Gene Ther Mol Biol Vol 3, 1-14. August 1999.

Routes of vector application for brain tumor gene therapy

Review Article

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Key words: adenovirus, brain neoplasms, gene therapy, gene transfer, herpes simplex virus, intra-carotid delivery, liposomes, plasmids, retrovirus.

Received: 25 September 1998; accepted: 5 October 1998

Summary

The development of highly efficient virus and non-virus vector systems for gene transfer to and gene therapy of brain tumors has advanced to the stage of clinical trials, but has still not successfully addressed some major limiting factors, such as the inability of a single delivery modality or therapeutic transgene to target a maximum number of tumor cells in diffuse or multifocal tumors, such as human glioblastoma, and to confer eradicating cytotoxicity to the whole neoplastic mass. Moreover, the choice of vectors and the route of their administration dramatically affect both the efficiency of tumor transduction and its spatial distribution, as well as the extent of transgene expression within a brain tumor and outside it, in the surrounding tumor cell-infiltrated tissue.

Three main routes of vector delivery to experimental brain tumors are reviewed in this paper: stereotactic or direct intratumoral inoculation; intrathecal and intraventricular injection; and intravascular infusion with or without modification of the blood-brain-tumor-barrier. The pros and cons of all these modes of application are discussed in respect to the specific and unique features of tumors in the central nervous system. We conclude that, at the present time, there is no ideal vector or unconditionally efficient application mode, and so the successful approaches to brain tumor gene therapy need to combine different application routes with different vectors and therapeutic genes designed to address the individual features of different tumor types. The intravascular vector delivery route, although at an early stage of development, seems to be the most pervasive and demonstrates the greatest therapeutic potential in animal experiments, but for human use it should be combined either with direct intratumoral vector injections or with CSF vector delivery.

I. Introduction

The advancement of gene therapy for brain tumors through the stage of animal models into clinical trials has not succeeded in eliminating major limiting factors, such as the inability of a single vector or delivery mode to target a pool of tumor cells large enough to confer cytotoxicity to the whole tumor (Ram et al., 1997). Even with the bystander effect elicited by many therapeutic genes, which is responsible for the killing of non-transduced cells surrounding transgene-expressing cells, still the transgene-bearing vector must be delivered to a substantial number of tumor cells (1-10%) throughout the tumor (Moolten, 1996; for review see Kramm et al., 1995, and Spear et al., 1998). The choice of vectors and the route of their administration have been demonstrated to affect both tumor transduction efficiency and spatial distribution, as well as the extent and stability of transgene expression within a tumor, in invasive tumor cells, and in the surrounding normal brain (Zlokovic and Apuzzo, 1997).

Three main modes of vector delivery to experimental brain tumors have been extensively studied and compared in animal models and, in some cases, in clinical trials: stereotactic intratumoral inoculation of virus suspension or vector-producing cells (VPC) (Badie et al., 1994; Boviatsis et al., 1994a and 1994b; Bramson et al., 1997; Culver et al., 1992; Eck et al., 1996; Izquierdo et al., 1995; Kramm et al., 1997; Mineta et al., 1994: Oldfield et al., 1993; Rainov et al., 1996; Ram et al., 1993 and 1997); intrathecal and intraventricular injection of virus or VPC (Bajocchi et al., 1993; Kramm et al., 1995, 1996, 1997; Oldfield et al., 1995; Oshiro et al., 1995; Ram et al., 1994; Rosenfeld, 1997; Vincent et al., 1996a); and more recently, intravascular application of virus vectors (Barnett et al., 1998; Chauvet et al., 1998; Doran et al., 1995; Kroll and Neuwelt, 1998; Muldoon et al., 1997; Neuwelt et al., 1991; Nilaver et al., 1995; Rainov et al., 1995 and 1998). The study of the modes of application and the factors which limit vector distribution and propagation in a brain tumor is of great importance to the improvement of present gene therapy strategies and the development of more efficient approaches. Therefore, the present paper will review the routes and methods for delivery of gene therapy vectors to malignant brain tumors, and will focus on strategies which may have the potential of improving the efficiency of gene transfer to brain tumors *in vivo*.

II. Intratumoral delivery of vectors

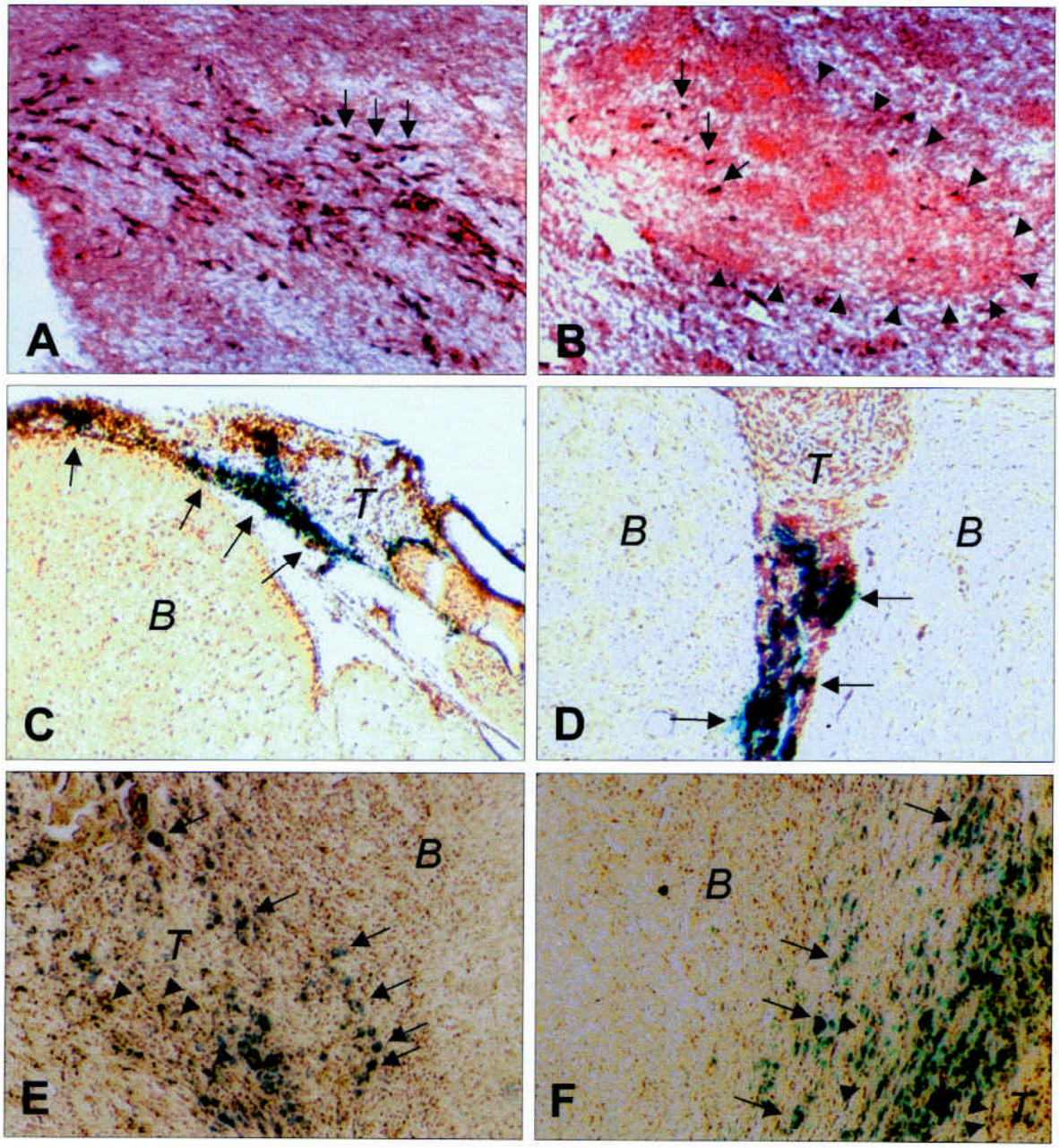
The earliest and most straightforward approach to delivery of gene therapy vectors to brain tumors is the stereotactic intratumoral injection (Short et al., 1990; Boviatsis et al., 1994a) or the direct injection after open surgery for brain tumor removal (Ram et al., 1997). It offers the advantages of low systemic toxicity, reduced vector loss, and high local vector concentrations, and can be employed either for application of concentrated vector suspension, as in the case of herpes-simplex-virus type 1 (HSV) (Andreansky et al., 1993; Boviatsis et al., 1994b; Kaplitt et al., 1994; Kramm et al., 1997; Martuza et al., 1991; Mineta et al., 1995); adenovirus (AV) (Badie et al., 1994 and 1998; Boviatsis et al., 1994a; Chen et al., 1994; Eck et al., 1996; Izquierdo et al., 1996; Le Gal La Salle et al., 1993; Maron et al., 1996; Perez-Cruet et al., 1994, Puumalainen et al., 1998); adeno-associated virus (AAV) (Mizuno et al., 1998; Okada et al., 1996); and liposome-DNA complexes (lipoDNA) (Gennuso et al., 1993; Yagi et al., 1994; Zerrouqi et al., 1996; Zhu et al., 1996); or for implantation of retrovirus (RV) producing cells (VPC) (Rainov et al., 1996; Ram et al., 1993; Culver et al., 1992; Takamiya et al., 1993; Tamiya et al., 1995). Since the life cycle of replication-conditional HSV and AV vectors is lytic or damaging to the host cell, there have been no available cell-based vector producer systems for *in vivo* use of these viruses (Kramm et al., 1995).

A. Direct intratumoral injection of vectors

Stereotactic surgery methods combined with 3D computer reconstruction and imaging databases provide powerful options for tumor gene therapy (Kelly, 1997). Volumetric stereotactic procedures can be modified for individually planned delivery of viruses or VPC to single or multiple tumor foci. Despite the potential for high spatial accuracy, direct intra- or peritumoral injections have several disadvantages, such as limited vector distribution to a few millimeters surrounding the injection site (Boviatsis et al., 1994a; Rainov et al., 1996; Lal et al., 1994), and the need of multiple injections of either virus or VPC suspension even with large volumes of inoculum of up to 0.5 ml per injection site (Muldoon et al., 1997; Ram et al., 1997) (**Fig. 1A** and **B**). Since the number of stereotactic injection sites is limited for practical reasons by length of surgery and increasing risk of hemorrhage with every new intracerebral puncture track, this mode of application can only provide vector delivery to small intracerebral foci or limited tumor areas (Spear et al., 1998).

Fig. 1: Microscopic appearance of 9L tumors in syngeneic Fischer rats. A. Photomicrograph of tumor tissue in an animal from the TK/GCV group, 7 days after intratumoral grafting of retrovirus-packaging cells (CRIP-MFG-TK) in a 1:5 ratio of producer cells to tumor cells. This section was stained immunohistochemically for HSV-TK. Note the high number of HSV-TK-positive cells (dark-brown, arrows) in the tumor (magnification 200 x, 20 µm frozen section, counterstained with hematoxylin). B. Photomicrograph of tumor tissue 12 days after grafting of CRIP-MFG-TK cells and 5 days after start of GCV application. Necroses (arrowheads) are visible inside the tumor area. HSV-TK-positive cells (arrows) are still detectable, but numbers are much lower (magnification 200 x, 20 µm frozen section, counterstained with hematoxylin). C. X-gal staining 10 days after implantation of 9L cells and 6 days after intrathecal injection of replication-conditional HSV vector bearing the *lacZ* gene demonstrates widespread distribution of vector in leptomeningial 9L tumor cells (blue, arrows) directly contacting the CSF. In the tumor parenchyma (T), only a few cells display X-gal staining, B = normal brain (75x magnification, 20 µm frozen section, counterstained with hematoxylin and eosin, H&E). D. Frontal tumor (T) in an animal 7 days after intracerebral and intrathecal implantation of 9L cells and 2 days after intrathecal inoculation with HSV vector used in C. Extensive X-gal staining (blue, arrows) is seen in tumor areas which have broken into the lateral ventricle, while other parts of the tumor (T) with no CSF contact show essentially no staining. Normal brain (B) is not affected by intrathecal HSV application (100x magnification, 20 µm frozen section, counterstained with H&E). E. Photomicrograph of intracerebral tumor in the BK/HSV group 24

hours after ipsilateral intra-carotid virus injection in the presence of bradykinin (BK). This section was double-stained for ß-gal (blue cells, arrows) and HSV-TK (dark-brown, arrowheads). Note the higher number of stained tumor cells at the tumor/brain border (T = tumor, B = brain) and the absence of transgene protein staining in normal brain (magnification 200x, 20 µm frozen section, counterstained with H&E). F. Photomicrograph of 9L gliosarcoma in the BK/HSV group 48 hours after virus injection in the presence of BK. This section was double-stained for ß-gal (arrows) and HSV-TK (arrowheads). Note the higher intensity of tumor staining, probably due to secondary spread of replication-conditional HSV vector and infection of neighboring tumor cells. Staining is limited to the tumor and does not extend into surrounding normal brain (T = tumor, B = brain) (magnification 200x, 20 µm frozen section, counterstained with H&E).



Although several reports have previously demonstrated that this mode of vector delivery may be efficient in rodents (Badie et al., 1994; Boviatsis et al., 1994c; Bramson et al., 1997; Culver et al., 1992; Izquierdo et al., 1995; Mineta et al., 1994; Rainov et al., 1996, Ram et al., 1994; Tamiya et al., 1995), it does not reach the same degree of efficiency in humans (Raffel et al., 1994; Ram et al., 1997). Part of the problem seems to be that human glioblastomas (GBM) are much larger, more randomly shaped, and more diffusely infiltrating than the rodent glioma models (Izquierdo et al., 1997; Kramm et al., 1995; Zlokovic and Apuzzo, 1997). Further, they have a lower fraction of dividing tumor cells which limits on site propagation of replication-conditional HSV and integration of RV (Ram et al., 1997, Harsh et al., in preparation, Puumalainen et al., 1998), and, since most vectors derive from common pathogens, the immune system may block infection of tumor cells. Herrlinger et al. (1998) have investigated the role of the immune system in HSV-mediated gene transfer and found that rats preimmunized to HSV had dramatic decrease in transduction efficiency to brain tumors.

Direct intratumoral injections into the walls of the tumor resection cavity, although they can be performed under direct visual control and with multiple vector depots very close to each other, have the same basic limitations as stereotactic procedures. Moreover, the depth of injection is limited to 10-15 mm from the resection border, which seems to be insufficient to reach tumor cells migrating away from the main tumor mass. Thus, both stereotactic and "free-hand" injection techniques are inefficient in cases of multiple tumor foci and diffusely infiltrating tumors.

B. Bulk convection-enhanced flow methods

An alternative method for efficient and widespread delivery of macromolecules and particles to tumors is convection-enhanced infusion, which is used to supplement simple diffusion and to improve vector distribution by bulk flow inside and outside the tumor (Bobo et al., 1994; Lieberman et al., 1985; Muldoon et al., 1997). Stereotactic injection and subsequent infusion by maintaining a positive pressure gradient is able to improve the distribution of large molecules in animal models (Lieberman et al., 1995). The volume of distribution seems to increase linearly with the infusion volume, if relative small molecules are used (Bobo et al., 1994). Kroll et al. (1996) used convection for delivery of MION, superparamagnetic iron oxide nanoparticles with a size comparable to that of viruses (Shen et al., 1993), to normal rat brain and found out that the concentration of the agent is of primary importance for the size of the distribution area. Infusion time did not affect distribution, and the volume infused was closely related to the size of the distribution area (Kroll et al., 1996). The same group (Muldoon et al., 1995) infused AV or HSV into normal rat brain for 2 hours at a rate of 0.2 µl/min and found widespread infection in tissue volumes of 40 mm^3 (replication-defective AV) and up to 200 mm^3 (replication-conditional HSV). When applied to rat brain tumors, this technique was able to mediate delivery of virus particles to tumors with an approximate volume of 100 mm^3, and also beyond the tumor borders into the surrounding brain tissue (Nilaver et al., 1995). Virus vectors, however, do not travel in the extracellular space of the brain solely by diffusion, since they bind to receptors and are taken up by cells, and because they are very large (e.g. HSV diameter = 150 nm). Tumor or brain cells near the injection or infusion site may take up many more virus particles than cells distant to it, which reduces the particle numbers of the suspension that diffuses further.

The above data demonstrate that convection-enhanced vector delivery is sufficient for targeting a relatively small and circumscribed rodent brain tumor implant. If this technique should be applied to human GBM, much larger tumor volumes have to be targeted and, since there is no selectivity in the delivery mode itself, normal brain tissue may be overloaded with vectors leaking out of the tumor mass.

III. Intrathecal and intraventricular vector application

The intrathecal gene therapy approach is attractive because access to the cerebrospinal fluid (CSF) is minimally invasive and distribution of virus vectors and VPC may be facilitated by CSF circulation, thus overcoming distribution barriers in solid tumors. Intrathecal delivery seems to be best suited for treatment of leptomeningial tumor manifestations. These are found in adults in secondary intracerebral tumors of carcinomas and lymphomas. The most frequent primary brain tumor in children, the medulloblastoma, often spreads from its primary location in the cerebellum and the fourth ventricle via the CSF pathways along the entire spinal cord down to the cauda equina. Moreover, leukemic leptomeningiomatosis is a frequent site of relapse of acute lymphoblastic leukemia, the most frequent pediatric cancer.

Retroviral vectors, as well as AV and HSV vectors, have been investigated for their use for gene therapy of leptomeningial tumors after intrathecal administration. Ram et al. (1994) implanted retrovirus producer cells into the leptomeningial space of rats, which have been intrathecally challenged with syngeneic tumor cells some

days prior. Prolonged survival was achieved by subsequent GCV treatment. Gene transfer was demonstrated in tumor foci growing in the *cistern magna*, the injection site of the VPC. Toxicity and gene transfer into normal cells was also evaluated in rats and non-human primates without tumors after single and repeated intrathecal application of retrovirus producer cells (Oshiro et al., 1995). Only choroid plexus cells, and no other normal CNS structures, showed transgene expression. Magnetic resonance imaging of brains of non-human primates revealed no pathological changes. In total, no significant toxicity was observed either in rats or in non-human primates, even after repeated intrathecal application of retrovirus producer cells with or without subsequent GCV treatment. Interestingly, measurable titers of retroviral particles were detected in lumbar, as well as in cisternal, CSF samples indicating an effective circulation of vector particles within the CSF. According to a former study, CSF does not inactivate retroviruses or lyse vector producer cells, as it occurs when these cells are incubated with serum of the same species (Russell et al. 1995). However, significant CSF retroviral titers could only be detected *in vivo* over 24 h (Oshiro et al., 1995). The retroviral studies in rats and non-human-primates by Ram et al. (1994) and Oshiro et al. (1995) were performed as preclinical studies for a clinical trial aiming to treat leptomeningial carcinomatosis by intrathecal application of retrovirus producer cells liberating retroviral particles bearing the *HSV-tk* gene for sensitization of transduced tumor cells towards subsequent GCV treatment (Oldfield et al., 1995). Despite the encouraging data in animals, this clinical trial was closed prematurely after toxic side effects occurred in the first patient (Anderson et al., 1995).

An interesting alternative to retroviral gene transfer was demonstrated by Vrionis et al. (1996a and b) who showed that therapeutic efficiency can also be achieved in a rat model of leptomeningial neoplasia by co-mixture of native and *HSV-tk* transduced cells in the same tumor with subsequent GCV treatment. This therapeutic approach relies mainly on the bystander effect describing that close proximity of non-transduced tumor cells with TK-positive tumor cells sensitizes non-transduced cells to GCV treatment. Vrionis et al. (1996a) demonstrated that an HSV-TK/GCV system which exploits the bystander effect at a relative low effector-to-target cell ratio (1:1) is more effective for treatment of leptomeningial neoplasia than the gene therapy approach with intrathecal application of retrovirus producer cells.

Intrathecal application of adenoviral vectors has also been used for gene therapy. Bajocchi et al. (1993) showed gene transfer into ependymal cells following direct injection into the ventricles. Ooboshi et al. (1995 and 1997) injected replication-deficient AV ($1x10^9$ pfu) intrathecally and demonstrated ß-galactosidase (*ß-gal*) gene transfer into ependymal and leptomeningial cells, as well as into cerebral blood vessels. No marked toxicity was observed in these studies. This was also true for two studies which investigated gene transfer into rodent leptomeningial tumor masses by replication-deficient AV vectors administered intrathecally. Viola et al. (1995) demonstrated AV-mediated gene transfer into the main tumor mass at the intrathecal injection site. Some limited gene transfer was also noted in tumor manifestations in the cauda equina and along the nerve roots emerging from the spinal cord. Vincent et al. (1996a) observed gene transfer into tumor cells along the entire neural axis after intrathecal administration of AV vectors. These authors achieved a significantly longer survival of treated animals by combining intrathecal delivery of AV vectors carrying the *HSV-tk* transgene with subsequent GCV treatment, but had no long-term survivors.

In contrast to this, when replication-conditional herpes vectors with the *HSV-tk* gene were injected intrathecally in a similar model of rodent leptomeningial neoplasia as above, long-term survival was achieved in approximately 90% of the animals treated with GCV (Kramm et al., 1996b). One reason for these diverging results may be that replication-conditional HSV vectors replicate in dividing tumor cells, and not in non-dividing normal cells, thereby producing and releasing new vector particles on site which move freely through the CSF. After intrathecal application of replication-conditional herpes vectors, Kramm et al. (1996a) also showed extensive gene transfer into leptomeningial tumors along the entire spinal axis (**Fig. 1C**), as well as into parenchymal brain tumors (**Fig. 1D**). Additionally, ependymal and endothelial cells, as well as neurons projecting to the ventricles, showed marked transgene expression during the first two days after injection of herpes vectors. Five and more days after vector application, normal cells no longer showed transgene expression. However, there was a high degree of toxicity to animals, probably due to inflammatory reaction to the virus, which was apparently absent after intrathecal application of retroviral and adenoviral vectors in rodents (Ram et al., 1994; Viola et al., 1995), but has been noted in humans (Ram et al., 1997; Eck, 1997, personal communication). The viral genesis of symptoms in the HSV study in rats is strongly suggested by the fact that GCV treatment, which blocks virus replication, significantly improved and curtailed this toxicity (Kramm et al., 1996b). The ambivalent potential of intrathecal delivery is emphasized by studies with a new generation of replication-conditional HSV vectors (Kramm et al., 1997, Mineta et al., 1995). The prototype of this new generation herpes vector was designed to be safer than the preceding vectors (ribonucleotide reductase-deleted) by deletion of viral neurovirulence genes (*gamma 34.5*). Application of this new vector intrathecally in

combination with GCV in the same rodent model of leptomeningial neoplasia as described above, was associated with no apparent toxicity or mortality, but also with no significant prolongation of survival of treated animals (Kramm et al., 1997).

Rosenfeld et al. (1995) used an adeno-associated virus (AAV) vector to transduce medulloblastoma cells in a nude rat model of leptomeningial disease. After intrathecal application, tumor cells transduced with the marker gene ß-galactosidase were detected in tumors, as well as in ependymal and subependymal cells, but not in normal brain parenchyma. No evidence of virus toxicity was noted during the course of the experiment.

In conclusion, leptomeningial neoplasia, which represents a main problem in the management of primary and secondary brain tumors, especially in children, is a good target for future gene therapy approaches for intrathecal delivery of therapeutic genes.

IV. Intravascular vector application

Intravascular methods of vector application make use of a natural and ubiquitously distributed network of arteries, veins and capillaries, which is present in every normal tissue and is even denser in malignant tumors. Intravascular applications, intra-arterial injection of virus vectors in particular, appear to have the greatest potential to date for delivering a vector to the largest proportion of tumor cells and surrounding tissues without afflicting mechanical injury to normal brain tissue or having other toxic consequences (Spear et al., 1998; Muldoon et al., 1997). Intra-arterial vector application with or without disruption of the blood-brain-barrier (BBB) or the blood-tumor-barrier (BTB) seems to offer a solution to the difficulties of vector distribution by employing the extensive tumor neovasculature for transgene delivery to all vascularized tumor foci (Muldoon et al., 1997). In contrast to the normal BBB, which consists of endothelial cells bound together with tight junctions and wrapped by astrocytic processes, and which limits the entry of substances into the interstitial and intracellular space of the brain, the brain tumor neovasculature has a somewhat more permeable barrier (Cox et al., 1976; Inamura and Black, 1994; Long, 1979; Yamada et al., 1982). Although the BTB may be more or less leaky, it still limits delivery of high molecular weight substances to tumor tissue and to immediately adjacent, partially tumor-infiltrated areas of the brain (Groothuis et al., 1991). The varying permeability found throughout the BTB in the majority of malignant brain tumors (Bergström et al., 1983; Burger et al., 1988) may restrict vector penetration, especially those with a larger size (HSV = 150 nm in diameter, AV = 70-100 nm). On the other hand, the existence of a tight BBB throughout the normal brain provides an advantage in terms of selective entry for CNS neoplasms (Rainov et al., 1995).

In addition to the BTB, some other factors limit intravascular vector delivery to brain tumors. In order to infect a maximum number of tumor cells, virus vectors must be delivered in sufficiently high titers and should not be inactivated by serum factors (Muldoon et al., 1997). High interstitial fluid pressure within tumors also acts to decrease entry of macromolecules and particles (Jain, 1987 and 1994). Larger tumors generally have a higher interstitial pressure than smaller tumor foci (Leunig et al., 1992), which theoretically limits efficiency of vector delivery to human brain tumors (Boucher et al., 1996). Human glioblastomas also have a variable degree of vascularization, and their microvasculature and hemodynamics vary considerably (Warnke et al., 1987). These obstacles for brain tumor vector delivery call for alternative strategies to circumvent them in order to make delivery of vectors more efficient by additional penetration-enhancing or barrier-modulating techniques.

A. Intravascular vector delivery without BTB modulation

The average size of a HSV particle is about 150 nm and that of an AV particle is 70-100 nm, and because they are so large, their penetration through normal brain capillaries or tumor neocapillaries is poor (Rainov et al., 1995). There are only a few studies being done with virus or non-virus vectors injected intravascularly without modulation of the BTB or the BBB. Chauvet et al. (1998) injected AV vectors into the middle carotid artery (MCA) of a dog with a benign intracranial meningioma and were able to achieve a high percentage of transduced tumor cells without any concomitant toxic effects to the CNS. Meningioma, however, unlike astrocytoma or glioblastoma, have excessive fenestration and leakiness of tumor capillaries, which probably facilitate virus vector entry (McDermott and Wilson, 1996). Our studies (Rainov et al., 1995) and those of other investigators (Neuwelt et al., 1991; Nilaver et al., 1995) have demonstrated increased transduction rates of tumor cells by HSV or AV after osmotic or pharmacologic barrier disruption, as compared to delivery of vectors across the intact BBB and BTB. Delivery studies of HSV particles and MION across the unmodified BBB and the BTB have shown that there is a small percentage (2-5%) of 9L gliosarcoma cells which can be targeted with HSV, presumably through the somewhat leaky BTB in 9L tumors, or by secondary spread from infected endothelial cells in neocapillaries producing replication-conditional vectors (Rainov et al., 1995). While HSV particles tend to infect cells in the periphery of the tumor and at the tumor/brain border, MION are delivered throughout the tumor and accumulate to some extent in the tumor center

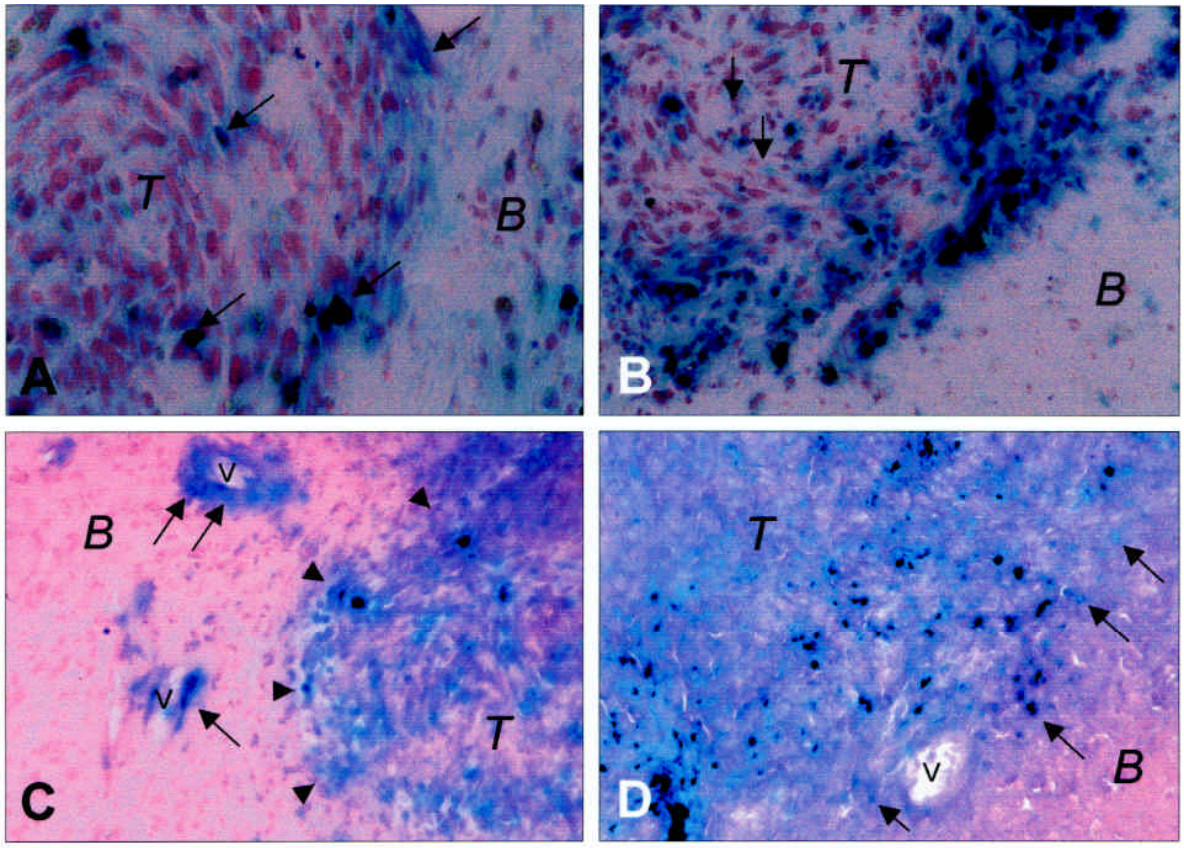


Fig. 2: Visual comparison of ß-gal expression in large and small 9L tumor foci in syngeneic Fischer rats injected with replication-deficient AV and liposome-DNA complexes (lipoDNA) bearing the *lacZ* gene with and without blood-tumor-barrier disruption by bradykinin (BK). A. Photomicrograph of a small intracerebral tumor focus (< 0.5 mm) in the AV group 48 hours after ipsilateral intra-carotid virus injection in the absence of BK. Note the relatively high number of X-gal stained cells (blue, arrows) in the tumor periphery and, to a certain extent, in the tumor center (T), B = tumor-infiltrated surrounding brain (magnification 300x, 20 µm frozen section, counterstained with hematoxylin). B. Photomicrograph of an intracerebral tumor focus in the AV group 48 hours after intra-carotid BK infusion and AV vector injection. An increased number of stained cells is distributed somewhat more evenly throughout the tumor (T), B = normal brain (magnification 200x, 20 µm frozen section, counterstained with hematoxylin). C. Photomicrograph of a tumor in the lipoDNA group 48 hours after vector injection in the absence of BK. Note the high number of X-gal stained cells (blue, arrowheads) throughout the tumor (T). Endothelial cells in capillaries (V) near the tumor/brain border are also stained positively (arrows), B = normal brain (magnification 200x, 20 µm frozen section, counterstained with NeutralRed). D. Photomicrograph of a tumor in the lipoDNA group 48 hours after intra-carotid BK infusion and vector injection. The number of X-gal stained cells (blue, arrows) throughout the tumor (T) is somewhat higher than in the absence of BK, V = tumor vessel, B = normal brain (magnification 200x, 20 µm frozen section, counterstained with NeutralRed).

(Rainov et al., 1995). Intra-carotid delivery of AV vector to 9L rat gliosarcoma without BTB disruption results in transgene expression in 3-10% of tumor cells, predominantly located at the tumor-brain border, as well as randomly distributed throughout the tumor (**Fig. 2A**) (Rainov et al., in press). Virus-mediated expression of marker gene products in this tumor model is particularly high in small tumor foci (<0.5 mm) away from the main tumor mass. In these foci, almost half of all tumor cells are transduced. A few endothelial cells in normal brain capillaries are also transduced by AV-mediated gene transfer. Intracarotid delivery of non-viral vectors, such as liposome-plasmid

DNA-complexes (lipoDNA), without BTB disruption renders more than 30% of the tumor cells positive for the marker gene (Rainov et al., in press). The pattern of distribution is homogenous throughout the tumor, with a slightly higher transduction rate in the tumor periphery (**Fig. 2C**). Although lipoDNA-mediated gene transfer without barrier modification has increased efficacy as compared to HSV- and AV-mediated gene transfer, it is less tumor-specific, since a considerable number of endothelial and glial cells also express the respective transgene. LipoDNA complexes represent alternative vehicles for gene transfer and avoid some of the unwanted features of virus vectors (Hug and Sleight, 1991). The route of *in vivo* administration may affect dramatically the uptake of liposomes by normal and tumor cells. Intravenously injected liposomes are taken up mainly by the reticulo-endothelial system (RES), particularly in the liver and spleen (Hug and Sleight, 1991). Intra-arterial application of lipoDNA to tumors has not been investigated extensively, and little is known about transduction efficiency in brain tumors *in vivo* (Gennuso et al., 1993).

B. Intravascular vector delivery with osmotic BBB and BTB disruption

BBB and BTB can be manipulated to increase permeability for gene therapy vectors, such as viruses or non-viral particles. Several studies have focused on transient osmotic disruption of the BBB and the BTB, and this technique has been well characterized in animal models and in humans as an enhancer of chemotherapeutic drugs and vector delivery to brain tumors (Doran et al., 1995; Neuwelt et al., 1987; Neuwelt et al., 1991b; Nilaver et al., 1995; Zünkeler et al., 1996). The mechanism of osmotic disruption of the barrier includes shrinkage of endothelial cells with subsequent opening of the capillary tight junctions, which is achieved by application of hypertonic solutions of sugars or salts into the arterial system (Rapoport and Robinson, 1986). Infusion of mannitol is most commonly used because of its relatively low toxicity and the applicability to humans (Muldoon et al., 1998). Mannitol offers the possibility of global delivery of drugs and virus vectors throughout the vasculature, which can reach even infiltrating tumor foci distant to the main mass (Neuwelt and Hill, 1987; Neuwelt et al., 1991a and b). With mannitol disruption of the BBB and BTB, however, delivery and uptake of therapeutic agents is less specific to the tumor and tends to spread the toxic agents throughout the whole affected hemisphere, which may increase toxicity to normal brain tissue (Zünkeler et al., 1996).

Studies of delivery of virus and non-virus vector particles across the BBB and BTB have demonstrated the high efficiency of osmotic barrier disruption. HSV and AV vector delivery to brain and intracerebral tumors was increased up to four fold by hypertonic mannitol (Neuwelt et al., 1991b; Nilaver et al., 1995). When virus was administered intra-arterially without barrier modification, virtually no infection was detected of either tissue type. MION can penetrate efficiently through the disrupted BBB in rats, and have the advantage of being imageable by MRI. After intra-arterial mannitol infusion, glial cells were predominantly infected by AV, while HSV and MION targeted neurons more efficiently (Muldoon et al., 1998). The degree of barrier opening correlated with the transduction efficiency of glial and neuronal cells (Doran et al., 1995). Osmotic BBB disruption in combination with intra-arterial administration of viral vectors may offer a method of global delivery to treat disseminated brain tumors (Nilaver et al., 1995), although its specificity is far from optimal.

C. Intravascular vector delivery with pharmacological BBB and BTB disruption

Vasoactive agents for modification of the BBB and BTB have been identified through studies of peritumoral brain edema and effects on systemic capillaries (Black, 1992; Chan et al., 1983; Cloughesy and Black, 1995). The BTB can develop transient increases in permeability with the intra-arterial delivery of vasoactive agents, while the normal BBB resists the effects of these compounds because of additional biochemical and physical barriers (Inamura and Black, 1994). Vasoactive compounds, including leukotrienes (Black and Chio, 1992; Chio et al., 1992), bradykinin (BK) and its analog RMP-7 (Barnett et al., 1998; Black et al., 1997; Doctrow et al., 1994; Elliott et al., 1996a and 1996b; Inamura et al., 1994a; Matsukado et al., 1996; Nakano et al., 1996; Rainov et al., 1995; Rainov et al., 1998), histamine (Inamura et al., 1994b; Nomura et al., 1994), and calcium antagonists (Matsukado et al., 1994) appear to selectively increase permeability in abnormal brain tumor capillaries.

BK, a nonapeptide hormone with peripheral vasodilatation effect, permeabilizes the vascular endothelium in brain capillaries at low concentrations (10 g/kg/min), when delivered intra-arterially in rodents, and its barrier-modifying effects are specific to brain tumor neocapillaries (Inamura and Black, 1994). BK exerts its effects by interaction with specific B_2 receptors (Hess et al., 1992) on endothelial cells, which mediate contraction of the endothelial cell cytoskeleton with subsequent temporary opening of the tight junctions (Doctrow et al., 1994; Inamura et al., 1994a; Sanovich et al., 1995) and may also increase the rate of pinocytosis/transcytosis in endothelial cells (Raymond et al., 1986). It has also been

demonstrated that BK and RMP-7 increase intracellular free calcium levels (Doctrow et al., 1994) and stimulate a nitric oxide-mediated pathway in tumor vasculature and/or in tumor cells itself (Nakano et al., 1996).

BTB disruption by low-dose BK can facilitate selective uptake of HSV vectors administered through the carotid artery to single or multiple tumor foci in the rodent brain, with essentially no infection of normal neurons and glia (Rainov et al., 1995). Transgene expression after intra-arterial BK infusion and HSV vector bolus injection is particularly intense in the periphery of the tumor, a zone with distinct biological and biomechanical properties such as high mitotic rate, angiogenesis, parenchymal invasion, and low interstitial pressure (Boucher et al., 1996). Up to 25% of tumor cells in this region express transgene proteins after BK/HSV administration, as compared to less than 0.1% of cells in normal brain tissue (**Fig. 1E** and **F**). In contrast, MION uptake is increased by BK predominantly in the tumor center and has less effect at the infiltrating edge (Rainov et al., 1995). Furthermore, this study demonstrated HSV infection of multiple bilateral tumor nodules by unilateral BK infusion and HSV injection, which suggests that BK may have generalized effects beyond the site of infusion in rat brain with extensive collateralization.

The increased rate of tumor infection by HSV after BK infusion has been exploited for eradication of intracerebral 9L tumors in syngeneic rats. In this model, virus vector concentration appears to influence survival rates in a dose-dependent fashion when GCV is given systemically starting three days after BK/HSV application and continued for 14 days (Rainov et al., 1998). A concentration of $1x10^{10}$ pfu HSV was able to eradicate tumors in 80% of the treated animals, while $1x10^{9}$ pfu eliminated tumors in 40% of the rats, and $1x10^{8}$ pfu was sufficient for prolonged survival, but not for permanent tumor cures. No apparent complications of intra-arterial HSV injection were encountered in this study (Rainov et al., 1998).

In another study, intra-carotid delivery of AV and lipoDNA to 9L rat gliosarcoma with and without BK-mediated BTB modification was compared (Rainov et al., in press). For AV-mediated gene transfer, BK infusion increased the amount of transgene-expressing tumor cells from 5 to 19 % (**Fig. 2B**) and enhanced expression in the center of larger tumor foci. BK infusion prior to lipoDNA injection was able to increase the number of transduced tumor cells from 30% to more than 50%, and to produce a more homogeneous pattern of transgene distribution in the tumor (**Fig. 2D**). The relatively low tumor specificity of lipoDNA transfer remains unchanged by BK application, with extensive delivery to normal tissue as well. These findings indicate that intra-carotid application of virus and non-virus vectors can preferentially and effectively transduce brain tumor cells, and that BTB modification by BK further increases the number of transgene-expressing tumor cells without apparent adverse effects (Rainov et al., in press). With AV and lipoDNA, it remains to be determined whether the increase in transduction following BK infusion will result in long-term survival in experimental brain tumor models.

To replace BK with a new, longer and more selectively acting derivative suitable for human studies, the synthetic nonapeptide RMP-7, H-Arg^{1}-Pro^{2}-HydroxyPro^{3}-Gly^{4}-Thi^{5}-Ser^{6}-Pro^{7}-Tyr(Me)-ψ-(CH_2NH)^{8}-Arg^{9}-OH, a BK analog with three amino acid substitutions, was designed by Alkermes, Inc. (Cambridge, MA). It is more resistant to angiotensin-converting enzyme (ACE) due to the replacement of alanine with 2-thienyl-alanine (Thi^{5}) and to neutral endopeptidase and carboxypeptidase I, due to replacement of phenylalanine with the Tyr(Me)- ψ-(CH_2NH)^{8} group (Elliot et al, 1996a). Replacement of proline with hydroxyproline (HydroxyPro^{3}) removes the undesirable action on the B_1 receptor. RMP-7 has a 5-10 times longer half-life in the blood circulation than BK, is 100-fold more potent in mice, and acts more selectively on endothelial cells by binding to the B_2 receptor only, without undesirable blood pressure drops (Elliot et al., 1996a and b). RMP-7 has been FDA-approved for human studies.

RMP-7 was tested extensively as an adjunctive therapeutic agent for primary and recurrent malignant gliomas. In humans, intra-carotid or intravenous infusion of low-dose RMP-7 (0.1 g/kg/min for 15 min) was able to increase the delivery to brain tumors of intravenously injected low and high molecular weight tracers, such as aminoisobutyric acid or dextrane, and of cytotoxic agents, such as methotrexate and carboplatin (Elliott et al., 1996b; Matsukado et al., 1996; Muldoon et al., 1998). There are data to suggest that RMP-7 is at least equivalent to BK in terms of enhancement of virus and non-viral particles to brain tumors (Barnett et al., 1998). This study compared BK and RMP-7 and, among other findings, demonstrated no significant difference in the enhancement of HSV delivery across the BTB, which confirms the potential of RMP-7 for application for gene therapy of human brain tumors (Barnett et al., 1998).

In conclusion, the issues of delivery of gene therapy vectors to tumors in the brain seem still to be underappreciated in the literature. Unfortunately, the development of new and more specific virus and non-virus vectors does not address the difficulties of accessing the maximum number of tumor cells in diffuse or invasive and multifocal tumors, such as human GBM. Since there are no ideal vectors or unconditionally efficient application modes or delivery routes, in the future, gene therapeutic approaches for brain tumors will be a combination of different application routes, vectors and transgene

combinations, designed to account for the individual features of different tumor types. The intravascular route of vector delivery should gain a much higher popularity and will be aided either by direct intratumoral injections, as in the case of adult multifocal GBM, or by widespread CSF vector delivery as in the case of pediatric brain tumors.

Acknowledgements

These studies were supported in part by grant 015VE1997 from the State Ministry of Culture and Education of Saxony-Anhalt, Germany, to NGR, and NCI grant CA69246 to XOB

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