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Short-term neuropathological aspects of in vivo suicide gene transfer to the F98 rat glioblastoma using liposomal and viral vectors

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Summary. To date, only few preclinical protocols on liposomal suicide gene transfer in tumors have been published, none of which directly compared viral to liposomal vectors in terms of immunoreactivity and efficacy. We thus studied the neuropathological alterations in 80 rats being treated for glioblastoma using liposomal and, for comparison, adenoviral and retroviral suicide gene transfer approaches to identify vector-associated efficacy and toxicity for further clinical studies. 62 rats served as controls. F98 tumors were established in Fisher rats and transfected in vivo with the thymidine kinase gene of herpes simplex virus (HSVtk) by a single intratumoral application and an implanted intratumoral continuous delivery system. Three days later ganciclovir was given intraperitoneally for 14 days. The animals were sacrificed 17 days post completed gene transfer. Brains were examined histologically and immunohistochemically using markers for immunocompetent cells. Ten animals showed complete tumor regression; they all belonged to the liposomal and adenoviral groups. In 6 of 10 experimental groups considerable numbers of lymphocytes along the margins of the regression cavities could be observed. Control animals of the liposomal and adenoviral groups showed only little lymphocytic infiltration, underlining the minimal immunogenicity of these carriers. In contrast, the retroviral control group featured a high lymphocyte infiltration. In summary, this study indicates that, in terms of both efficacy and immunoreaction, liposomes are as appropriate as adenoviruses in the treatment of rat glial tumors using suicide gene transfer strategies.

Key words: F98 tumor model, Ganciclovir, Gene therapy, Glioblastoma, Liposomal gene transfer, Rat

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Introduction

To date, no effective therapy is available to cure malignant gliomas such as the glioblastoma multiforme (GBM). Mean survival time is only eleven months after diagnosis in spite of surgery, radiation therapy, and chemotherapy. Efforts have been taken to cure experimental brain tumors in rats using a suicide gene transfer paradigm in which the herpes simplex virus thymidine kinase (HSVtk, EC 2.7.1.21) gene is transferred in vivo to neoplastic cells. Subsequently ganciclovir (GCV) is administered to animals and thought to be processed by HSVtk to a mono-phosphate which thereafter is transformed to the toxic metabolite ganciclovir-triphosphate through cellular kinases. This concept was first published and shown to be effective in subcutaneous tumors in 1986 (Moolton, 1986) and later performed successfully in intracerebral neoplasms (Culver et al., 1992). Since the toxic GCV metabolite is supposed to cross gap junctions between tumor cells not all cells have to be transfected for total tumor eradication. This effect is known as the "bystander effect" (Ishii-Morita et al., 1997). Only few clinical studies have been published to date with disappointing results (Ram et al., 1997; Klatzmann et al., 1998; Shand et al., 1999; Packer et al., 2000; Trask et al., 2000).

Different vectors have been shown to deliver genes sufficiently for transfection. These include viral and non-viral vectors. Retroviral vectors have long been considered an ideal vector for suicide gene therapy in cerebral tumors since they only transfect replicating cells (Culver et al., 1992). A number of studies have shown promising results in suicide gene transfer with retroviral vectors in cerebral tumors (Culver et al., 1992; Ram et al., 1993a; Barba et al., 1994; Izquierdo et al., 1995; Cool et al., 1996; Rainov et al., 1996; Vincent et al., 1996b; Berenstein et al., 1999). Adenoviral vectors show a high transfection efficiency (Chen et al., 1994; Vincent et al., 1996a) but do transfect non-replicating cells as well (Boviatsis et al., 1994) and are associated with a

high degree of immunoreactivity (Kajiwara et al., 1997). Liposomes and other non-viral vectors are considered less-immunogenic and less-toxic (Kramm et al., 1995) and can hence be delivered to the tumor site in a repetitive or continuous manner. Because detailed neuropathological studies on liposomal gene transfer are lacking, we here thoroughly examined and compared histopathologically the extent of total tumor destruction, immunogenicity, and toxicity of various liposomal gene delivery systems in the F98 rat glioblastoma.

Material and methods

Surgical procedures and time schedule

The operative procedures have been described in detail elsewhere (Zhu et al., 1996). $4x10^4$ cells of the rat glioblastoma cell line F98 (Ko et al., 1980) were stereotactically implanted into the right hemisphere of adult male Fischer rats 2.6 mm lateral and 1.0 mm frontal to the bregma, 5.0 mm deep using a Hamilton syringe. From a total of 175 animals eleven experimental and ten control groups were created, controls receiving either irrelevant control genes or subsequent placebo instead of GCV or a combination of both (Table 1). On day seven after tumor implantation gene constructs were administered intratumorally using the same coordinates described above either by a single administration or by an implanted continuous intratumoral vector delivery system over three days (Alzet Brain Infusion Kit/3-5 mm, Alza, USA). Three days after completed gene transfer GCV was administered intraperitonally 100 mg/kg body weight/d for 14 consecutive days. On day 24 or 27 respectively, depending on single or continuous pump application, animals were sacrificed, the brains were removed, perfused with 3% paraformaldehyde in ice-cold phosphate buffered solution (PBS), and processed histopathologically.

Plasmids and vectors

Three different plasmids, all containing the HSVtk gene, were used for liposomal transfection, which are pG1TkSvNa (Genetic Therapy Int./Novartis, USA), pUT649 (Cayla/Eurogentech, B), and Bk-Tk (Dr. Th. Blankenstein, MDC Berlin, FRG). LacZ (pUT651, Cayla/Eurogentech, B), encoding for ß-galactosidase, was used as a control gene. As gene taxis, we used DMRIE (Felgner and Ringold, 1989), DC-Chol (Gao and Huang, 1991), DAC-Chol (Reszka et al., 1995), and DOCSPER (Groth et al., 1998). DOPE (Sigma, USA) was used as helper lipid in all cases. The molecular ratios used were as follows: DMRIE/DOPE 1:1, DC-Chol/DOPE 3:2, DAC-Chol/DOPE 6:4 (w/w), DOCSPER/DOPE 1:1 (w/w). The preparation of liposomes is described in detail elsewhere (Reszka et al., 1995; Groth et al., 1998). For liposomal gene transfer a DNA/liposome complex of 10μg/30μl was injected, whereas for continuous liposomal application we used a complex of 35μg/110μl. For retroviral gene transfer we

Table 1. Experimental groups (upper part, groups A-K) and control groups (lower part, a-j).

GROUP	TUMOR	GENE CONSTRUCT	VECTOR	GCV	n_1	n_2
A	F98	pG1TkSvNa	DMRIE/DOPE, s	GCV i.p.	12	12
B	F98	pUT 649 (tk)	DMRIE/DOPE, p	GCV i.p.	7	6
C	F98	Bk-TK	DMRIE/DOPE, s	GCV i.p.	12	11
D	F98	pG1TkSvNa	DMRIE/DOPE, p	GCV i.p.	12	11
E	F98	pG1TkSvNa	DC-Chol/DOPE, s	GCV i.p.	11	7
F	F98	pUT 649 (tk)	DAC-Chol/DOPE, p	GCV i.p.	14	10
G	F98	pUT 649 (tk)	DOCSPER/DOPE, p	GCV i.p.	6	5
H	F98	AdCMV-TK	adenovirus, s	GCV i.p.	9	9
I	F98	AdCMV-TK	adenovirus, p	GCV i.p.	5	4
J	F98	TK	retrovirusVPC, s	GCV i.p.	6	4
K	F98	pUT 649 (tk)	DAC-Chol/DOPE, s	GCV i.t.	5	5
a	F98	pG1TkSvNa	DMRIE/DOPE, s	PBS i.p.	8	6
b	F98	LacZ	DMRIE/DOPE, s	GCV i.p.	13	9
c	F98	LacZ	DMRIE/DOPE, s	PBS i.p.	8	7
d	F98	LacZ	DMRIE/DOPE, p	GCV i.p.	11	9
e	F98	AdRSV-gal	adenovirus, s	GCV i.p.	2	2
f	F98	AdRSV-gal	adenovirus, s	PBS i.p.	2	2
g	F98	TK	retrovirusVPC, s	PBS i.p.	5	5
h	F98	./.	PBS, s	PBS i.p.	19	15
I	F98	./.	DMRIE/DOPE, s	PBS i.p.	3	3
j	F98	./.	DAC-Chol/DOPE, p	PBS i.p.	5	5
Σ					175	147

Abbreviations as follows (in alphabetical order): ./.: no application of vectors; i.p.: intraperitoneal application of GCV; i.t.: intratumoral application of GCV; n_1: total number of animals; n_2: number of animals surviving surgery and entering the study; p: vector application via microosmotic pump; s: single vector application via stereotactic apparatus. For abbreviations of gene constructs and vectors please refer to the Material and Methods section.

used 10^6 vector producing cells in 50 μl with a viral load of $4x10^5$ plaque forming units (pfu); the adenoviral transfer was done with 10^9 pfu.

Histopathological and immunohistochemical evaluation

Brains were removed, stored at -70 ºC, and cut on a cryostat into 7 μm coronal slices at -20 ºC. Slides were pretreated with poly-L-lysine (Sigma, USA). Slices from the site of the surgical procedure were stained following a standard hematoxylin and eosin (HE) protocol, embedded in Eukitt® (Kindler GmbH, FRG), and analyzed for size and morphology of the tumor. Every other slice was also stained immunohistochemically using the APAAP method (alkaline phosphatase-anti alkaline phosphatase). The following primary monoclonal antibodies were used in the given dilution: anti-CD4 (Ox35, 1:50), anti-CD8 (Ox8, 1:400), anti-CD11b/c (Ox42, 1:100), anti-MHC I (Ox18, 1:400), and anti-MHC II (His19, 1:200, all Pharmingen, USA); anti-T (B115-1, 1:1600) and anti-B (B115-9, 1:800, both Holland biotechnology bv, NL); anti-GFAP (1:100, Eurodiagnostika, FRG); anti-connexin 43 (Chemicon International Inc., USA); anti-proliferation (Ki67, 1:100, Novocastra Laboratories Ltd, UK); and anti-HSVtk (1:100, Dr. W. C. Summers, Yale University, USA, only group G). Sections were incubated at 4 °C in a humid chamber with 100 μl solution for 16 hours. After rinsing sections were incubated with 50 μl goat anti-mouse antibody (1:50, Jackson ImmunoResearch Laboratories, USA) as secondary antibody at RT for 30 min. Incubation with the antibody-enzyme complex (APAAP mouse monoclonal D 651, 1:50, DAKO, DK) was done for 30 min at RT with 50 μl solution per section. Sections were developed in a bath consisting of solutions A, B, and C for 10 min at RT. Component A was composed of 0.1 g levamisole (Sigma, USA), 62.5 ml 0.2M propandiol (Merck-Schuchardt, FRG) and 175 ml 0.5M tris (pH 8.7, Serva, FRG). Component B consisted of 0.12 g naphthol As Bi phosphate (Sigma, USA) solved in 1.7 ml dimethylformamide (Serva, FRG). Component C contained 0.05 g sodium nitrite (Merck, FRG) in 1.25 ml Aq. dest. gently stirred for 60 s with 500 μl 5% new fuchsin solution in 2N HCl (Neufuchsin-Certistain®, Merck, FRG). Component C was stirred into component A, and component B was added. After developing, the slides were rinsed thoroughly, counterstained with hematoxylin, and embedded in a watery medium (Aquatex®, Merck, FRG). Tissue sections without application of the primary antibody served as negative controls for the immunoreactions, sections from rat lymph nodes served as positive controls.

Tumor size was defined as tumor area in a coronal section in per cent of the right hemisphere. The slice for evaluation was taken exactly from the site of implantation, using the cortical entry of the needle track as marker. The same HE-stained slice was used to describe pattern of growth and pathologies. Hemorrhages were assessed using a six-point semiquantitative scale ('0' = no hemorrhages, '1' = low grade hemorrhages into tumor or into tumor cavity, '2' = high grade hemorrhages into tumor or into tumor cavity, or low grade hemorrhages into brain parenchyma, '3' = high grade hemorrhages into brain parenchyma of the contralateral hemisphere, '4' = any intratumoral hemorrhage with a mass effect, '5' = any intraparenchymal hemorrhage with a mass effect, or any intraventricular hemorrhage). Necroses were judged accordingly. Criteria to describe histological parameters in malignant gliomas were adapted from other recent publications (Karkavelas et al., 1995; Coons et al., 1997). The number of immunocompetent cells was assessed histologically and immunohistochemically using a five-point semiquantitative scale ('-' = 0 cells, '+' <50 cells, '++' <250 cells, '+++' <1000 cells, and '++++' ≥ 1000 cells in a single coronal slide).

Statistical calculations were performed using GraphPad Prism 2.0. Tumor sizes were compared using the unpaired t-test. Infiltration of cells and other histological alterations were analyzed using the Mann-Whitney algorithm. A 95% confidence interval was chosen for statistical significance ($p<0.05$).

Results

Tumor implantation was survived by 147 out of 175 animals. All deaths were due to surgery or anesthesia. Clinical chemistry was done on all animals and is described elsewhere (Zhu et al., 1996). Five animals were excluded from further analysis because of a lack of tumor growth.

Controls and tumor histology

Animals in the main control group (group h) were only treated with PBS. On day 24 the tumor measured more than 60% of the right hemisphere on the average, exhibiting a large scale mass effect with compression of the right lateral ventricle (Fig. 1a). In the other control groups reductions in tumor size were non-significant when compared to the PBS group (Fig. 2a).

Tumors were highly cellular with infiltration of peritumoral vessel sheaths (Fig. 1c) and metastasized to ipsilateral and contralateral subarachnoid spaces. In some animals residual tumors were found along the needle track of the inoculation procedure (Fig. 1c). Small to mid-sized intratumoral necroses were always visible. Hemorrhages into the tumor tissue were found regularly. However, there was no bleeding into the ventricles or into the subarachnoid space. The neoplastic cells were fusiform in shape and moderately polymorphic. Many cells showed atypical mitoses. Regularly, few macrophages were detectable, mainly in the neighborhood of small vessels. In some animals a few CD8+ lymphocytes were found. Few granulocytes were seen in one animal. Tumors were weakly positive for GFAP.

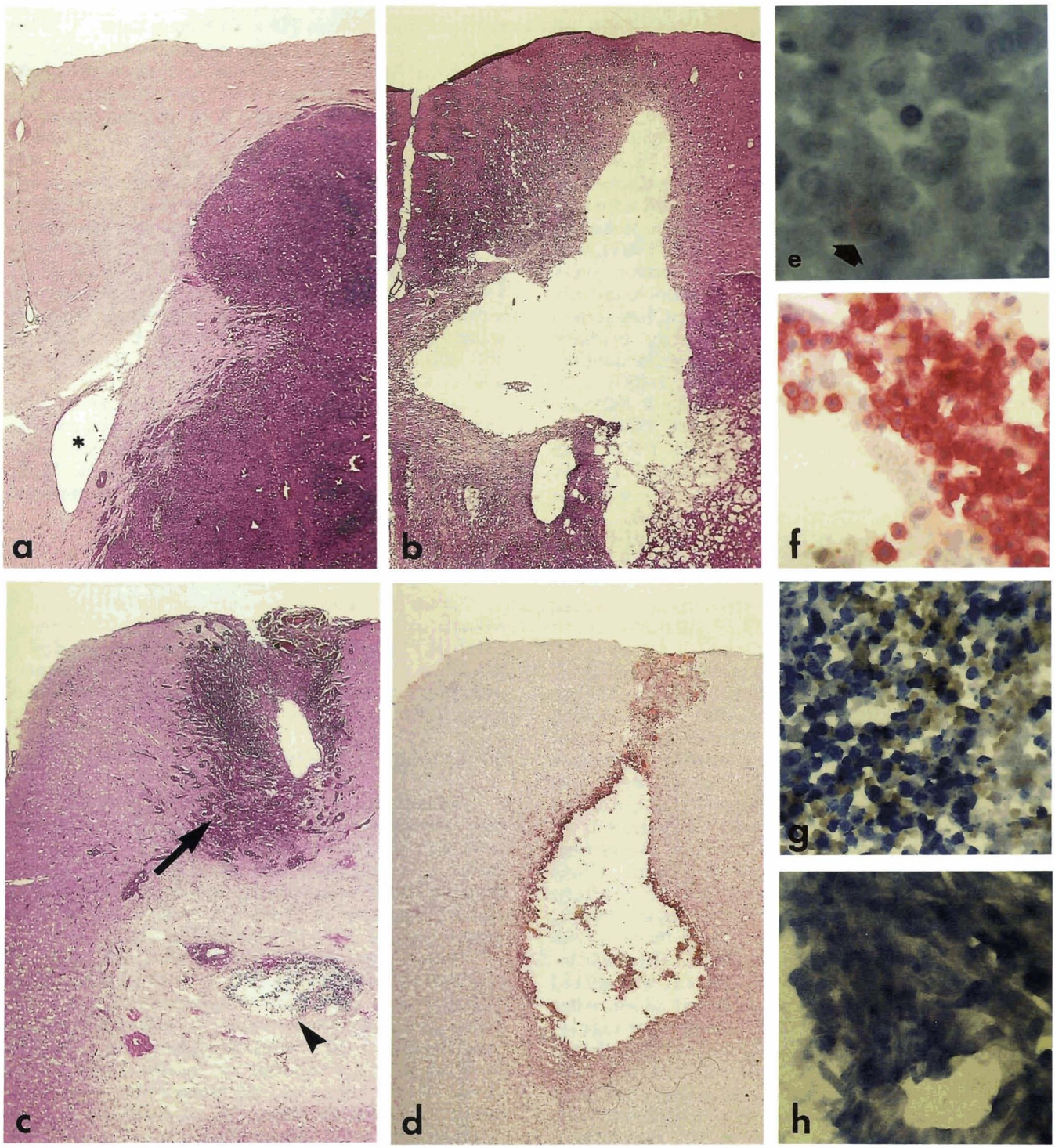

Fig. 1. a-h. Histopathological results. **a-c.** Therapeutic success. HE. **a.** Group h. Single, large tumor of the right hemisphere in a coronal slice. The asterisk is placed in the right ventricle. **b.** Group D. Total tumor regression without any residual tumor cells in high power field examination. **c.** Group D. A small residual tumor is left along the needle track (arrow). Note the infiltration of the peritumoral vessel sheaths. Very clearly visible also is a therapeutic regression cavity in the central area of the brain parenchyma, filled with detritus and lymphocytes (arrowhead). **d-e.** Immunohistochemical results. **d.** H anti-T, group C. T-cells along the margin of the regression cavity of a treated animal. **e.** IH anti-HSVtk, group G. HSVtk is found on day 24 post transfection. The arrowhead marks an HSVtk-positive cell. **f-h.** Controls of immunogenicity of the different vectors used. IH anti-T. **f.** Group g. Marked immunoreaction in the retroviral control group. **g.** Group a. No immunoreaction in the liposomal control group. **h.** Group e. No immunoreaction in the adenoviral control group. a-d, x 20; e, x 1,000; f-h, x 400

Size of tumor

In a total of ten animals, all in three of eleven experimental groups, large regression cysts without any residual tumor cells were found (Fig. 1b). The percentage of animals with total tumor regression was higher in the adenoviral group H (5 of n=7 animals; 71.4%) than in the liposomal groups B (2 of n=6 animals; 33.3%) and D (3 of n=11 animals; 27.3%). Tumor sizes were also significantly reduced in these groups when compared to the PBS control group h (adenoviral group H, n=7, $p<0.01$; liposomal group B, n=6, $p<0.001$; liposomal group D, n=11, $p<0.001$; Fig. 2a). Liposomal groups A, C, and E did not show a significant tumor size reduction. Application of the vector via an implanted microosmotic pump (liposomal

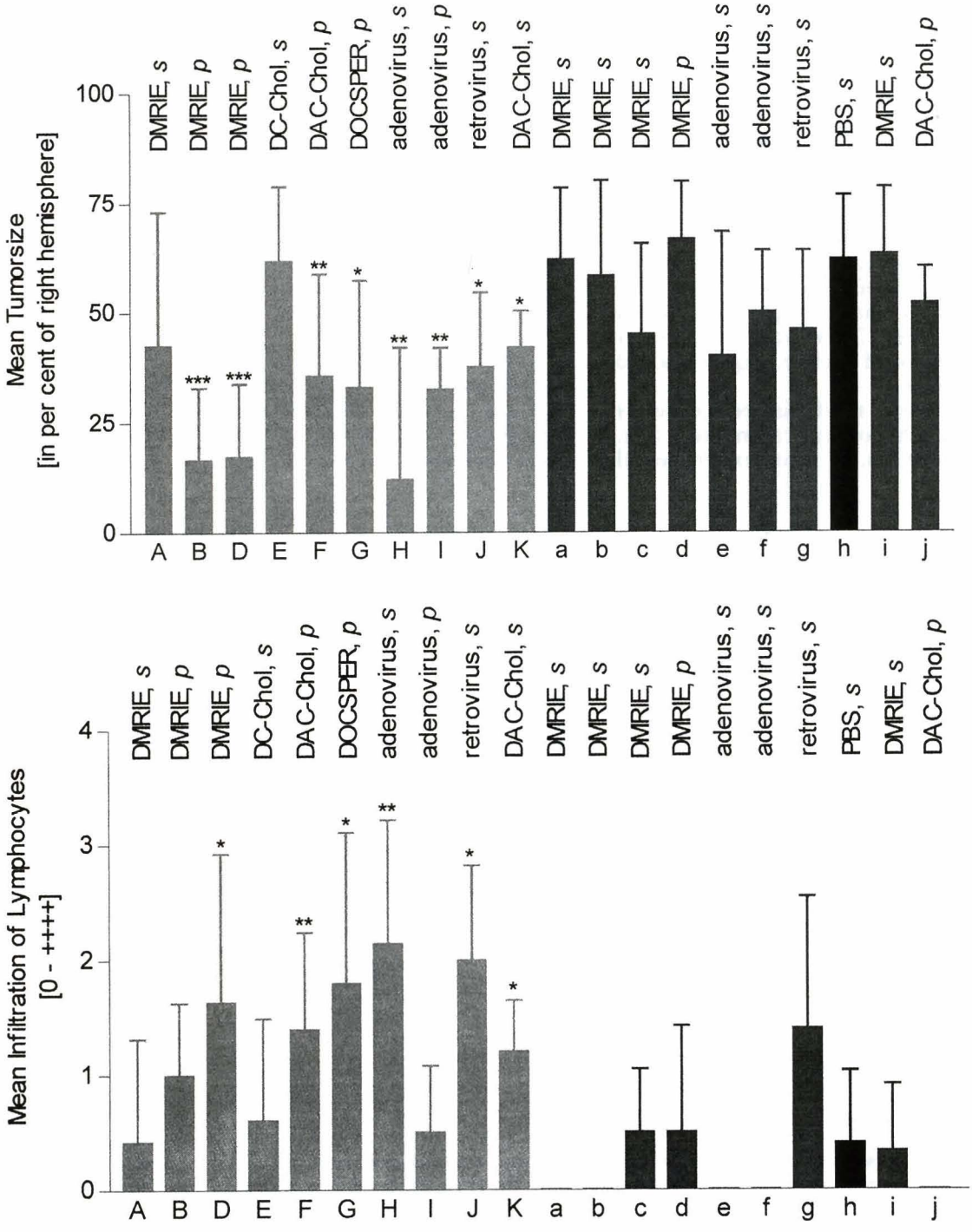

Fig. 2. Histograms on the histological and immunohistochemical results. Values are given ± SD. Experimental groups A-K are given on the left hand side (light columns), control groups a-j are given on the right hand side (dark columns) of the illustrations. Group C is excluded because a different gene construct is used. Column h (black column) features the only true control since only PBS was used for injection. Columns are marked with vectors used. For complete therapy protocols please refer to Table 1. Groups differing significantly from group h are marked as follows: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$. **Upper graph.** Mean tumor size in per cent of right hemisphere. **Lower graph.** Mean lymphocyte infiltration on a semiquantitative scale.

group D) in comparison to single application (liposomal group A) yielded a significantly smaller tumor ($p<0.05$, Fig. 2a).

Immunogenicity and infiltration of immunocompetent cells

Lymphocytes were detected by histological and immunohistochemical stainings with a variety of markers (anti-T, anti-B, anti-CD4, anti-CD8, anti-MHCI, and anti-MHCII). In six experimental groups the mean semiquanitative lymphocyte count was significantly elevated compared to the PBS control group h (liposomal group D, n=11, $p<0.05$; liposomal group F, n=10, $p<0.01$; liposomal group G, n=5, $p<0.05$; adenoviral group H, n=9, $p<0.01$; retroviral group J, n=4, $p<0.05$; liposomal group K, n=5, $p<0.05$). Among the controls only the retroviral control group g with a retroviral vector (n=5, $p>0.05$) featured a high lymphocyte infiltration that was non-significantly elevated compared to control group h (Fig. 2a). Typically, lymphocytes gathered along the margin of the regression cavity in treated animals (Fig. 1d). In almost all cases lymphocytes were CD8+, only occasionally were CD4+ lymphocytes found in close neighborhood to granulocytic accumulations.

Macrophages were seen in most specimens either by HE stainings or immunohistochemical markers (e.g. anti-CD11 and anti-MHCII). In three experimental groups the mean macrophage infiltration score was significantly elevated compared to control group h (liposomal group F, n=10, $p<0.05$; adenoviral group H, n=7, $p<0.05$; liposomal group K, n=5, $p<0.05$, data not shown). The other groups did not show significant differences. Granulocytes were found in some experimental groups; the mean infiltration score was not significantly elevated in any group.

Other neuropathology

In two experimental groups treated with liposomal and adenoviral gene transfer, animals with intraventricular bleeding were found; the hemorrhage score was significantly higher in these groups compared to PBS control group h (lipsomal group B, n=6, $p<0.05$; adenoviral group I, n=4, $p<0.05$). Other differences regarding hemorrhages and necroses were non-significant.

Immunohistochemically HSVtk was found in tumor cells on day 24 post transfection (Fig. 1e).

Discussion

Our results are based on microscopic assessment of the brains of 142 rats in eleven experimental and ten control groups. Due to resources, groups did significantly differ in size. Groups of n<4 were excluded from statistical analysis and served as non-significant

Table 2. Efficacy of gene therapy in in vivo HSVtk/GCV suicide gene transfer studies in intracerebral and leptomeningeal tumors of rodents. Listed in chronological order and in comparison to our recent results.

STUDY	TUMOR	VECTOR	RESULTS
Ram et al., 1993a	9L	VPC	76% total regression
Barba et al., 1994	9L	VPC	40% total regression
Boviatsis et al., 1994	9L	HSV	48% survive five months
Chen et al., 1994	C6	ADV	20% total regression
Perez-Cruet et al., 1994	9L	ADV	100% survive 98 days, 75% survive 120 days
Tapscott et al., 1994	9L	VPC	no significant success
Izquierdo et al., 1995	C6	VPC	100% total regression
Maron et al., 1995	C6	in vitro †	100% survive 30 days, 33% survive 80 days
Ross et al., 1995	9L	ADV	significant longer survival
Cool et al., 1996	9L	VPC	no significant success
Kramm et al., 1996b	9L	HSV	90% survive 100 days
Maron et al., 1996	C6	ADV	28-fold tumor reduction
Okada et al., 1996	U-251SP	AAV	11-fold tumor reduction
Rainov et al., 1996	9L	VPC	65% survive 26 days
Vincent et al., 1996a	9L	ADV	significant longer survival
Vincent et al., 1996b	9L	VPC	significant longer survival
Kruse et al., 1997	9L	in vitro ‡	53% total regression
Quillien et al., 1997	9L	ADV	67% total regression
Berenstein et al., 1999	C6	VPC	33% total regression
Sandmair et al., 1999	BT4C	VPC	no significant success
group B	F98	DMRIE/DOPE	33.3% total regression
group D	F98	DMRIE/DOPE	27.3% total regression
group H	F98	ADV	71.4% total regression

†: tumor cells were transfected with HSVtk-DNA prior to implantation; ‡: tumor cells were transfected with retroviral particles prior to implantation; AAV: adeno-associated vector; ADV: adenovirus; DMRIE/DOPE: liposomal preparation; HSV: herpes simplex virus; VPC: vector-producing cells. For therapy protocols of groups B, D, and H please refer to Table 1.

trials only. Due to limited resources, PBS control groups for each experimental group were not done. However, the lack of influence of PBS on either tumor size or survival has been shown by a number of other investigators (e.g., Zhu et al., 1996).

According to others, the F98 tumor model is only weakly immunogenic compared to other widely used malignant glial tumor models, e.g. C6 and 9L, and has a high invasive nature and a reliable lethality in Fisher rats. It is valued as one of the most reliable, highly malignant, and non-immunogenic rodent brain tumor models (Barth, 1998).

Size of tumor and treatment efficacy

We used the size of the tumor after sacrifice of the animals on day 24 or 27, respectively, as marker for therapeutic efficacy. Tumor size was expressed in per cent of the right hemisphere in the coronal slice. The slice for evaluation of the tumor size was taken from the site of implantation, the entry of the needle track was clearly visible on the cortex while preparing the frozen sections. Tumor growth was spherical in serial cuts of individual animals, hence this procedure yields a representative value of the tumor volume.

For the first time, liposomes were used in our laboratory as gene taxis in HSVtk/GCV suicide gene therapy for malignant brain tumor (Zhu et al., 1996). Here we present a detailed neuropathological analysis of this study. It extends the scope of earlier studies on viral transfection strategies to a significant degree (c. Table 2). Promising results were especially achieved in liposomal groups B and D using DMRIE/DOPE liposomes. Liposomal preparations DAC-Chol/DOPE and DOCSPER/DOPE also showed a significant tumor regression, however, to a somewhat lesser degree. Application of the vector via an implanted microosmotic pump yielded additional therapeutic power.

On the other hand, we also found a significant tumor regression in one of the two adenoviral groups. Nevertheless, this approach was only non-significantly superior to the liposomal approaches. Other groups that used adenoviral vectors for transfection as well yielded comparatively good results (Chen et al., 1994; Perez-Cruet et al., 1994; Ross et al., 1995; Maron et al., 1996; Vincent et al., 1996a,b; Quillien et al., 1997).

In the small group that was treated with a retroviral vector (group J) only a slight tumor regression was noted, which stands in contradiction to other publications (Ram et al., 1993a, Barba et al., 1994; Izquierdo et al., 1995; Rainov et al., 1996; Vincent et al., 1996b, Berenstein et al., 1999) but is supported by others (Tapscott et al., 1994; Cool et al., 1996; Sandmair et al., 1999). Although we cannot exclude that the virus titer might have been too small for highly successful transfection in this experiment, a retrial with VPC with a confirmed virus titer of 10^8/ml did not complete tumor regression either (data not shown, Dr. R. Reszka).

Two studies were designed to directly compare retroviral and adenoviral transfection, but no significant differences were found (Boviatsis et al., 1994; Vincent et al., 1996b). In one study tumor cells were transfected with a herpes virus vector and excellent results were found (Kramm et al., 1996a).

Differences between our results and those of others can partly be explained with the missing similarity of parameters for successful therapy (Table 2). Two of the listed studies used tumor cells that were already transfected prior to implantation (Maron et al., 1995; Kruse et al., 1997). Two other studies report survival rates or complete tumor regression in 100% of the animals (Perez-Cruet et al, 1994; Izquierdo et al., 1995). While the former of these reports is based upon magnetic resonance imaging (MRI) and therefore neglects the possibility of small residual tumors, the latter one draws its conclusion from an experimental group with only four animals and therefore has to be treated with caution. Two studies did not show any significant therapeutic success (Tapscott et al., 1994; Cool et al., 1996). However, the authors found significant reduction of a subcutaneous tumor in an otherwise identical experimental setting and hence postulated that the blood-brain barrier reduces the GCV concentration in the intracerebral tumor (Cool et al., 1996). Two studies examined the role of intrathecal metastases (Kramm et al., 1996a; Vincent et al., 1996a). In addition, the different time schedules have to be taken into account. In a couple of studies long-term survival rates have been evaluated, while in others, like in our study, animals were killed on a fixed day post tumor implantation.

Histopathological alterations and infiltration of lymphocytes

Only few studies demonstrate immunopathological alterations in the specimens (Ram et al., 1993b; Barba et al., 1994; Wood et al., 1994; Ross et al., 1995; Kramm et al., 1996b; Shine et al., 1997). Some authors describe a moderate edema and gliosis (Ram et al., 1994), or activation of microglia (Wood et al., 1994), infiltration of macrophages and lymphocytes (Barba et al., 1994; Wood et al., 1994; Shine et al., 1997), and generally marked leptomeningeal inflammation (Kramm et al., 1996b). One study showed that a tumor resistance against a second implantation of tumor cells developed after therapy (Barba et al., 1994). The only neuropathological long-term study of CNS-1 tumors revealed a marked chronic inflammation and signs for loss of myelin after adenoviral-mediated transfer of HSVtk and subsequent administration of GCV (Dewey et al., 1999). Inflammation could have various causes in our experimental setting. The implanted tumor itself can be immunogenic (Tapscott et al., 1994; Barth et al., 1998); this was not relevant in our study since control animals received the same tumor. During surgical manipulation, bacteria and viruses can be inoculated into the cranium. Applied vectors can induce an

inflammatory immunoreaction; this was taken as a sign for immunogenicity of the respective vector when found in control animals. Lastly, when killed successfully, dead tumor cells and the detritus have to be removed from the tumor site, which also involves an immunoreaction.

In our study, we have focused on the immunogenicity of the vectors used. We were especially interested in the immunogenicity of liposomal vectors. The lymphocyte infiltration in the liposomal controls was clearly similar to the score of the PBS control group h (groups a, b, c, d, i, and j), indicating that the liposomal preparations themselves did not elicit any detectable immunoreaction after day 24 or 27, respectively, and hence justifying a continuous application via a microosmotic pump. This concept is important for effective tumor control in malignant gliomas, because only about a third of all cells in human GBM are proliferating at a given time (Hoshino and Wilson, 1979).

In treated animals, lymphocytes were only found along the needle track and the regression cavity. In some animals periventricular lymphocytes were detectable. No marked encephalitis was seen in any animal.

Adenoviral vectors, HSV-vectors and allogenic VPC are known to be immunogenic, whereas liposomes are supposed to be non-immunogenic and non-toxic (Kramm et al., 1995). We did not find any immunoreaction in our adenoviral controls in terms of infiltration with lymphocytes (groups e and f), although the groups were too small to give this finding any statistical significance. We have to assume that the adenoviral vector titer was high enough since animals with total tumor regression were successfully treated with the same vector (group H). This finding stands in contrast to a publication mentioned above (Shine et al., 1997), but is supported by others, which do not consider adenoviral vectors to be immunogenic (Boviatsis et al., 1994; van der Eb et al., 1998). The adenoviruses used by us were cleared from any additional protein which might contribute to the low immunogenicity of the adenoviral vectors in our study.

The first vector to be used in suicide gene transfer was a retroviral vector, in which only edema and gliosis were described as a reaction to the transfer of a marker gene (Ram et al., 1994). This again stands in contrast to our findings, since we noted marked infiltration of both macrophages and CD8+ lymphocytes in the retroviral control (group g).

In comparison to the PBS control group h, the experimental groups B (DMRIE/DOPE) and I (adenovirus) showed a high tendency for intraventricular hemorrhages and a significantly higher hemorrhage score. The hemorrhages in the liposomal group B are unclear to us. An explanation in regard to group I (adenovirus) is the possible transfection of the endothelium of intraparenchymal vessels and subsequent destruction and rupture following GCV administration, for it is known that adenoviral vectors can transfect all types of cells (Boviatsis et al., 1994; Perez-Cruet et al., 1994). We however did not find transfection of endothelial cells.

Ganciclovir

The amount of GCV administered is still a matter of debate. For successful tumor control a sufficient concentration of GCV has to cross the blood-brain barrier to be metabolized by the intratumorally-expressed HSVtk. In this study 200 mg/kg body weight/d GCV for 14 days was used; in clinical studies usually 10 mg/kg body weight/d GCV is given. This might explain the unsatisfactory results of recent clinical studies. Cerebral toxicity of intraperitoneally-applied GCV has never been reported in previous experimental settings. We do not see any signs for microscopically evident toxicity to GCV in our study (groups b and c). Although direct intratumoral administration of GCV yielded a significant tumor regression (group K), the outcome was similar to experimental groups with intraperitoneal administration.

Concluding remark

Taken together, in our experimental setting, only tumor cells were found to have synthesized HSVtk protein; structures other than tumor cells were not damaged. Liposomes, e.g. DMRIE/DOPE, used as vectors for tumor gene therapy are as efficient as adenoviruses are. They are non-immunogenic and hence can be applied via a microosmotic pump or any other continuos application system for an even better and safer tumor therapy.

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