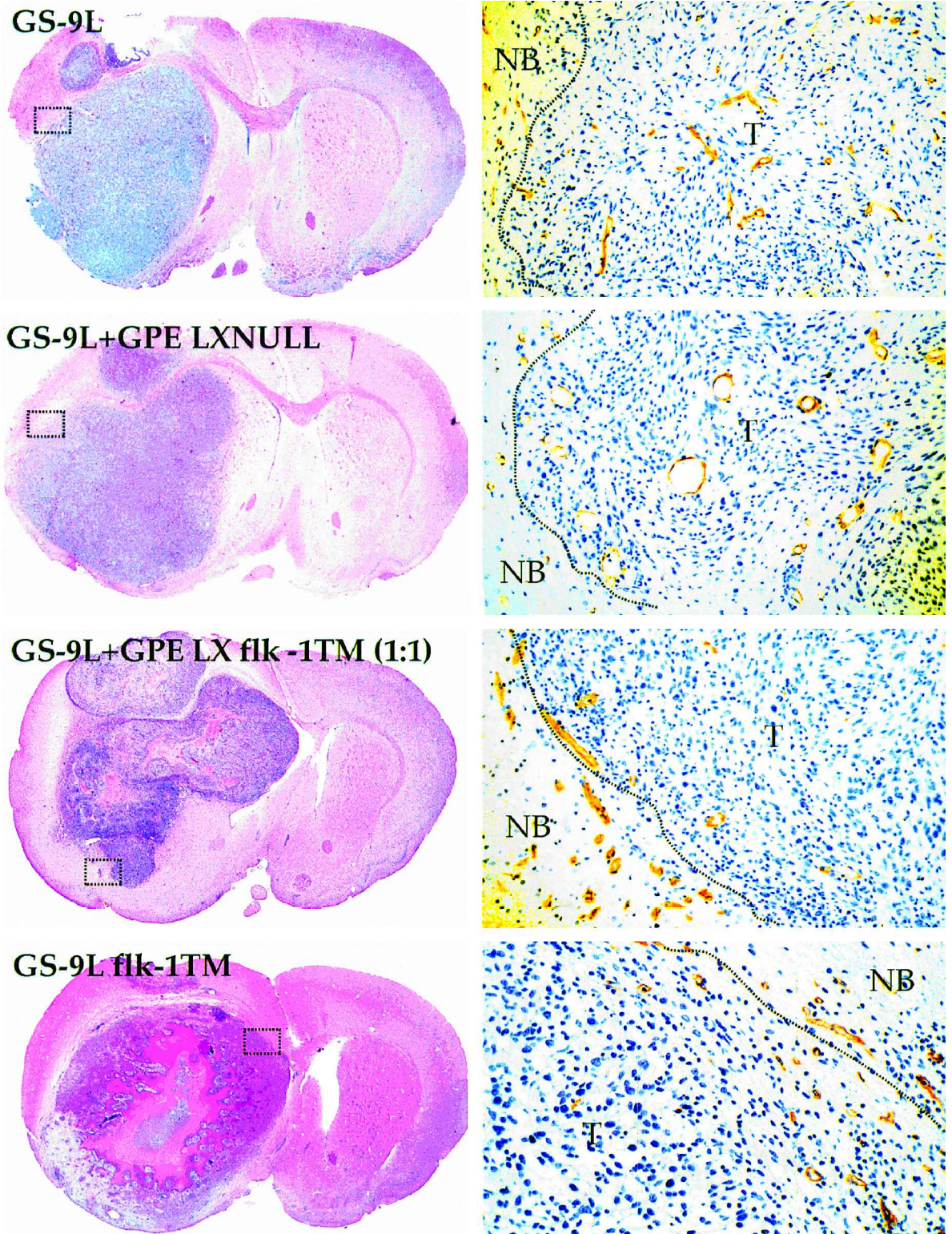


FIG. 3. Hematoxylin and eosin staining, left (original magnification, $\times 7$), and immunohistochemistry for von Willebrand factor, right (original magnification, $\times 125$), of control and treated tumors. Control animals (implanted with GS-9L alone and coinjected with GPE LXNULL) showed large tumors, with well-vascularized tissue. Necrotic areas were absent. In contrast, GPE LXflk-1TM-treated tumors showed extensive necrosis with reduced tumor vascularization. Animals injected with GS-9L stably transfected with flk-1TM also developed large tumors, with central necrosis. NB, Normal brain; T, tumor.

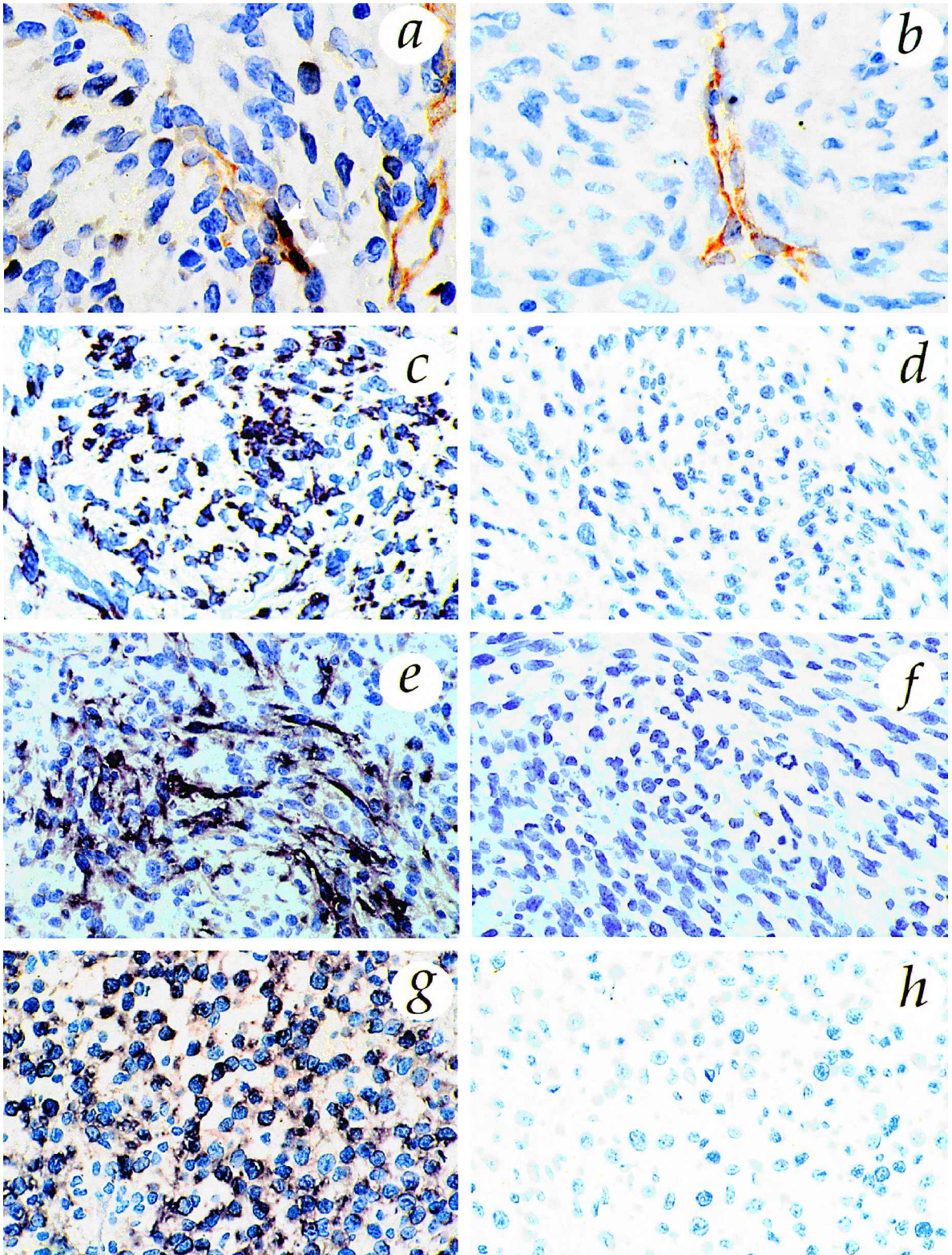


FIG. 4. Expression of gene marker *neo'* in endothelial cells and tumor cells. (a) *In situ* hybridization with von Willebrand immunohistochemistry of GS-9L tumor sample coimplanted with GPE LXflk-1TM, showing endothelial cells positive for *neo'* (arrowheads); (b) sense control (original magnification, $\times 500$). (c-f) Coexpression of *neo'* (c), sense control (d), and VEGFR-2 (e) in clusters of tumor cells; sense control (f) (original magnification, $\times 312$). (g) GS-9L flk-1TM tumors, showing flk-1 mRNA expression; (h) sense control (original magnification, $\times 312$).

implanted with GPE LXflk-1TM only, we found *neo^r* sequences predominantly in lung and spleen at 3 months postinjection. At 6 months postinjection no specific amplification of *neo^r* gene was found in any tissue (Fig. 5). To identify the cells positive for retroviral vector sequences, *in situ* hybridization analyses were carried out on tissues that were strongly positive for *neo^r* gene on PCR analysis. None of these tissues showed detectable mRNA levels for *neo^r* (data not shown). Histological analysis of these tissues showed no morphological abnormalities (data not shown).

DISCUSSION

Our study shows prolonged survival when GS-9L tumor cells and virus-producing cells expressing a truncated VEGFR-2 were coinjected into rat brains. The morphological features of tumors, such as necrosis and decreased tissue vascularization, suggest that angiogenesis was successfully impaired in this model. On the other hand, tumors injected with control vector virus producer cells were highly vascularized, indicating that tumor neovascularization was not affected by the presence of these cells or by infection with retrovirus. Moreover, null vector virus-producing cells did not have any protective effect on survival of the rats.

The survival was prolonged in a dosage-dependent manner in coimplantation studies. Virus-concentrated supernatant also suppressed tumor growth and prolonged survival time, but to a lesser extent as observed when virus-producing cells were coimplanted. Under these experimental conditions, virus-producing cells or retroviruses were in close contact with target cells and transduction efficiency was likely to be high.

We next implanted virus producer cells or virus-concentrated supernatant in established intracerebral tumors, a experimental setting that mimicks more closely the clinical situation in humans. On day 5 after tumor cell inoculation, rats received an

intratumoral injection of GPE LXflk-1TM or VCS. No changes in survival time were observed in the treated groups. Using *in situ* hybridization with a *neo^r* probe, we observed a low level of transduction that was restricted to a small tumor zone. It is well established that the transduction of target cells using retroviral vectors occurs in the immediate vicinity of virus-producing cells, since the diffusion of virus particles is limited in the tumor extracellular matrix (Puumalainen *et al.*, 1998). Thus, at the time point of treatment tumors might already be too large to achieve efficient transduction. Even if optimal gene transduction could be obtained in this setting, a syngeneic 9L intracerebral glioma may grow too fast (median survival, 12 days) for cytostatic rather than cytotoxic therapy.

We further examined the influence of tumor size on the therapeutic efficacy of VEGFR-2 dominant-negative retroviral treatment in a subcutaneous tumor model. Significant growth inhibition was observed when an equal ratio of tumor cells and virus-producing cells was used. Delayed treatment starting on day 3 or on day 9 also suppressed tumor growth (67.5 and 45%, respectively). In the later treatment groups (treatment commencing on day 18), the failure of tumor growth inhibition most likely represents inadequate gene transfer since in large tumors, a large fraction of tumor and endothelial cells escaped transduction. Taken together, these results emphasize the limits of retrovirus-mediated gene therapy protocols.

Relatively few endothelial cells were found to coexpress *neo^r* and von Willebrand factor. The majority of transduced cells were tumor cells. This observation raised the possibility that in addition to dominant-negative receptor inhibition, VEGF depletion by VEGFR-2-expressing tumor cells could participate in the antiangiogenic effect. We therefore implanted GS-9L cells stably transfected with the truncated VEGFR-2. In this setting, which mimicks an optimal situation of *in vivo* gene transfer to tumor cells, but will leave endothelial cells untransduced, rats exhibited a rate of survival similar to that of animals treated

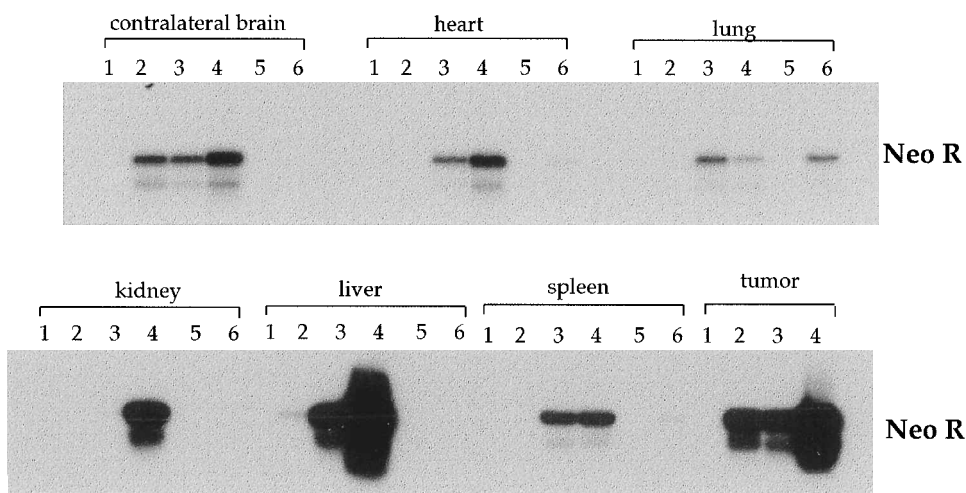


FIG. 5. PCR analysis for *neo^r* in peripheral organs after inoculation with GPE LXflk-1TM, performed using *neo^r* primers and 1 μ g of DNA. Negative control tissue DNA (lane 1) was obtained from rats implanted only with tumor cells. Rats were implanted with GS-9L and GPE LXflk-1TM at ratios of 1:0.1 (lane 2), 1:1 (lane 3), and 1:8 (lane 4). Rats implanted only with GPE LXflk-1TM were sacrificed 3 months (lane 6) and 6 months (lane 5) after intracerebral inoculation.

with high numbers of GPE LXflk-ITM. Morphologically, tumors showed a similar phenotype of impaired angiogenesis with large necrotic areas and few tumor capillaries. GS-9L flk-ITM expressed the extracellular domain from VEGFR-2 at the cell surface and could therefore bind VEGF, reducing the amount of VEGF available to endothelial cells.

Several gene therapy strategies have been developed in order to interfere with the normal function of endothelial cell receptors (Goldman *et al.*, 1998; Lin *et al.*, 1998). Kong *et al.* (1998) showed that an adenovirus encoding soluble VEGFR-1 substantially suppresses tumor metastasis. The authors suggested that the soluble VEGFR-1 might interact with endogenous VEGFR-1 and VEGFR-2, forming inactive heterodimeric receptors; alternatively, the soluble receptor could sequester VEGF.

Thus, dominant-negative strategies for VEGFR-1 and VEGFR-2 may act not only in a *cis* fashion, which would require gene transfer to all endothelial cells within the tumor, but also in a *trans*, "bystander" fashion: depletion of VEGF may induce vessel regression, since VEGF has been shown to act as a vascular survival factor (Alon *et al.*, 1995; Benjamin and Keshet, 1997; Hanahan, 1997). Our results are consistent with this hypothesis and suggest that VEGF sequestration may be an important mechanism *in vivo* when retrovirus encoding a truncated VEGFR-2 is inoculated within tumors. The use of vectors with endothelial cell-specific promoters or vector delivery directly into the blood stream may improve endothelial cell transduction and therefore the participation of receptor heterodimerization in the VEGFR-2 dominant-negative strategy.

Toxicity studies

Replication-defective retroviral vectors have been extensively used in animal models and in more than 100 early-phase human clinical trials to deliver the transgene of interest (for reviews see Anderson, 1992; Miller, 1992; Ross *et al.*, 1996). Retroviral vectors are incorporated selectively into the genome of dividing cells. Within the brain, intratumoral injection of retroviruses will transduce only the proliferating cell pool, e.g., tumor and endothelial cells (Culver *et al.*, 1992). However, one theoretical concern associated with the use of retroviral vectors for gene therapy is the possibility that these vectors induce transduction of remote, nontarget tissues.

In this study promiscuous transduction of nontarget tissues was monitored using Southern blot analysis and PCR assay. Southern blot analysis of the samples did not reveal any vector sequences in peripheral organs. By using PCR, vector sequences were detected in all tissues examined (contralateral brain, heart, lung, kidney, liver, and spleen). Rats sacrificed 6 months after inoculation of GPE LXflk-ITM did not exhibit vector sequences in the organs studied. To verify the cell types that were positive for vector sequences, *in situ* hybridization analysis was performed. No specific signal was found in any tissue. It is possible that mRNA transcripts levels for *neo^r* were too low to be detected by *in situ* hybridization. Alternatively, the LTR promoter could be shut down by the host cell.

The blood-brain barrier could minimize the spread of retroviruses or virus-producing cells, but it is well known that the barrier is significantly disrupted in brain tumors. Murine virus-producing cells could pass through the blood-brain barrier into

the peripheral blood. It has been shown that murine retroviruses or murine cells are not destroyed in serum from nonprimate species, including rats (Rollins *et al.*, 1996). Thus, promiscuous transduction can occur, owing either to circulating virus-producing cells or to circulating retroviruses in a rat model. However, this situation is not expected to be found in humans, since in peripheral blood of primates the survival of murine cells and retrovirus vectors is limited owing to inactivation by the complement-mediated immune response (Rother and Squinto, 1996). Several studies dealing with the safety of retroviral vectors in gene therapy showed no retroviremia or pathology in long-term follow-up of primates exposed to murine amphotropic retrovirus (Cornetta *et al.*, 1989). We also did not find any morphological change in the peripheral organs that could be related to the integration of retrovirus sequences. Still, a further concern associated with the use of retroviral vectors in gene therapy is the possibility that these vectors can induce oncogenesis by insertional mutagenesis (Temin, 1990). Additional long-term studies on this subject are certainly warranted.

In conclusion, antiangiogenic gene therapy using a dominant-negative VEGFR-2 strategy is a feasible approach to control brain tumor growth if gene delivery can be improved. Our results suggest a dual mode of function, namely inhibition of endogenous VEGFR-2 function and VEGF depletion. The use of other vector systems or intravascular delivery of cationic liposome-DNA complexes may improve endothelial cell transduction. Although inadvertent transduction of nontarget organs could be detected in a rat model, no pathological changes were observed in transduced tissues. Therefore, the dominant-negative VEGFR-2 strategy in brain tumors is not expected to be associated with significant toxicity.

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Address reprint requests to:

Dr. Karl H. Plate

Neurozentrum, Abteilung Neuropathologie

Universitätsklinikum Freiburg

Breisacherstr. 64

D-79106 Freiburg, Germany

E-mail: Plate@nz11.ukl.uni-freiburg.de

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